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Approaches To Fracture Healing Under Inflammatory Conditions: Infection And Diabetes

Sean Vincent Cahill

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Approaches to Fracture Healing Under Inflammatory Conditions: Infection and Diabetes

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

Sean Vincent Cahill

2020
Abstract

Non-union is a devastating complication of fracture and can be precipitated by abnormal inflammatory states including infection and diabetes.

This thesis focuses on four related research problems that are addressed through original scientific investigation and literature review. In addressing these questions, this dissertation presents evidence for the following conclusions through in vivo animal models and using methods including bacterial cell culture and counting, histology, radiography, and micro-computed tomography:

1. Rifampin-loaded hydrogels decrease bacterial load and improve fracture healing in a MRSA-infected open fracture model.
2. MRSA-infected nonunion is characterized by impaired chondrocyte maturation and is associated with IL-1 and NF-KB activation.
3. Local teriparatide improves radiographic fracture healing in a type 2 diabetic mouse model, but is inferior to systemic treatment.
4. Systemic administration of teriparatide, along with systemic antibiotics, improves fracture healing in a diabetic, MRSA-infected mouse tibia fracture model.

This current work is not without limitation, and many aspects of this work are still in progress. Nevertheless, the author hopes that this dissertation will serve as providing meaningful, foundational data for future laboratory and clinical studies to improve our understanding of inflammatory fracture healing and arrive at new therapies to advance the practice of fracture care.
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Lee lab members, spring 2019. From left: Hyuk-Kwon Kwon, PhD; Jungho Back, PhD; Zichen Hao, MS; Minh-Nam Nguyen, PhD; Francis Lee, MD, PhD; Sean Cahill, BA; Kareme Alder, BS; Kristin Yu, BS; Yeon-Ho Cheung, PhD.
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Introduction

I. Overview: Fracture healing is essential to human health

Unlike repair mechanisms of nearly every other human tissue, bone fracture healing has the potential to restore the original structure and physical properties without leaving functional deficits, scar, or other evidence of previous injury [1]. The biologic process of fracture healing is complex and requires mechanical stability, growth factors, stem cells, and other factors in order to restore structure and function [2].

Successful fracture healing is essential to human health, as fracture is one the most common traumatic injuries to humans [1,3]. Fracture nonunion and delayed union results in pain and disability, and can be devastating for patient’s quality of life [4-5]. Specifically, in a 2013 study, tibia shaft non-union resulted in a negative effect on mental and physical health that was worse than congestive heart failure and equivalent to end-stage hip arthrosis [4]. In a similar study, Schottel et al found that femoral fracture nonunion demonstrated a reduced quality of life similar to type 1 diabetes, stroke, and acquired immunodeficiency syndrome [6]. Forearm and clavicle nonunion resulted in the greatest degree of impairment, compared to femur, tibia, fibula, and humerus fracture [6].

Fracture non-union also poses a major burden to our healthcare and economic systems. An estimated 100,000 fractures result in non-union in the United States every year [7]. In the US, additional healthcare costs due to tibia fracture nonunion range from $11,333 to $13,870 [8-9]. Indirect costs of nonunion, most notably productivity loss, account for the majority of the economic burden resulting from fracture nonunion. Among Canadian and European healthcare systems, these indirect costs make up for an estimated 67-79% and 82-93% of total costs burden, respectively [7].
The overall fracture nonunion rate is cited to be approximately 5-10% in the orthopaedic literature [10-11]. In a 2016 study of open long bone fractures, 17% progressed to nonunion and an additional 8% demonstrated delayed union [12]. Non-union risk is variable and depends on injury factors such as site, mechanism, and severity; and patient factors such as age, sex, and comorbidities [13-14]. The incidence of non-union and delayed union is proposed to have increased over the past decades due to improved patient survival and advances in medical and surgical care following major injuries [15].

Many approaches to improving fracture healing have been investigated, from biologic and surgical approaches to traditional medicine practices [16]. This dissertation will discuss translational science approaches to improve fracture healing in altered inflammatory environments including diabetes and infection. It will discuss the use of a locally-applied hydrogel to deliver antibiotics and teriparatide under inflammatory conditions, using mouse models of infected nonunion and diabetes. It will also identify key cellular processes and potential avenues for targeted therapies. It is the author’s hope that these findings will enhance our understanding of fracture non-union and move the field of orthopaedic surgery forward by providing a basis for future clinical investigations.

II. **Bone quality in health and disease**

Successful fracture healing and underlying bone quality are closely related. Mesenchymal stem cells, chondrocytes, osteoblasts, osteocytes, and osteoclasts form a tightly-regulated cellular network that performs in the tasks of building and maintaining bone as well as fracture healing.

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1 Based on: SC, Lee, FY. “Orthopaedic Tissues,” Orthopaedic Knowledge Update 13, AAOS 2020. All text and figures in this thesis, including hand drawings, are original and were prepared by the author, unless explicitly noted. The author acknowledges Dr. Lee’s guidance in preparing and revising this portion of the text.
Hormonal regulation is essential, with mesenchymal progenitor cells playing major signaling roles. This section will investigate the normal workings of this cellular network of orthopaedic tissues and how it can fail in diseased states such as smoking and cancer. This section will present basic components of bone biology that are relevant to fracture healing, diabetic bone disease, methodologies, and findings presented in this dissertation.

Figure 1. Major transcription factors and regulators of bone cell differentiation

*Mesenchymal stem cells differentiate via a stepwise progression into chondrocytes, adipocytes, osteoblasts, osteocytes, tenocytes, and myocytes. Osteoclast arise from the monocyte lineage of hematopoietic stem cells. A host of transcription factors, genes, and growth factors regulate*
differentiation. Activation via RANK-L and inhibition by OPG, both expressed by pre-osteoclasts, are major regulators for osteoclastogenesis.

Orthopaedic tissues are derived from pluripotent stem cells which become increasingly more specialized (figure 1). Bone is unique in that regulation of cellular and metabolic processes occur primarily at the level of the stem cell. Osteoblasts, chondrocytes, adipose cells, fibroblasts, and myocytes share the mesenchymal stem cell as the common precursor, while osteoclasts are derived from the macrophage/monocyte lineage of hematopoietic stem cells. Altered development and function of these precursor lineages underly many of the processes that alter bone quality and fracture healing potential.

Variable expression of transcription factors facilitates stem cell differentiation into terminal lineages to form orthopaedic tissues as cellular migration and ossification take place [17]. Runx2 and osterix are essential for differentiation of the osteoblast lineage. Sox5, 6, and 9 are markers of chondrocyte development, with Sox9 having been identified as an essential regulator [18] (Figure 1). These signaling pathways involved in mesenchymal stem cell differentiation have important consequences for fracture healing under infected conditions (pages 75-80, 97-100). The Wnt/β-catenin pathway is one of the most important signaling pathway for regulating bone formation, leading MSCs towards osteoblastic differentiation and suppressing adipose development, and is altered in diabetes (page 42).

Ossification is a foundational principle that underlies both skeletal development and fracture healing. During human development, contact between mesenchymal cells and epithelial cells triggers pre-osteoblastic differentiation and intramembranous ossification, during which mesenchymal cells differentiate directly into periosteum and osteoblasts [19]. During
endochondral ossification (Figure 2), mesenchymal tissue develops into bone from a cartilage template [20]. Chondrocytes proliferate and undergo hypertrophy and apoptosis, and the remaining matrix is mineralized and invaded by vasculature. Systemic factors, such as growth hormone and thyroid hormone, and local factors such as Indian hedgehog and PTHrP, promote and regulate these processes (Figure 2). Woven bone, secreted by osteoblasts, is eventually replaced by lamellar bone. After a rudimentary skeleton is formed, osteoblasts and chondrocytes undertake skeletal modeling to shape the skeleton and improve its strength and resilience. More information about the ossification process as it relates to fracture healing can be found on pages 23-26.

Secondary ossification widens bones, with peripheral growth from the apophysis. In contrast to primary ossification, which begins in the embryonic stage and continues through adolescence, secondary ossification only begins during the post-natal period.

**Bone Cellular Biology.** Bone is a rich, biologically active tissue. Osteoblasts, osteocytes, and osteoclasts maintain and renew the bony matrix and are involved in systemic processes such as mineral metabolism. An understanding of bone cellular biology is essential for understanding the mechanisms behind fracture healing and diabetic bone disease.

Mature osteoblasts contain abundant rough endoplasmic reticulum for collagen synthesis, as well as an extensive Golgi apparatus. Osteoblasts synthesize bone through type I collagen secretion and production of osteoid (unmineralized matrix). Parathyroid hormone stimulation and Runx2 expression induce the expression of alkaline phosphatase, type I collagen, and bone sialoprotein II in the preosteoblast stage [21] (Figure 1). Transcriptional activation of RUNX2 and osterix result in osteoblast differentiation, allowing for matrix mineralization and expression of other proteins such as osteocalcin to occur. Experimental evidence for the importance of RUNX2
in fracture healing is given on page 75-81. Osteoblasts create a basic environment with alkaline phosphate that helps catalyze calcium-phosphate crystal deposition.

Figure 2. Endochondral Ossification and Cartilage Differentiation

Endochondral ossification occurs at the physis, during which chondrocytes undergo proliferation, hypertrophy, and apoptosis. The matrix left behind is mineralized and invaded by blood vessels. Growth factors, including growth hormone, thyroid hormone, and FGF3, promote osteogenesis. Indian hedgehog and PTHrP create a feedback loop to modulate and regulate chondrocyte proliferation and hypertrophy. Fracture healing, as discussed in this thesis, relies on this process.
Osteocytes, which comprise over 90% of all bone cells in adults, are differentiated from osteoblasts [22]. Our understanding of osteocytes has dramatically increased over the past decade, especially our understanding of the mechanisms of response to mechanical loading and their role in bone metabolism. The process of the osteoblast becoming embedded in bone lacunae induces changes genetic expression to induce osteocyte differentiation, including participation in mineralization regulation and development of dendritic processes [22]. The expression of membrane type 1 matrix metalloproteinase is necessary for canaliculi formation, through which osteocytes form an extensive intercellular network of dendritic processes that directly communicate via gap junctions [23]. The lacunar-canicular system contains circulating fluid that provides osteocytes with oxygen and nutrients and allows osteocytes to sense acute deformation of the bone matrix, inducing release of anabolic factors to increase bone mass in response to strain. These lacunar networks are significant in the setting of osteomyelitis, as it will be shown (Page 75) that the lacunae are the main harboring site of bacteria following MRSA-infected fracture.

Osteocytes are important regulators of bone metabolism. In addition to sensing fluid microcurrents and responding to bone matrix strain, osteocytes produce sclerostin and Dkk1, which are potent Wnt inhibitors and therefore key negative regulators of osteoblastogenesis [22]. Sclerostin and Dkk1 monoclonal antibodies are under investigation as a potential means of increasing bone mass and improving fracture healing, especially in diabetes [24]. A more thorough discussion of the roles of sclerostin and Dkk1 in the altered inflammatory environment of diabetic bone disease is provided on page 42.

Osteoclasts, derived from hematopoietic stem cells, appear as large, multinucleated cells housed in Howship’s Lacunae, microscopic grooves on the bone surface. The ruffled border seals
the bone surface, creating a closed microenvironment in which degradation products such as acid (produced by carbonic anhydrase and H+ ATPase) and cathepsin K degrade the bone matrix [25].

Osteoclast development and differentiation is tightly linked to the osteoblastic lineage, and dysregulation leads to bone pathology (Figure 1). Commitment to the osteoclastic lineage from hematopoietic precursors is induced by macrophage colony stimulating factor, c-fos transcription factor, and RANK expression [26]. RANK-ligand, expressed by osteoblasts, is required for pre-osteoclast differentiation into osteoclasts. Osteoprotegerin (OPG), produced by cells of the osteoblast lineage as well as some hematopoietic cells, serves as a decoy receptor for RANK-L and competes with osteoclast RANK receptor; increased OPG decreases osteoclast differentiation and activation. Systemic factors help to regulate RANK-L and OPG production, including TNF-α, a catabolic factor, and PTH and estrogen, anabolic factors. Factors that contribute to osteoclast activation are found in the setting of infected fracture (pages 27-29).

Although osteoclast differentiation is tightly regulated by the osteoblastic lineage, osteoclast activity is also influenced by hormones. Resorptive activity is increased by vitamin D, PTH, PTHrP, and prolactin, and decreased by estrogen, calcitonin, and TGF-β. Cytokines also regulate osteoclast activity. IL-17 has been shown to decrease bone resorption; IL-6 and TNF-α increase resorption [26]. Parathyroid hormone is the main anabolic agent studied in this dissertation, and is used to improve fracture healing in normal and diabetic mice. More detail about the action of parathyroid hormone can be found on pages 49-50.

Marrow adipocytes have long considered inert place holders in the marrow space of long bone, ribs, sternum, and vertebrae. However, marrow fat has been increasingly recognized as an important, active element of the bone cell milieu and is considered a distinct type of adipocyte (as opposed to white, brown, or beige fat found elsewhere in the body). As discussed in a 2017 study,
it has been shown that marrow adipocytes increase with age and are tightly correlated with reduced
bone mass [26]. These regulatory effects on bone mass are thought to be induced through
modulation of PPARγ and RUNx2 proteins [27]. Thus, marrow adipocytes and associated
signaling pathways have become sought-after targets for bone disease therapies. In addition to
bone metabolism, it is thought that marrow adipocytes influence other cell populations within the
marrow and negatively affect hematopoiesis [26]. The adipocyte lineage is important to the
pathogenesis of diabetic bone disease, as discussed on page 44.

   Matrix Composition. The cellular components produce and maintain a mineralized bone
matrix, which serves as the functional component of bone. The extracellular matrix is
heterogeneous and structured. Mineral and organic components together impart strength and
rigidity (Table 1a). The composition of bone varies with age, gender, and ethnicity. Health status
can also affect matrix composition, and alterations of the matrix underlie diseases such as
osteogenesis imperfecta and osteoporosis [28].

   The mineral calcium hydroxyapatite, Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}(OH)\textsubscript{2}, is the most abundant substance in
bone, 60-70% of its mineral composition. Other abundant minerals include sodium, magnesium,
and bicarbonate [28]. The organic component of bone, or osteoid, stabilizes the extracellular
matrix, facilitates calcification and mineralization, and provides tensile strength. Type I collagen
is the dominant organic substance of bone, comprising 90% of total protein and the second most
abundant substance following hydroxyapatite. The collagen triple helix, characterized by glycine-
X-Y repeating sequence, is highly crosslinked, providing elasticity. Fibronectin, another important
structural protein of the bony matrix, helps develop and maintain the structure of the collagen
network. Non-collagenous proteins, such as proteoglycans and osteocalcin, also play various roles
[29] (Table 1). The quality of bony matrix is determined by a number of factors, but one crucial
factor is accumulation of advanced glycosylation end products (AGEs) that occurs in the setting of diabetes; a detailed discussion is provided on page 40-41 and figure 9.

**Bone metabolism:** As impact and activity imparts microscopic damage, osteoclasts and osteoblasts work in concert to remove old bone and replace it with new bone to maintain strength and integrity. This process of remodeling is ongoing, working to replace approximately 10% of the skeleton every year and replacing the entire bone mass every ten years [30].

Remodeling is a tightly regulated process, as osteogenesis is intimately coupled to osteoclastogenesis. Remodeling imbalances can result in systemic disease such as osteoporosis as well as local bone destruction as in cancer metastasis. Bone loading affects the rate of bone remodeling as stated by Wolff’s Law, that mechanical stress results in greater bone density and strength. Systemic and hormonal factors, such as parathyroid hormone and 1,25-dihydroxy vitamin D, modulate remodeling by inhibiting bone resorption and promote differentiation of osteoblasts and osteoclasts [31-32]. Direct communication between osteoblasts and osteoclasts is also achieved through release of local factors from the bone matrix itself. As osteoblasts degrade the bony matrix, proteins including TGF-β, platelet-derived growth factor, and fibroblast growth factor are released to stimulate osteoblasts and thus enhance bone formation. Micro-RNA modulation has also been identified as a significant regulator of bone remodeling [33]. Bone remodeling is essential not only in normal skeletal maintenance, but also in the later stages of fracture healing; more detail can be found on pages 24-25.

**Anatomy and Structure.** Long bones allow for mechanical motion of the extremities and are formed by endochondral ossification (Figure 2). The epiphysis, covered by articular cartilage, forms joint surfaces (Figure 3). The physis, distal to the epiphysis, is the location of endochondral
ossification, and is located between the epiphysis and the metaphysis. The apophysis, a feature of both long and flat bone, is an area of secondary ossification.

Periosteum is a thin, membranous tissue that surrounds both long and flat bones, and endosteum is the corresponding inner surface. The periosteum contains a rich vasculature that provides primary blood supply to bone, making it essential for fracture healing. It is also the site of muscle and tendon attachment.

Cortical bone is dense, compact tissue which functions to provide mechanical rigidity. Haversian canals run parallel to the diaphysis along the mechanical axis of bone. These spaces house nerves and microvasculature that supply the bone tissue. Laminae are discreet, concentric sheets of bone that surround the Haversian canal. Volkmann canals allow for communication between periosteal vessels and Haversian system. Osteocytes are housed in lacunae and their dendritic processes communicate via small canaliculi. Cutting cones comprise the remodeling unit of cortical bone, with osteoclasts forming a canal along the longitudinal axis of bone and osteoblasts following to close the gaps [32]. The mouse, an animal model used to fracture healing under inflammatory conditions throughout this dissertation, lacks the haversian system found in human bones.

Trabecular (or cancellous) bone is a lower-density tissue. Trabecular bone has a porous, sponge-like structure which houses marrow elements, including hematopoietic stem cells and marrow fat. Remodeling occurs directly on the surface of the trabeculae, with osteoclasts forming lacunae which are subsequently filled by osteoblasts [32]. The trabecular network can be assessed by micro-computed tomography, a major method used to assess the quality of fracture healing in this dissertation; please see page 62 for more information.
The major regions of long bone include the epiphysis (nearest to the joint), physis, metaphysis, and diaphysis. Blood supply via nutrient arteries is derived from the periosteum, which is also a major source of stem cells in fracture healing. Endochondral ossification occurs at the physis.
Cartilage, synovial membrane, and synovial fluid compose synovial joints. Together, they provide lubrication and protection of joint surfaces and allow for movement along a virtually frictionless surface. Inflammation and degradation of these tissues underlie arthritic processes and result in debilitating pain and deformity. Interleukin 1 (IL-1), is a major inflammatory mediator that has been demonstrated to cause cartilage destruction in osteoarthritis. A more complete discussion of the proposed role of chondrocyte differentiation and IL-1 in fracture healing and infected nonunion is provided on page 97.

The chondrocyte is the cellular component of cartilage, and is crucial to fracture healing. A major portion of this dissertation is devoted to characterizing changes in chondrocytes and cartilage in the setting of infected fracture (pages 75-80, 97-100). The chondrocyte comprises only a small amount of total articular cartilage mass, approximately 5% of its dry weight. Cartilage is composed primarily of water and cross-linked type II cartilage, secreted by chondrocytes (Table I). Proteoglycans, most abundant in the deep layer, trap fluid and contribute to pressurization and compressive tissue strength. SOX5 and SOX6 expression, along with interactions with CEBP/p300, stimulate chondrocytes to secrete type II collagen and proteoglycans [34]. In addition to secretion, chondrocytes regulate cartilage homeostasis by expression of metalloproteinases that break down the cartilage matrix. Components of the cartilage extracellular matrix, especially type XI collagen, have been identified in a 2017 study as playing a role in inducing chondrogenesis [35].

Chondrocytes undergo terminal differentiation through the process of hypertrophy, marked by cellular swelling, decreased proliferation, and eventual apoptosis. While chondrocyte
hypertrophy and death allow for bone formation in developing bone, a 2018 study reported that hypertrophy has been shown to be an essential step in the pathogenesis of osteoarthritis and marks the beginning of irreversible degradation to the cartilage matrix [34].

The quality of tissues including bone, cartilage, and ligaments plays a significant role in prevention and treatment of skeletal disease. Physiologic causes, such as age, and pathologic states, such as diabetes mellitus and metastatic bone disease, can decrease the integrity of these tissues, resulting in increased fracture incidence and decreased surgical outcomes. A host of drugs, such as corticosteroids, can also affect bone quality.

Bone health and bone density can be affected by a host of physiologic, pharmacologic, and demographic factors that affect the balance of bone resorption and formation (Figure 4). While a comprehensive review of causes of impaired skeletal health are out of the scope of this introduction, a few key subjects, including aging, malignancy, and smoking are included here, as they are common in the clinical setting affect fracture risk and healing potential. A thorough review of the role of diabetes, a major focus of this dissertation, is provided on pages 37-51.

**Aging:** Aging bone is characterized by decreased bone mass due to progressively dysregulated remodeling. With increasing age, the amount of new bone formed with each remodeling cycle decreases slightly, while the amount of resorbed bone remains constant. This is associated with increased osteocyte apoptosis and decreased number of precursor cells. Other factors, such as decreased mechanical loading of bone and accumulation of reactive oxygen species, may also contribute to decreased osteocyte function. In addition to decreased bone mass, the bone quality changes with aging. As discussed in a 2017 study, collagen becomes increasingly crosslinked, resulting in bone that is more brittle with a disrupted mineralized matrix [35]. Bones
become slenderer with decreased trabecular bone mass, thinning cortices, and changes in center of gravity [36]. Overall, these changes contribute to greater risk of fracture in the elderly.

**Figure 4. Bone Quality Determinants**

Bone mass is determined by a balance between bone formation and resorption. Peak bone mass is achieved in early adulthood, and slowly declines due to decreased bone synthesis during each remodeling cycle.

Osteoporosis is a bone disease distinct from aging that is marked by decreased bone mineral density, especially in trabecular bone. The hallmark of osteoporosis is fracture

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2 Original figure was prepared by Dr. Lee and modified by the author.
predisposition, including non-traumatic fractures and vertebral body fractures. While osteoporosis is an age-associated disease, it is generally not considered a disease of aging, as osteoporosis can (although rarely) affect the young and does not affect all elderly individuals. Treatment generally is targeted against bone resorption, and including bisphosphonates, hormone therapy, and calcitonin. Anabolic agents, such as teriparatide (recombinant parathyroid hormone), are also used. Preventative measures, including weight-bearing exercise and vitamin D supplementation, is also recommended for patients at risk of developing osteoporosis. Patients with diabetes can present with osteoporosis, especially type 1 diabetics early in life. Please see pages 37-40 for more information on diabetes-induced osteoporosis.

**Malignancy:** Bone destruction can be a devastating source of morbidity in cancer. Two major causes of bone destruction will be covered here: radiotherapy and metastasis to bone.

Radiation therapy, common bone and soft tissue tumor treatment, can cause inflammation, vascular fibrosis, and reduced tissue circulation. Bone necrosis can result from hypoxic and hypovascular conditions, tissue breakdown, and disrupted wound healing [46]. These conditions raise the risk for pathologic fracture and allow for development of chronic infections that are poorly responsive to systemic antibiotics. A case presentation on fracture and infection following tumor resection and radiation therapy can be found on pages 29-35.

Malignant bone metastases, an incurable progression of a primary tumor, can lead to pain, fracture, and hypercalcemia due to dysregulated bone remodeling [47]. Lung, breast, and renal cancer, as well as multiple myeloma, are best known for causing osteolysis. A host of pathways contribute to tumor-driven bone destruction, including increased expression of RANK-L, matrix metalloproteinases, and PTHrP. Tumor cells can also undergo osteoclastic mimicry by fusing with osteoclast precursors, gaining the ability to participate in bone resorption. Furthermore, osteolytic
matrix destruction facilitates cancer progression via TGF-B release, increase calcium, and hypoxia. Prostate cancer bone metastasis, on the other hand, is marked by pathologic production of immature, woven bone. Tumor cell epithelial-to-mesenchymal transdifferentiation and osteomimicry are the major osteoblastic pathways involved.

Smoking: Smoking is a leading cause of preventable morbidity and mortality worldwide, and has significant, deleterious effects on bone health. Smoking generates reactive oxygen species, impairs mitochondrial activity, impairs fibroblast migration, and reduces blood flow to sites of injury [48]. Smoking is a risk factor for low bone density, with recent animal models demonstrating increased osteoclast numbers and impaired bone growth in response to long-term cigarette smoke exposure [49]. Smokers demonstrate greater time to union and impaired chondrogenesis following fracture, as well as higher rates of spinal fusion failure and pseudarthrosis compared to non-smokers [48,50]. In addition to impaired bone integrity, smokers carry significantly increased risk of infection following trauma [51].

III. Normal fracture healing is an inflammation-dependent process.

Fracture healing is a complex process that results from tightly-ordered interactions of cells, growth factors, and extracellular matrices [52]. Fracture healing is broadly classified as secondary or primary, delineations that refer to underlying healing mechanisms dependent on rigidity of the fracture construct. Primary fracture healing occurs when bone fragments are in direct contact with one another and are highly stable with minimal strain [52]. Haversian remodeling facilitates healing of the fracture site, dependent on osteoclasts and osteoblasts to directly repair the bone fragments. Secondary healing is more common, and is characterized by four steps: 1) the initial

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3 The author acknowledges the assistance of Yeun-Ho Cheung, PhD, in revising the first two paragraphs of this section.
inflammatory phase, 2) soft callus formation, 3) hard callus formation, and 4) remodeling [52-54] (Figure 5).

**Figure 5. Phases of Normal Fracture Healing.**

*Schematic of secondary fracture healing, which progresses through step-wise phases to restore original strength and shape to bone without leaving a scar. The inflammatory phase is crucial, during which the presence of hematoma from disrupted blood vessels forms a clot, attracting immune cells and mesenchymal progenitor cells via inflammatory signaling. Soft-callus, primarily made of cartilage, bridges the bone gap. New bone formation follows the process of endochondral ossification. The bone’s normal shape is returned by remodeling via osteoclasts osteoblasts.*

The early inflammatory phase of secondary fracture healing, prior to soft callus formation, is considered to be crucial to the entire healing process [55]. Blood vessel disruption in the bone and surrounding soft tissue results in hematoma formation, which incites an inflammatory reaction. Conversion of fibrinogen to fibrin occurs in the hematoma, allowing for trapping of innate immune
cells. These immune cells release chemokines that attract additional cells to the site [55]. Various immune cells that are attracted to the injury site secrete growth factors and cytokines such as TNF-α, IL-1β, IL-6, and MCP-1, recruiting mesenchymal stem cells which originate from the periosteum, bone marrow, and systemic circulation [56-57]. The hematoma and inflammatory response support chondrogenic and osteogenic differentiation, eventually resulting in soft callus formation [53]. Several animal model studies have demonstrated that impairing this initial stage can inhibit the entire fracture repair process [58-59].

The soft callus is comprised of cartilage and granulation tissue that provides the biologic scaffold onto which new bone is formed. Chondrogenic maturation progresses in a step-wise fashion to facilitate bone formation, in similar manner to secondary ossification at the physis. Chondrocytes undergo phases of proliferation, hypertrophy, and apoptosis, followed by vascular invasion. Osteoblasts produce woven bone upon the scaffold of dead chondrocytes, resulting in a hard callus. The fourth phase of secondary fracture healing, the remodeling phase, occurs over a longer period of time as osteoclasts and osteoblasts shape and strengthen the bone, eventually renewing its original strength, histologic appearance, and, in many cases, shape.

By its very nature, then, fracture healing is an inflammatory process. Secondary bone healing requires the hypoxic, fibrin hematoma to incite an inflammatory phase to recruit immune cells and mesenchymal stem cells to the fracture site in order for secondary fracture healing to occur [59-60]. Without inflammation, secondary fracture healing could not occur.

The rest of this dissertation will focus on fracture healing under altered inflammatory conditions, where the immune response is changed with abnormal inflammatory signaling. The altered inflammatory environment may arise due to inherent disruptions in metabolic and cellular processes, such as diabetes, or as a mechanism of defense, as due to infection. Therefore, studying
strategies to prevent infection and improve fracture healing in the setting of diabetes will, hopefully, lead to improved outcomes in fracture care.

IV. Infection and osteomyelitis: mechanisms and the inflammatory response

Musculoskeletal infection – osteomyelitis and septic arthritis – is a major source of morbidity among orthopaedic patients. This section will focus on osteomyelitis, infection of bone, and briefly discuss infection mechanisms and the inflammatory response as related to findings in this dissertation.

Osteomyelitis can occur via hematogenous spread, when bacteria from a distant site travels through the blood and seeds in bone, or contiguous spread, when bacteria is inoculated from contact with the outside environment, through surgical procedure or trauma [61]. Staphylococcal infections, including Staph. aureus Staph. epidermidis, are most common [62-63]. Antibiotics and surgical debridement are gold-standard therapies, but osteomyelitis is difficult to treat with a high rate of treatment failure [64]. Osteomyelitis is characterized by formation of sequestrum, an area of necrotic bone colonized by bacteria along canaliculi [65]. Staphylococcal bacteria also are difficult to eradicate due to ability to cause intracellular infection thus evading immune cells, as well as formation of biofilms that form a thick, protective physical barrier around bacteria (figure 6) [66]. Many antibiotics, such as vancomycin, are ineffective against biofilms and intracellular Staph [66].
Figure 6. Schematic of mechanisms of chronic *Staphylococcus* osteomyelitis.

*Staphylococcus* infections account for the majority of orthopaedic infection and can seed bone via hematogenous spread or direct inoculation. Biofilm formation on bone and orthopaedic implants, as well as intracellular infection, allow Staph to evade host immune defenses. *Chronic osteomyelitis* is characterized by sequestrum, an area of devitalized bone colonized by bacteria. Successful treatment typically requires aggressive sequestrum debridement and antibiotic therapy.

Innate immune mechanisms provide a non-specific, first line of defense against infectious organisms, such as *Staph* in osteomyelitis. These innate immune mechanisms also function against non-microbial insults, such as implant wear nanoparticles, making the diagnostic distinction
between infectious and non-infectious inflammation difficult [67]. These inflammatory pathways act as primary means of defense, but also can promote destruction of host tissues [68]. There are various components to this defense including mechanical barriers, cellular defenses, and chemical defense. Mechanical barriers include skin, mucus, and cilia that provide physical separation of the host from the outside environment. Cellular components include macrophages and neutrophils. Monocytes, which share stem cell lineage with osteoclasts, are also involved in the innate immune response [69]. A host of proteins and chemical factors, such as cytokines and chemokines, also play an important role in response to infection.

The first step of bone invasion by *Staph* involves adhesins that bind to the bone extracellular matrix components and facilitating uptake into osteoblasts via endosomes [70-71]. A variety of toxins and proteins facilitate the escape of *Staph* from the endosome [72-75]. Bacterial recognition occurs by pattern recognition receptors (PRRs), expressed by osteoblasts, osteoclasts, and precursor cells [76]. Nod proteins and Toll-like receptors are the best studied PRRs. They recognize bacterial components called pathogen-associated molecular patterns (PAMPs), such as cell wall molecules including lipopeptides, peptidoglycan, lipoteichoic acid [77-79]. Specific receptors have specific signaling properties that result in a variety of downstream inflammatory effects. For example, TLR9 functions within the osteoblast endosome to recognize bacterial DNA, and downstream signaling results in oxidative damage to induce bacterial killing [80]. TLR2, on the other hand, recognizes peptidoglycan and lipoteichoic acid in osteoblasts, and downstream effects include release of antimicrobial peptides and cell death [79].

While different TLR and Nod receptors may have distinct downstream effects, there are common inflammatory signaling pathways of the innate immune system that mediate host defense. A crucial pathway is interleukin (IL) 1 signaling, which induces neutrophilic response. Other
common inflammatory mediators, including TNF-alpha and IL-6, promote osteoclast differentiation and function, via upregulation of RANK-L [81]. IL-1 is well-known for its negative effects on bone and was initially named osteoclast activating factor [82]. Other downstream effects include NF-KB activation and the MAP-kinase pathway, which mediate a broad array of cytokines and chemokines involved in the immune defense and bone destruction [83].

While an in-depth discussion of inflammatory pathways involved in Staphylococcal infection is out of the scope of this dissertation, these inflammatory pathways, especially PAMP recognition and IL-1 and NF-KB signaling, have importance in the findings of this dissertation. Please refer to the results (page 75) and discussion (page 97) sections for further discussion and their relevance to infection and inflammatory fracture healing.

V. Consequences of infected fracture: case presentation and treatment approaches

Infection is a devastating complication following fracture, posing a significant burden on patients, including increased time and cost of treatment and decreased quality of life. The following case illustrates the morbidity associated with infection, highlighting the possibility of loss of limb. In this case, infection occurred in the setting of poor bone quality and repeated fracture secondary to radiation therapy. First, the case will be presented. Second, the case will be used to comment on management of osteomyelitis and infected nonunion.

This case is illustrative of how diminished bone quality (in this case, due to radiation therapy for soft tissue sarcoma treatment) resulted in significant morbidity for the patient, and ultimately required thigh resection and limb salvage using a rotational tibio-pelvic constrained hip

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4 Based on SC et al, “Rotational tibio-pelvic constrained hip arthroplasty: a surgical technique and case report,” JBJS Case Connector 2019; 9(4):E0404. The author acknowledges Kristin Yu, BS, for her assistance in editing and revising the text, and Christopher Dussik, BS, for his assistance in obtaining and formatting the photographs represented in the figures. Case report and images used with permission from the patient.
arthroplasty. Radiation therapy can lead to the development of chronic infection [84]. Osteonecrosis can also occur in the setting of radiation therapy, and is characterized by hypoxia, hypovascularity, tissue breakdown, and disrupted wound healing [85]. These mechanisms are similar to hypovascularity and disrupted wound healing seen in diabetes mellitus. As seen in our patient, these conditions allow for chronic infection and increase the risk of pathologic fracture [86].

The patient in this case gave consent to present and publish this case report.

A twenty-five-year-old male presented with pain, exposed bone cement, and purulent discharge through a massive soft tissue defect of the right thigh. Five years prior to presentation, the patient had been diagnosed with multi-compartmental epithelioid sarcoma of the proximal right thigh with metastasis to the lungs. He had no other prior medical conditions. He was initially treated with wide tumor resection and reconstruction using an intercalary allograft bone, as well as radiation to the right thigh and chemotherapy. Metastatic lung lesions were treated with chemotherapy. Although epithelioid sarcoma is known to be fatal, the disease had been stable following primary sarcoma resection. His initial post-operative course was uneventful until one year later, when he developed delayed infection caused by methicillin-sensitive *Staphylococcus aureus* (MSSA) leading to bone resorption, fracture, and mechanical instability. This required several revision surgeries using an antibiotic-loaded cement spacer, nails, and implants. After a blunt trauma over the operated thigh, he developed a massive soft tissue defect through which the antibiotic-loaded cement was exposed (Figure 7A).
**Figure 7. Right thigh before and after femur resection.** (A) *Photograph demonstrating patient’s condition on presentation, with massive soft tissue defect and wound dehiscence with exposed bone cement. The entire right femur was resected along with non-viable tissue, leaving the sciatic nerve and femoral vessels intact* (B). *Photographs by Dr. Lee.*

After discussion of the risks and benefits of available surgical options, the patient decided to undergo right femur resection with rotational arthroplasty in attempt to debride non-viable, infected tissue while avoiding hip disarticulation. A major concern was that absence of ligaments and muscle attachments around the reconstructed hip joint that would most likely lead to hip
dislocations. Therefore, we decided to also perform a constrained hip arthroplasty. During the procedure, the proximal femur bone quality was extremely poor, and it was evident that the hip joint could not be preserved. We resected the entire right femur, leaving the sciatic nerve and femoral vessels intact (Figure 7B). Pathology confirmed the presence of osteomyelitis of the proximal femur, including femoral head and neck, without evidence of recurrent tumor. Cultures confirmed the continued presence of MSSA, consistent with previous cultures.

Next, we rotated the remaining limb externally 180°. We then performed a constrained, cemented hip arthroplasty, placing the femoral component stem into the externally rotated proximal tibia (Figures 8). Although porous ingrowth would have likely led to an acceptable result, cement was used for fixation due to history of lower extremity radiation and infection. Additionally, we were able to achieve good positioning of the stem in the tibia without any mantle defects, allowing for optimized cement fixation. Antibiotic-containing cement was not used.

Following rotational arthroplasty, we sutured the remaining hip abductor muscles and hamstring muscles to the knee joint capsule and patellar ligament distally to function as an abductor. We also sutured the gastrocnemius muscles to the rectus muscle as a hip flexor. The patella was removed, as it caused a pressure point at the hip.

Post-operatively, the patient achieved good wound healing. Over 18 months of follow-up, he was maintained on antibiotic therapy, and there were no signs of recurrent infection with normal laboratory studies. Radiographs have shown no evidence of component loosening or implant failure (Figure 8). While the patient will continue indefinitely on antibiotic therapy, we will consider stopping antibiotics only after carefully weighing the risks with the patient. We will
pursue a full workup including joint aspiration if pain or other symptoms concerning for recurrent infection develop.

The patient was fitted with a prosthetic device 3 weeks post-operatively that is placed over his right foot, with the rotated ankle functioning as the knee joint. He underwent a course of physical therapy to strengthen the right gastrocnemius, which functions as the extensor for the prosthesis. On physical exam, he exhibits good flexion, extension, and abduction of the reconstructed limb and the new functional knee. With the prosthesis in place, there is no external deformity and leg lengths are equal. The ankle joint is at the level of contralateral knee joint. (Figure 8). He ambulates very comfortably without a walking aid and can hop up and down. The patient can also run, although it does not feel entirely normal, owing to femur resection. The patient remains cancer-free seven years after his initial diagnosis.
Figure 8: Follow-up radiographs. Antero-posterior radiographs one year following rotational tibio-pelvic hip arthroplasty. (A) shows well-positioned acetabular and femoral prosthesis components with weight bearing, without evidence of loosening or failure. (B) is a leg-length study demonstrating orientation of the reconstructed right hip with tibial rotationplasty. The right ankle, well-aligned with the left knee, acts as function knee joint with leg prosthesis in place. The gastrocnemius and soleus act as the leg extensor mechanism. Functional leg lengths are equal with the prosthesis in place.
Case notes and treatment of osteomyelitis and infected nonunion.

Standard treatments for osteomyelitis include wide surgical debridement and intravenous antibiotics. However, these measures are often ineffective, as demonstrated in this case [87]. Large tissue resection of necrotic tumor tissue is complicated by postoperative infection in 20% of cases, and the rate of infection associated with tumor prosthesis ranges from 8-15% [88].

Hip disarticulation is an accepted option for chronic infection of the proximal thigh [88-89]. As in our patient case, poor soft tissue integrity is a significant risk factor for failed limb salvage [88]. Since recurrent infection of tumor prostheses often results in extended hospitalization and high amputation, repeated revision surgery is discouraged [88].

Devastating for a patient’s quality of life, proximal lower extremity amputation both significantly limits mobility and is associated with negative psychosocial effects [89-91]. Although prostheses for proximal lower extremity amputations are available, the majority of patients following hip disarticulation reject these devices due to awkwardness or intolerance [90-93]. The function of our patient’s prosthesis allows the patient to walk, run, and hop with daily use is superior to the likely functional outcome had we proceeded to hip disarticulation. Nevertheless, studies have determined that increased energy expenditure due to hip disarticulation prostheses is not excessive [94]. Little difference in energy consumption parameters, including oxygen uptake and consumption, has been identified between patients with prostheses for hip disarticulation and those who have undergone rotationplasty [95]. However, it was determined that rotationplasty does allow for greater walking speed [95].

Winkelmann conducted a case study in 1986 employing his modification of the Van Nes procedure for proximal tumors of the femur and similarly found excellent functional results [96].
While these patients were treated for malignancy and not chronic infection as in our case, Winkelmann reported no recurrence of primary tumor or pulmonary metastasis at two years follow-up. Following femur resection, our patient has had no evidence of recurrent infection for over eighteen months of follow-up.

VI. Open fracture: minimizing infection risk with systemic and local strategies

While the previous case illustrates the challenges surrounding treating chronic osteomyelitis, prevention strategies are at the forefront of orthopaedic research. In the trauma setting, open fracture poses a crucial area for infection prevention, as wound contamination and tissue disruption result in a high risk of post-traumatic, contiguous infection.

Infection rates following open fracture have been reported to be as high as 13.9%, which decreases to 2.3% with antibiotic treatment [97]. Infection risk largely depends on the degree of soft tissue disruption [98]. Timely administration is the most important factor in improving the efficacy of systemic antibiotics [98-99]. While the global infection rate is low following open fracture, diabetics, smokers, and patients with high-grade injuries are at increased risk of fracture site infection [100-101].

*Staphylococcus* is the most common infectious agent following infection, with MRSA being particularly difficult to eradicate [102]. As discussed previously, intracellular infection and biofilm formation are major mechanisms by which *Staphylococcal* infections become established. Unlike vancomycin, which is typically used to treat MRSA infection, rifampin is effective against biofilms and can target intracellular *Staphylococcus* [103-106].

While early administration of systemic antibiotics is crucial for infection prevention in for open fracture, local strategies have been implemented to prevent and contain infection. These
strategies are particularly important when vascular supply to the injury site is poor, as in the case above or in diabetes. Local infection prevention strategies have become common in addition to systemic antibiotics, fixation, irrigation and debridement [107]. Antibiotic-loaded cement was first advocated for in the 1970s [108-109]. Antibiotic-loaded PMMA beads are widely used in high-grade, open injuries [110]. Antibiotic powders have also proven effective in reducing infection in animal and clinical studies [111-113].

Hydrogels, typically comprised of a cross-linked collagen network, have recently been investigated as a local antibiotic delivery option and have several distinct advantages over powders and PMMA. Hydrogels are injectable, do not require full wound exposure, and release antibiotics over time [114]. Hydrogels are biodegradable and do not require retrieval, as opposed to PMMA. This dissertation investigates the use of hydrogel as an antibiotic delivery mechanism in a mouse open fracture model, as well as for local application of teriparatide.

VII. Diabetes is a pro-inflammatory condition that increases fracture risk

Diabetes mellitus (DM) is a major health care problem. Hyperglycemia, the hallmark of DM, can lead to cardiovascular, nerve, eye, and kidney damage [115]. DM also damages the musculoskeletal system by altering bone metabolism, increasing fracture risk, and impairing fracture healing [116-119].

In 2015, the CDC estimated that over 9% of the US population, or 30.3 million people, had diabetes [120]. DM can be classified as type 1 (T1DM) or type 2 (T2DM), with T2DM accounting

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5 This section and the following section (Diabetic fracture healing) are based on Henderson et al, “Bone quality and fracture healing in type 1 and type 2 diabetes mellitus,” JBJS 2019; 101(15):1399-1410. Dr. Henderson provided text outline and select references. Dr. Ibe participated in the initial draft of the text and editing. The author performed comprehensive literature review, original writing, and all revisions (based on reviewer feedback) of the text represented here, with guidance from co-authors, unless explicitly stated. Figures and tables presented in this section are original and were prepared entirely by the author.
for 90-95% of cases [116,119]. T1DM results from an inability to produce insulin due to autoimmune destruction of pancreatic beta cells [115]. Decreased insulin sensitivity and β-cell dysfunction are characteristic of T2DM [121]. While genetic factors play a role, obesity is considered a critical factor in T2DM pathogenesis [121]. The mouse models used in this work are of T2DM.

Patients with diabetes have a higher risk for vertebral and non-vertebral fracture when compared to nondiabetic counterparts. People with T1DM have been found to have relative risk of fractures of nearly 7%, while those with T2DM have a lower, but still elevated RR of 1.4% [123-127].

Falls are a particular concern in DM, especially among the elderly. Diabetes is associated with impaired physical function that increases fall risk such as peripheral neuropathy, poor balance, and heart disease [128]. Among elderly women, diabetes was associated with poorer physical performance parameters, including one leg standing and get up and go tests [129]. Other potential contributing factors to increased fall risk include hypoglycemic episodes, neuropathy, retinopathy and neuromuscular impairments [123,130].

A common consequence of T1DM is bone loss that can progress to osteoporosis [131]. Young, pre-menopausal women may present with reduced bone mass upon clinical diagnosis of T1DM, with the incidence of osteoporosis in T1DM patients being 20% [131-132]. Although patients with T2DM are also at increased risk of fracture, they often demonstrate normal or increased bone density (T2D, spine 0.41 ± 0.01; hip 0.27 ± 0.01), albeit with lower quality bone [127,133-135]. It is theorized that this increased bone density in T2DM may result from obesity and increased weight placed on an individual’s bones in the context of decreased bone turnover [118].
Fracture risk assessment can be challenging for patients with DM. Bone mineral density (BMD), the trabecular bone score (TBS), and the Fracture Risk Algorithm (FRAX) score are used to identify patients at higher risk for fracture [136]. Prospective studies have demonstrated that BMD can be useful to assess fracture risk in older patients with diabetes, although BMD underestimates fracture risk in diabetic patients compared to non-diabetic controls [137]. The FRAX score has also been previously shown to underestimate fracture risk among diabetic patients [138], although a more recent version of the tool now includes additional criteria that includes diabetes. TBS, a measure of bone microarchitecture, can be used in conjunction with BMD and FRAX scoring. Lower TBS scores, which predict fracture risk in osteoporosis, reflect low trabecular connections and increased space in the trabecular network [139]. TBS has been shown to be decreased in patients with secondary osteoporosis, including T1DM (1.309 ± 0.125 vs. 1.370 ± 0.127, \( P = 0.04 \)) and T2DM (1.228 ± 0.140 vs. 1.298 ± 0.132, \( p = 0.013 \)) [125-126,140]. Recent prospective data demonstrates that FRAX, or FRAX adjusted with TBS, is more highly correlated to vertebral fracture risk among postmenopausal diabetic patients compared to BMD alone and is favorable due to its low cost and wide availability [139,141].

**Diabetic Bone Biology**

The hallmark of diabetic bone disease is decreased bone turnover and functionally weaker bone, contributing to increased fracture risk [142-143]. This occurs via a combination of mechanisms that alter bone metabolism by disrupting normal balance between osteoclasts and osteoblasts, which is normally regulated by mesenchymal precursor differentiation, local growth factors, and mechanical stress as discussed previously in this introduction (Figure 1). Limitation of osteoblastic differentiation in combination with an increased adipogenesis in the bone marrow
leads to micro architectural changes [118]. Additionally, decreased RANKL expression (65–70%) observed in diabetes limits bone turnover [124].

**Figure 9:** Effects of hyperglycemia and advanced glycation end products on diabetic bone quality. Accumulation of advanced glycation end products (AGES) occurs as the result of non-enzymatic coupling of glucose to lipids and proteins. AGES bind to the receptor for AGES (RAGE), resulting in downstream activation of the MAPK/NF-κB pathway; overexpression of cytokines including TNFα, IL-1β, IL-6, and reactive oxygen species; decreased osteoblast
differentiation and increased osteoblast apoptosis; and increased osteoclast activity. AGES also act directly on osteocytes to increase sclerostin expression, a potent inhibitor of Wnt and osteoblast differentiation. AGES directly disrupt the collagen and mineral matrix, resulting in weaker, more brittle bone. Finally, AGES are the underlying cause of microvascular complications such as retinopathy and neuropathy, which can contribute to overall fall risk.

AGEs – Advanced glycation end products; RAGE – Receptor of advanced glycation end products; ROS – Reactive oxygen species; SOST – Sclerostin.

Accumulation of advanced glycation end products (AGEs) disrupts bone cellular biology and microarchitecture (figure 9). AGEs, which are produced as a result of the patient’s hyperglycemic state, are formed when excess circulating glucose undergoes non-enzymatic reactions with various proteins and lipids [118,123]. Clinical studies have demonstrated correlations between AGEs and increased fracture risk [144-145]. AGEs accumulation and non-enzymatic glycosylation in bone directly disrupts the bone extracellular matrix by causing excessive cross-linking of type I collagen fibrils, increasing bone stiffness and fragility and diminishing its biomechanical properties [146].

DM is a pro-inflammatory state mediated by AGE accumulation and binding to the receptor for advanced glycation end products (RAGE) [147-148]. RAGE activation trigger mitogen-activated protein (MAP) kinase signaling cascade which leads to the activation of NF-kB [149]. RAGE promotes the expression of proinflammatory genes including TNF-α, IL-1β, IL-6, and reactive oxygen species [150], which results in a vicious cycle of chronic inflammation and bone resorption [151]. AGE-mediated activation of RAGE stimulates osteoclasts, which is involved in

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6 The author acknowledges the assistance of Dr. Yeon-Ho Cheung in revising this paragraph.
diabetic bone loss [152-154]. On the other hand, in vitro resorption assay show that AGEs alter the structural integrity of bone matrix proteins and inhibit osteoclastogenesis, resulting in decreased osteoclastic bone resorption [155]. RAGE signaling also induces osteoblast apoptosis via the MAP kinase and cytosolic apoptotic pathways [156] and limits the proliferation of mesenchymal stem cells and differentiation into osteoblasts [157]. The endogenous secretory receptor for advanced glycation end products (esRAGE), which blocks RAGE, has been demonstrated to be clinically associated with lower risk of fracture [158].

Importantly, the Wnt signaling pathway is also disrupted by DM. Wnt signaling regulates bone metabolism by increasing proliferation and differentiation of osteoprogenitor cells and decreases osteoblast apoptosis [159]. Osteocytes are major regulators of the normal balance between osteocytes and osteoclasts, and release molecular regulators including sclerostin, a potent Wnt inhibitor. Sclerostin suppress bone formation through binds osteoblast surface receptors and inhibits the Wnt/β-catenin pathway via downstream effects [160]. Sclerostin is upregulated in diabetes, due to the effect of hyperglycemia and AGEs on osteocytes [161-164]. Increased sclerostin levels have been demonstrated in both T1DM (1.18-fold) and T2DM (1.3-fold) [161-163]. Furthermore, a 2013 study demonstrated that sclerostin levels in patients with T2DM have been directly associated with increased fracture risk [165]. Sclerostin has also been implicated as a factor contributing to insulin resistance in addition to its role abnormal bone biology [161,166].

**Clinical management of diabetic patients to optimize bone health and reduce fracture risk**

The following sub-section will discuss current evidence regarding management of diabetic patients with a focus on musculoskeletal health.

*Lifestyle alterations and vitamin supplementation*
Considering the increased fracture risk among patients with T1DM and T1DM, bone health should be optimized for all diabetics. Diabetic patients, especially post-menopausal women and older men who are already at risk for osteoporosis, should be provided vitamin D and calcium supplementation. In addition to being standard of care for osteoporosis, vitamin D supplementation has been shown to decrease symptoms of diabetic neuropathy [167-168]. Weight bearing exercise should be encouraged for all diabetic patients regardless of age, in order to improve bone health in addition to improving insulin sensitivity. Fall risk assessment should be considered, especially among elderly diabetic patients. Regular eye exams, which can reduce the risk of visual impairment and therefore reduce fracture risk, should also be standard of care among diabetic patients [169].

Osteoporosis and Antiresorptive Therapy

Diabetic patients who are diagnosed with osteoporosis should be given standard anti-osteoporotic therapies as non-diabetics would receive. There is strong evidence showing bisphosphonates and raloxifene are as effective in diabetic compared to non-diabetic patients with osteoporosis in clinical trials and retrospective studies [170-173]. Animal models have also demonstrated the efficacy of bisphosphonates and SERMs in treating bone loss in the context of diabetes and osteoporosis [174].

Glycemic Control

HgA1C targets may vary patient to patient in the management of T2 DM, while intensive glycemic control is standard of care in T1DM [175]. While poor glycemic control has been associated with increased fracture risk in T1DM [176-177], several studies state that there is little, if any, correlation with glycemic control in T2DM [176]. According to the ACCORD randomized trial, over 7,000 older adults with T2DM randomized to intensive or standard
glycemic control groups (median A1C 6.4 and 7.5%, respectively), had no difference in in fracture and fall outcomes, with an average of 1.3-year follow-up [178]. Patients with T2DM should be managed with at least standard glycemic control to reduce the long-term risk of microvascular complications and promote bone health.

VIII. Diabetic fracture healing and the need for new treatment approaches

Mechanisms of impaired fracture healing in diabetes

In addition to an increased risk of fracture, diabetic patients’ bone healing is impaired (figure 10), with a higher rate of delayed union and nonunion than nondiabetic patients [123,179]. Normal fracture healing is a process dependent on complex interactions of cells and signaling molecules, with stability a requirement, as discussed previously in this introduction (figure 5).

In diabetes, fracture healing is impaired due to decreased fracture stability, altered bone metabolism, and an abnormal inflammatory response (figure 10). Stability is at risk, as comorbid obesity and abnormal joint loading can reduce stability of the diabetic fracture site, especially among lower extremity fractures. Diabetic peripheral neuropathy may also contribute to poor protective sensation following injury and physiologically inappropriate weight bearing.

Diabetes is marked by increased adipogenesis that leads mesenchymal stem cells to preferentially differentiate into adipocytes rather than osteocytes [123,180]. The increased fatty tissue at the site of the fracture limits the fracture callus and the healing process.
Figure 10: Mechanisms of impaired fracture healing in diabetes mellitus.

Fracture healing is marked by the inflammatory, soft callus, hard callus, and remodeling phases. Endochondral ossification facilitates this process. Diabetes disrupts multiple steps of this process. Global factors such as poor vascular supply, peripheral neuropathy, obesity, and abnormal joint loading affect multiple steps of the process and may decrease fracture stability. Decreased growth factor production, including VEGF, BMP2, TGF-β, and PDGF limit angiogenesis and cellular proliferation. Increased inflammation, mediated by TNF-α, leads to chondrocyte apoptosis and...
decreased callus size. The number of mesenchymal precursor cells (MPC) is decreased, and MPCs demonstrate preferential adipogenesis in diabetes. Remodeling is impaired due to altered bone biology, including AGE-induced reduction in osteoblast activity and increased sclerostin production. Apop – apoptosis, MPC – mesenchymal precursor cell, Ages – Advanced glycation end products.

A rich blood supply is also necessary to facilitate fracture healing. The negative effects of diabetes on the microvasculature cannot be overlooked, as the hallmark of end-stage organ damage in diabetes is microvascular compromise [115]. Insulin resistance in T2DM is directly related to microvascular complications [181], and microvascular changes are also seen early in children and adolescents with T1DM [182]. Vascular calcification and smooth muscle cell apoptosis underlie diabetic vascular disease, and in vitro studies have demonstrated the significance of AGEs and RAGEs in these processes [183-184]. The diminished blood supply impedes the perfusion and distribution of important nutrients that encourage fracture healing [185].

Overexpression of TNF-α, an inflammatory mediator, is an often-reported cause of impaired diabetic fracture healing [186-187]. TNF-α triggers chondrocytes apoptosis by upregulating the expression of apoptotic genes and reduces proliferation of mesenchymal stem cells through activation of FOXO1 [188-192]. TNF-α mRNA levels were 3.7-fold and osteoclast numbers were 2.5-fold higher in the diabetic fracture than the normoglycemic animals, leading to premature resorption of cartilage. Furthermore, TNF-α contributes to reduced angiogenesis and VEGFA expression in endochondral bone formation. Inhibition of TNF-α or treatment of insulin rescued the negative effect of diabetes on cartilage loss, increased osteoclastogenesis and impaired

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7 This and the following paragraph were co-written with Yeon-Ho Cheung, PhD.
angiogenesis [190-192]. TNF-α also contributes to reduced angiogenesis and VEGFA expression in endochondral bone formation. These negative effects of TNF-α ultimately lead to a decrease in callus size, delayed endochondral ossification, and bone formation. In animal models of DM, inhibition of TNF-α or treatment with insulin rescued the negative effect of diabetes on cartilage loss [179,191].

Altered expression patterns of other chemokines and other inflammatory mediators in DM have been reported. These include bone morphogenic protein – 2 (BMP2), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), transforming growth factor beta - 1 (TGF-Beta1), Monocyte chemotactic protein (MCP) 1, and chemokine c-x-c motif ligand -13 (CXCL 13) [193-196]. Important transcription factors like core binding factor alpha 1/runt related transcription factor 2 (Cbfa1/RUNX2) and distal-less homeobox 5 (Dlx5) are also decreased [117,180].

Management of Diabetic Fracture

Diabetes leads to a high risk of complications following fracture including nonunion, malunion, infection, and poor wound healing [119,197-198]. Delayed fracture healing and nonunion are important orthopaedic problems, and this patient population often requires more complex treatment and management than non-diabetic patients [199-200]. Unfortunately, most interventions to improve fracture healing lack strong, high-quality evidence among normal and diabetic patients. This section outlines potential strategies for enhancing diabetic fracture healing, with glycemic control having the most evidence for clinical practice. Table 1 summarizes these strategies. Future research for experimental interventions is needed, and this is the impetus of a large part of this dissertation.
Table 1: Grades of recommendation for proposed therapies to improve fracture healing in diabetes. While several interventions have been proposed to improve diabetic fracture healing, most have little to no quality evidence to support them. A=good, level I studies with consistent findings; B=fair, level II or III studies with consistent finding; C=conflicting or poor-quality evidence (level IV or V studies); I = investigational/animal studies only.

**Glycemic Control**

Poor glycemic control has been shown to be a significant predictive factor for contributing to poor fracture healing. Clinical studies have demonstrated that hemoglobin A1c levels A1C ≥ 6.5% were correlated with poorer radiographic outcomes and increased risk of complications and revision [201]. Studies in animal models of type I and type II diabetic fractures demonstrate significant improvement in biomechanical properties and bone formation between diabetic rats treated with insulin to those untreated [202-205]. The anabolic effect of local insulin is facilitated by increased osteogenesis, angiogenesis, and callus formation, and suggests insulin’s direct, beneficial effect on fracture healing in addition to contributing to glycemic control [206]. It should
be noted that there is little prospective clinical data on whether improving glycemic control at the time of fracture can improve fracture healing in long-standing diabetes. Nevertheless, glycemic control should be the cornerstone of management for all diabetic patients.

Furthermore, glycemic control has been shown to reduce infection risk in both diabetic and non-diabetic patients. Several randomized control trials have demonstrated that perioperative glycemic control reduces surgical site infection rates [207-211]. Although various protocols have been used, these trials have maintained patients with target random blood glucose levels less than 150 mg/dl, using subcutaneous regular insulin or regular insulin infusion. Retrospective studies have identified perioperative glucose control to be predictive of increased surgical site infection risk in the orthopaedic setting [212]. HbA1c also correlates with surgical infection risk [212], with one study demonstrated that for every 1% HbA1c increase, the odds of surgical infection increased by a factor of 1.59 (95% CI 1.26-1.99) [213]. Currently, however, there is no consensus on the target preoperative HbA1c levels among diabetic patients for preventing surgical site infection.

*Teriparatide and Parathyroid Hormone*

Teriparatide is a peptide comprised of the active portion of parathyroid hormone (PTH). PTH is a regulator of calcium and phosphate homeostasis. Teriparatide acts as an anabolic agent by stimulating pre-osteoblasts to differentiate and proliferate. Conditions of chronically-elevated levels of PTH are associated with hypercalcemia, bone resorption, and phosphate wasting due to upregulated osteoclast activation in bone and PTH activity on the kidney (*Figure 11*). Intermittent PTH exposure, however, has selective anabolic properties without excessive osteoclast activation.
Although not first-line therapy for osteoporosis, teriparatide has been shown to reduce the risk of vertebral and nonvertebral fractures and increase BMD in postmenopausal women in randomized, clinical trials [214-215]. Teriparatide administration has also been demonstrated to accelerate fracture healing in postmenopausal women in some clinical trials [216-217], whereas others have showed no benefit [218]. Non-diabetic animal models have demonstrated improved fracture healing with teriparatide [219]. However, clinical evidence of teriparatide use among diabetic patients is lacking. Animal studies evaluating local and intermittent systemic parathyroid hormone administration in diabetic models have shown an improvement in the fracture healing response.
In addition to improving fracture healing, intermittent teriparatide therapy has also been shown to partially reverse bone mineralization and trabecular defects in T2DM animal models without influencing blood glucose control. However, the benefits of intermittent teriparatide were greater in non-diabetic animals compared to the T2DM phenotype, suggesting relative resistance to PTH in diabetes [221].

**Bone Stimulation**

Bone stimulation exposes bone to electrical, electromagnetic, or ultrasound energy to accelerate fracture healing [222]. Evidence is mixed as to whether these strategies improve union in operatively and non-operatively managed fractures in non-diabetic patients [223-226]. Data is especially lacking for use of bone stimulation in the diabetic population. Small, prospective studies have demonstrated potential benefit among diabetic patients in foot and ankle surgery [227] and treatment of Charcot foot [228], and studies in diabetic animal models demonstrated improved bone anabolism under surgical implant and bone defect conditions [229-230].

**Experimental approaches**

Other approaches to improving fracture healing have been investigated and may become more prevalent in the future management of diabetic fractures as more clinical evidence is gathered. Studies in animals and humans have demonstrated that antibodies against sclerostin decrease bone resorption and increase new bone formation in nondiabetic (26% at LV and 17% at femur–tibia) [231-232] and diabetic conditions [233]. There is evidence demonstrating that local insulin therapy improves fracture healing and bone regeneration in non-diabetic [234-235] and diabetic [206] animal models. Direct administration of the deficient factors like BMP, CXCL13, human platelet-derived growth factor and recombinant human bone morphogenic protein-2 have been shown to improve bone healing in *in vitro* diabetic animal models [236-239].
approach has been the inhibition of factors like tumor necrosis factor alpha, which can enhance MSC numbers and prevent MSC apoptosis [189].

IX. The role of murine models to study fracture healing and musculoskeletal disease

There are many biological systems in which fracture healing has been studied, including biopsy sampling of human specimens; in vitro studies including progenitor cells, immune cells, and osteoblasts; small animal models, including mice and rats; and larger animal models such as rabbits, dogs, and horses. All have advantages and disadvantages.

Mice are the most common small animal model in all biomedical research. They are readily obtained, breed and mature quickly, and are relatively cheap to house, feed, and maintain owing to their small size. Mice also are available in a huge array of strains – the Jackson Laboratory, one of the main providers of laboratory mice, report more than 7,000 different mouse strains available for purchase. Certain strain-dependent characteristics are desirable in bone and fracture healing studies are necessary to model musculoskeletal conditions in certain diseases. For example, nude/athymic mice, which lack T cells, are widely used in studies modeling metastatic bone cancer, in which tumor cells are directly implanted into bone. A variety of mouse strains with genetically-acquired diabetes, such as DB/DB (leptin receptor deficient) and Tallyho (multigenic) strains, have been used to study diabetic bone disease. Additionally, transgenic and knockout mice can be obtained with relative ease for studying specific molecular pathways that may affect fracture healing, allowing for hypothesis testing of genetic or molecular pathways.
Purpose and Specific Aims

The overarching purpose of this dissertation is to use animal models of inflammatory fracture healing, including infection and diabetes, to identify new therapeutic strategies for promoting bone healing. Although the aims of this dissertation are broad, the ultimate purpose is to provide a basis of animal studies on which future clinical studies can be undertaken to implement these strategies to improve patient outcomes.

Throughout the course of the work on these investigations into inflammatory fracture healing, more questions have arisen than have been answered. Efforts have been undertaken to begin to attempt to address them, which have been included within this dissertation but are by no means complete. A description of ongoing investigation and plans for future investigation are provided at the conclusion of this work.

Primary research questions of this project are:

1) Can a locally-applied, antibiotic-containing hydrogel reduce bacterial load and improve fracture healing in the setting of MRSA infected fracture?

2) What cellular and molecular changes occur that inhibit fracture healing in the setting of Staphylococcal infection?

3) Can a high-fat, high-sugar diet be used to model diabetic fracture healing and the propensity for infection in mice?

4) Can locally-applied teriparatide, via hydrogel delivery, improve fracture healing in diabetic mice? How does it compare to systemic, intermittent administration?

5) Can systemically-delivered teriparatide improve fracture healing in cases of acute post-traumatic infection?
6) What accounts for the observation that fracture healing in diabetic mice is improved with MRSA infection and treatment with vancomycin/rifampin, compared to sterile fracture?
Methods

The following section details the methods used throughout this dissertation. Since many of the methods (animal care, surgical model, histology preparation, etc.) are used among various components of this dissertation, the overall experimental designs of each component of the project are presented first, followed by detailed methods.

Summary of Experimental Designs

1) **Local antibiotic delivery to reduce bacterial load and improve fracture healing in a MRSA-infected open tibia fracture mouse model.**

   The overall experimental design is given in **Figure 12**. Normal c57 mice age 10-12 weeks were subjected to open tibia fracture surgery. Mice in the infection group were directly inoculated with MRSA at the time of surgery. Hydrogel treatment containing PBS only (control group) or rifampin were applied after a 10-minute incubation period during the surgical procedure. Mice were sacrificed at 3, 14, and 28 days. Colony-forming unit analysis was obtained after 3 days. Histology was performed at days 14 and 28. Radiographic analysis including x-ray and micro-computed tomography (micro-CT) were performed at day 28. Blood was collected for CBC and serum analysis at 7, 14, and 28 days.
2) Local and systemic teriparatide to improve fracture healing in diabetic mice.

Mice were kept on a high-fat, high sugar diet (diabetic model) or lean diet (control) for 6 months, with body weight, glucose tolerance test, and fasting blood glucose measured at various timepoints. Mice underwent sterile fracture surgery. Mice in the local treatment group had teriparatide-containing hydrogel applied directly to the fracture site. Mice were sacrificed at 14 and 28 days. Primary analysis included x-ray and micro-CT analysis at 28 days following surgery. Histology was obtained after 14 days; histologic results are pending at the time of dissertation submission.

3) Diabetic fracture healing under infected conditions: the effect of antibiotics and systemic teriparatide in a MRSA-infected, open tibia fracture mouse model.

Diabetic and control mice were used. Mice underwent sterile fracture surgery and those in the infection groups were inoculated with MRSA at the time of surgery. Mice were treated with

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9 The author acknowledges the assistance of Dr. Hyuk-Kwon Kwon in preparing this figure.
systemic vancomycin/rifampin combination (3 days), and systemic vancomycin/rifampin combination (3 days) plus teriparatide (once daily for 5 days per week over 28 days). Mice were sacrificed at 28 days. Primary analysis included x-ray and micro-CT analysis at 28 days following surgery.

**Detailed Methods**

**Animals**

Experiments were approved by the Yale University School of Medicine Institutional Animal Care and Use Committee. Male C57BL/6J mice aged 10-12 weeks were purchased from Jackson Laboratories (Bar Harbor, ME, USA) for use in infection/rifampin hydrogel experiment. Male C57BL/6J mice aged 4 weeks were purchased from Jackson Laboratories Animals for the diabetic model. Animals were housed at 22 ± 3°C on a 12-hour light/dark cycle in ventilated cages and were given food and water *ad libitum*. The author acknowledges the support from the Yale Animal Resource Center, especially Mark Chernyak, for their dedication to our animals’ well-being.

**Type 2 Diabetic Mouse Model and Metabolic Testing**

Male C57BL/6J mice aged 4 weeks were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Animals were housed at 22 ± 3°C on a 12-hour light/dark cycle in ventilated cages and were given food and water *ad libitum*. Type 2 diabetes was modeled by providing mice

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This work required efforts from a large investigational team in the Lee lab and the Department of Orthopaedics and Rehabilitation. Experiments were carried out by the author, unless noted in the text. The author would like to acknowledge Dr. Kwon and Dr. Back for their teaching in nearly all methods used in this investigation and for helping to facilitate all experiments. The author would like to thank Dr. Lee for providing resources and financial support for this work. The author would like to acknowledge Karem Alder, Inkyu Lee, and Saelim Lee for their assistance in carrying out animal experiments and their help as mouse surgery assistants.
with a high fat, high sugar diet (40% fat by weight with added sucrose), mimicking a Western diet. Wild-type, age-matched controls were provided standard chow. Mice underwent glucose tolerance testing after 3 months on the HFHS diet. Age-matched Tallyho mice were tested as positive controls. Mice were fasted overnight before the tolerance test. Baseline fasting blood glucose levels were measured with a commercially available glucometer using blood collected from the tail vein (maximum reading 600 mg/dL). During tail vein collection, efforts were made to minimize mouse stress, and anesthesia was not used. An intraperitoneal injection of 20% glucose solution was given, with a total glucose load of 2g/kg. Blood glucose levels were measured at 15, 30, 60, and 120 minutes following injection. Fasting blood glucose levels were measured at 6 months. Animals underwent surgery at 7-8 months. The author performed all metabolic testing.

Hydrogel Preparation

A 2% weight/volume collagen-based hydrogel was made using the HyStem™ hydrogel system (ESI BIO, Alameda, CA, USA) by reconstituting and combining Glycosil and Extralink-Lite per manufacturer’s instructions, at a 2:1 ratio. 10 µL of gel was allowed to polymerize for 20 minutes. An equal volume of phosphate buffered saline (PBS) containing rifampin of doses 0 µg (sterile fracture and MRSA infection), 60 µg (low-dose), and 300 µg (high dose) were added to the hydrogel. For the teriparatide hydrogel preparation, a dose of 40 µg/kg was loaded into the hydrogel in a 10 µL solution.

Surgical Open Fracture Model

The tibia fracture surgical procedure was based on previous fracture healing studies and were developed by the author for the purpose of these experiments [20-21]. The author (SC)
performed all surgeries. Anesthesia was achieved with ketamine (50 mg/kg) and xylazine (5 mg/kg). Depth was assessed by pedal withdrawal. Buprenorphine (0.1 mg/kg intraperitoneal) and bupivacaine (4 mg/kg subcutaneous) were given pre-emptively. Hair of the right leg was removed with depilatory cream and skin was disinfected with povidone-iodine and alcohol. An incision was made over the right tibia. The patella was lateralized and a 25-gauge needle was used to pre-drill a hole in the cortical bone of the proximal tibia shaft. A scalpel was used to create a transverse, non-comminuted, mid-shaft fracture just distal to the tibial prominence (figure 13). The fracture was stabilized with a 0.35mm insect pin inserted at the proximal tibia. Care was used to minimize bleeding and surrounding tissue damage. 5 μL of PBS containing $1 \times 10^6$ CFU MRSA (USA 300) was applied to the fracture site. The author acknowledges Dr. Kwon for handling and preparing the bacteria sample. An incubation period of 10 minutes was allowed. The fracture site was dried of excess moisture with sterile gauze, and hydrogel was then applied. The wound was closed using sutures.

Mice in systemic rifampin therapy groups were given 25 mg/kg subcutaneous injections, based on previous mouse studies investigating Staphylococcal implant and bone infection [251-253]. Mice were treated over a three-day period as per clinical guidelines [254-255], with one injection immediately following surgery and then 24 and 48 hours later.
Figure 13. Mouse MRSA-infected, open tibia fracture model. (a.) Skeletonized mouse lower extremity, demonstrating bony anatomy including the fracture site, tibial prominence, patella, fibula, and ankle joint. Skeleton was prepared by the author. Flesh was cleared with dermestid beetles and bones were bleached by soaking in 3% hydrogen peroxide for 12 hours. (b.) An incision was made over the anterior right tibia, and 0.35mm stainless steel insect pin was pre-inserted into the medullary canal. A transverse mid-shaft fracture was made and stabilized by advancing the pin. MRSA (USA 300) was directly applied to the fracture site and allowed to penetrate tissue for 10 minutes before applying hydrogel and closing with sutures. (c.) Post-operative day 1 radiograph demonstrating midshaft tibia fracture and placement of the intramedullary pin.
For mice receiving systemic intermittent teriparatide treatment, a subcutaneous injection containing 40 µg/kg was given immediately following surgery 5 days per week over 28 days, for a total of 20 injections.

Blood was obtained retro-orbitally at 7, 14, and 21 days. Complete blood counts were measured using VetScan HM5 (Abaxis, Union City, CA, USA).

Mice were euthanized via cardiopuncture under anesthesia at post-surgery day 3, 14, and 28. The author acknowledges Dr. Kwon, Inkyu Lee, Kareme Alder, and Saelim Lee for their assistance with surgical procedures and animal harvesting and specimen collection.

**Bacterial Colony-Forming Unit Analysis**

Tibiae were harvested in a sterile fashion after 3 days. Intramedullary pins were removed, placed in 1.00 mL PBS, and vortexed at medium speed for 10 minutes. 100uL of the resulting solution was plated onto MRSA-specific Mueller-Hinton agar (Sigma-Aldrich, St. Louis, MO, USA) containing oxycyclin (6 µg/mL; Sigma-Aldrich Co.). Tissue samples containing bone and soft tissue from the fracture site were isolated, manually homogenized, then incubated in 1.00 mL 0.25% collagenase solution (Stem Cell Technologies, Vancouver, BC, Canada) for 1 hour at 37°C. 1 × 10⁵ cells were plated and allowed to incubate for 36-48 hours at 32°C. Plates were imaged with ChemiDoc Touch (Bio Rad, Hercules, CA, USA). Colonies were counted using ImageJ software (NIH, Bethesda, MD, USA). The author acknowledges Hyuk-Kwon Kwon for assistance in carrying out these experiments.

**Radiographic and Histologic Analysis**
Anterior-posterior (AP) and lateral radiographs were obtained by the author using *In-Vivo* Multispectral FX Pro (Bruker, Madison, WI, USA) after 28 days. Healing was assessed by the radiographic union scale in tibia fractures score (RUST score, Table 1) by two individual researchers (The Author, Kwon) [256-257]. A score of ≥10 was considered radiographic union. Tissues were randomly assigned to histologic analysis or μCT, with additional histology tissues harvested at 14 days.

**RUST Scoring System**

<table>
<thead>
<tr>
<th>Score (per cortex)</th>
<th>Radiographic Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Callus absent, fracture line visible</td>
</tr>
<tr>
<td>2</td>
<td>Callus present, fracture line visible</td>
</tr>
<tr>
<td>3</td>
<td>Callus present, fracture line not visible</td>
</tr>
</tbody>
</table>

4 cortices are scored between antero-posterior and lateral views.

Total possible score = 12.

Radiographic union = RUST score 10 or greater.

**Table 2. Radiographic Union Scale in Tibia Fracture (RUST) Score.**
Histology tissues were fixed in 10% neutral buffered formalin and decalcified in 0.5 M solution ethylenediaminetetraacetic acid, pH 8.0 (American Bio, Natick, MA, USA) for at least days. Intramedullary pins were removed. Tissues were dehydrated with graded alcohols, cleared with xylene, and paraffinized using the Tissue Tek VIP tissue processor (Sakura Finetek, Torrance, CA, USA). The author acknowledges the assistance of Nancy Troiano, MS, and the Yale Orthopaedics Histology and Histomorphology Laboratory for their assistance in preparing and staining specimen slides. Tissues were cut into sections 5 microns thick, mounted onto slides, and stained with Gram, hematoxylin and eosin, and Safranin O stains. Histology images were obtained using Cytation 5 (BioTek Instruments, Winooski, VT USA).

Whole tibiae were used for μCT analysis of cancellous bone inside the callus with the Scanco μCT 50 (Scanco Medical, Brüttisellen, Switzerland) system [258]. 10µm voxel size, 55KVp, 0.36 degrees rotation step (180 degrees angular range) and a 1000 ms exposure per view were used for the scans which were performed in 70% alcohol. The Scanco μCT software (HP, DECwindows Motif 1.6) was used for 3D reconstruction and viewing of images. After 3D reconstruction, volumes were segmented using a global threshold of 400 mg HA /mm³. Tissue (in this case Bone) mineral density (TMD), directly measured bone volume fraction (BV/TV), connectivity density, surface to volume ratio (BS/BV), thickness (Tb.Th), number (Tb.N) and separation (Tb.Sp) were calculated for the trabecular bone formed inside the callus. Imaging and analysis were performed by the bone imaging core at Columbia University.

**Immunohistochemistry**

Immunohistochemistry was conducted by using Rabbit-specific HRP/DAB Detection IHC Detection Kit – Micro-polymer (Abcam, ab236469). Slides were first deparaffinized by washing
three times in Xylene (Fisher chemical, Belgium) for five minutes and in graded alcohol (Decon Laboratories Inc, Pittsburg, PA) concentrations — 100%, 95%, 70% — for five minutes each. Antigen unmasking was performed utilizing a heated 0.1 M sodium citrate solution (J. T. Baker, Phillipsburg, NJ) for ten minutes. Following antigen unmasking, slides were washed in 1x TBST (American Bio, Canton, MA) three times for five minutes each. Hydrogen Peroxide Blocking was performed for 30 minutes using the IHC detection kit at room temperature. Slides were then rewashed in 1 x TBST solution three times five minutes each. Sections were then incubated with protein block from the kit for 30 minutes and then incubated in the chosen primary antibody at a concentration of 1:200 overnight at 4° C. Primary antibodies used included: IL1b: ab9722, Pp65: ab86299, Sox9: ab185966, Runx2: BNP1-77461.

After overnight incubation, slides were washed in 1 x TBST three times for five minutes each. Then, immunohistochemistry was performed by applying Goat Anti-Rabbit HRP Conjugate for 30 minutes along with 1 x TBST washing after. Samples were then visualized by diaminobenzidine (DAB) substrate. DAB-visualized sections were then counterstained with Harris’ Hematoxylin, 1% acid alcohol, and ammonium water. Following counterstaining, the tissues were rehydrated in 100 % Ethanol three times five minutes and Xylene three times five minutes before adding the slide cover. The author acknowledges Inkyu Lee for performing immunohistochemistry staining procedures for these experiments.

Slide images were captured using a Gen5 3.02 (BioTek CYTATION5 image reader); positive cells were counted using QuPath software.

**Biomechanical testing**
Following µCT, samples were placed in PBS before undergoing biomechanical testing. Flexural rigidity was evaluated using a nondestructive 3-point bending test. Each sample was loaded to a maximum force of 1.5 N and flexural rigidity (EI) was calculated from the slope (k) of the linear region of the load–deflection curve. Since the callus was not always located in the middle of the supports, the distances between the load vector and the proximal (a) and distal (b) supports were considered for calculating $EI = k \frac{ab^3}{3l}$ (N mm$^2$).

The proximal and distal ends of the bone were then potted in brass cylinders. Femora were subjected to torsion at 1°/s. The gage length was measured for each specimen. Torque and displacement data were recorded at a rate of 20 Hz. Maximum torque, torsional rigidity, the twist to failure and twist to max load (units: radians normalized by gage length), and toughness were calculated using a custom routine in LABVIEW (National Instruments, Austin, TX, USA). Torsional rigidity was defined as the slope of the initial portion of the torque-twist curve, where twist was normalized by gage length, and toughness was defined as the area beneath the curve up until the maximum torque was reached. The author acknowledges Steven Tommasini, PhD, for carrying out these biomechanical tests.

**Statistics**

All analyses were performed by the author with Prism8 (GraphPad Software, La Jolla, CA, USA). Means and standard deviation (SD) were calculated for all data sets. Differences among groups were compared with ANOVA with multiple comparisons, to assess fracture healing parameters (micro-CT, RUST score, torsional strength) among treatment condition groups. For glucose tolerance testing, geometric area under the curve (AUC) was calculated for each group to compare glucose tolerance. Differences between diabetic and wild-type groups for blood glucose
testing, glucose tolerance testing, body weight, and uninterrupted fracture healing parameters were calculated with unpaired T-test. P < 0.05 was considered statistically significant.
Results

1. **Rifampin-loaded hydrogels decrease bacterial load and improve fracture healing in a MRSA-infected open fracture model.**

   96 mice were used in this study. Two died spontaneously within 24 hours after surgery. No signs of systemic infection were observed. No animals were euthanized prematurely. On CBC analysis, MRSA infection trended to increased total and percent neutrophil count, as well as total monocyte count. However, no significant differences among groups were observed, including sterile fracture versus MRSA infection.

   Untreated MRSA infection resulted in mean tissue CFU count of 6296 colonies (**figure 14**). Low-dose rifampin hydrogel reduced this count to 3149 (p=0.022). High-dose rifampin hydrogel recovered a mean of 2 CFU (p<0.0001) and systemic rifampin 0.4 CFU (p<0.0001). Untreated MRSA infection resulted in mean detection from implant of 1657 CFU, low-dose rifampin hydrogel of 356 CFU (p=0.14), high-dose rifampin hydrogel of 0 CFU (p<0.041) and systemic rifampin 0 CFU (p<0.041).

   Among sterile fractures, radiographic union was achieved in 88% of cases (mean RUST 11; **Figure 15**). Among untreated infections, zero samples achieved union (mean RUST 6, p<0.0001; **Figure 3**). A statistically significant improvement of mean rust score was identified between MRSA infection and the high-dose local rifampin group (mean 8.7, p<0.024). Systemic rifampin and combined systemic/hydrogel treatments resulted in recovered fracture healing (mean 10, p=0.0005; mean 10.7, p<0.0001, respectively). No significant differences were observed among the sterile fracture, high-dose hydrogel, or systemic treatment groups. On tissue harvest at 14 and 28 days, gross pus and inflammation were observed at the fracture site of infected cases (**Figure 15c**).
Figure 14. Bacterial colony forming unit analysis of fracture site tissue and implants after 3 days\textsuperscript{11}. Following tissue harvest, implants were sterily removed, vortexed in an aliquot of 1 mL PBS for 10 minutes. 100 uL of the resulting solution was plated onto MRSA-specific medium. Fracture site tissues were isolated, homogenized, and digested with collagenase. 1x10\textsuperscript{5} cells were plated onto MRSA-specific medium. Plates were allowed to incubate for 36-40 hours. Representative images from each treatment condition are shown in (a) for tissues and (b) for

\textsuperscript{11} The author acknowledges Dr. Kwon for assistance in obtaining the cell culture plate images used in this figure.
implants. Quantitative analysis of bacterial loads is shown in (c) for tissues and (b) for implants. 

\[ n = 4-5 \text{ per group. Mean with 95% CI shown. Differences were assessed by ANOVA with multiple comparisons, MRSA infection as control group.} \]

\[ *p<0.05, ****p<0.0001, \text{ ns } p>0.05. \]

**Figure 15.** Radiographic and gross healing of fracture sites after 28 days. (a) Representative lateral (LAT) and antero-posterior (AP) views from each treatment condition are shown. (b) RUST scores were calculated, \( n = 6-8 \) per group. Mean with 95% CI shown. Differences were assessed ANOVA with multiple comparison test. \[ *p<0.05, ***p<0.001, ****p<0.0001, \text{ ns } p>0.05. \] (c) Photographs of gross sterile and infected fracture sites after 28 days. Infected cases were obvious, with gross pus at the fracture site and inflammation at the knee joint.
µCT demonstrated a well-healed callus among sterile fractures and samples demonstrating radiographic union (figure 16a). Infected fracture sites were marked by nonunion and demonstrated involucrum, with “bone within bone” appearance typical of osteomyelitis [259]. A significant increase in bone volume at the fracture site was calculated between sterile controls and MRSA infection (4.52 mm$^3$ vs 13.77 mm$^3$, p=0.034). Bone volume, total volume, bone density, and trabecular thickness were all significantly decreased with systemic rifampin therapy compared to untreated MRSA infection (p values <0.05, figure 16b).

Figure 16. µCT images and analysis of fracture sites after 28 days. (a) Select 3-dimensional reconstructions of healed tibiae, with bone surface (BS) and cut surface (CS) views. (b) Analysis of callus area total volume (TV), bone volume (BV), bone density (BV/TV), trabecular thickness (Tb.Th), bone surface/bone volume (BS/BV). n =4 per group. Mean with 95% CI, n = 3-4 samples per group. Differences assessed by ANOVA with multiple comparisons. *p<0.05, **p<0.01.
Biomechanical strength testing between sterile fracture and MRSA infection samples after 28 days demonstrated significantly reduced flexural rigidity (mean 973.6 N mm$^2$ to 248.4 N mm$^2$, $p<0.05$) and significantly reduced maximum torque (21.2 N mm to 4.8 N mm, $p<0.01$, figure 17). Nonsignificant trends of increased biomechanical properties among local and systemic treatment groups compared to MRSA infection were observed for parameters including flexural rigidity, torsional rigidity, maximum torque, and toughness to maximum torque.

Figure 17. Biomechanical strength testing of fractured tibiae after 28 days. Tibiae were isolated and underwent biomechanical strength testing. Flexural rigidity, torsional rigidity, max torque, and toughness to max torque were calculated. MRSA-infected samples showed significantly decreased flexural rigidity and max torque compared to sterile controls, and trends toward increased biomechanical parameters were observed among treatment groups compared to MRSA infection. Mean and 95% CI shown, $n = 3$-$4$ samples per group. Differences were assessed using ANOVA with multiple comparisons. *$p<0.05$, **$p<0.01$. Mean with 95% CI, $n = 3$-$4$ samples per group. Differences assessed by ANOVA with multiple comparisons. *$p<0.05$, **$p<0.01$. 

...
On histology, sterile callus areas were well-demarcated and organized (figure 18). Sterile fracture samples demonstrate bridging soft callus with chondrocyte proliferation at 2 weeks, with soft callus spanning the fracture site. At 28 days, bony bridging and mineralized tissue was present. Cement lines clearly demarcated necrotic and healthy bone. No bacteria were observed at the fracture site on Gram stain.

**Figure 18.** Select histology of sterile and infected fracture sites after 14 and 28 days. *Slides were stained with hematoxylin and eosin (H&E), safranin O (inlet/magnified view), and Gram stain (inlet/magnified view) at 14 and 28 days. Select representative histology samples are shown here for clarity. Vertical intramedullary pin tracts are seen in the low-power H&E views at 14 and 28 days. Sterile fracture cases demonstrated soft, cartilaginous callous at 14 days, and bony...*
callus with remnant cartilage matrix at 28 days. Cartilage matrix is identified as red staining area on safranin O. Areas of necrotic bone with empty lacunae (*) abutting healthy bone were observed at the sterile callus sites. No bacteria were present on Gram stain. MRSA-infected samples demonstrated sequestrum with bone necrosis and bacteria within the lacunar space (arrows), and involucrum formation (+). Scant cartilage and dense lymphocytic infiltrate were also observed. Histology samples from high-dose hydrogel and systemic therapy groups in which infection was eliminated are shown, resembling sterile healing. n = 3-4 per group.

MRSA-infected fractures demonstrate dense lymphocytic infiltrate with scant cartilage formation at 2 and 4 weeks, with a disorganized callus area and lack of soft and bony bridging. Involucrum was identified in infected cases by 14 weeks, with profuse development of mineralized, but disorganized, tissue by 4 weeks. Extensive areas of bone necrosis of cortical bone at the fracture site were observed, with lacunae empty of osteocyte nuclei. Bone resorption at the fracture site was also observed. Gram-positive organisms were observed on Gram stain infiltrating the lacunae of cortical bone, as well as within the medullary space, by 4 weeks in infected cases.

Cases treated with antibiotic therapy, including systemic rifampin and local rifampin, resulted in binary morphologic appearance by 14 and 28 weeks, resembling either sterile fracture cases or untreated MRSA-infection. Briefly, successfully treated cases demonstrated well-organized cartilaginous and bony callus by 14 and 28 weeks, respectively. No differences in morphology were observed between cases successfully treated by local or systemic antibiotics. Treated cases that did not successfully clear infection (e.g., hydrogel antibiotic delivery) were marked by lymphocytic infiltration, involucrum formation, and extensive bony necrosis and Gram-positive organism infiltration of lacunae.
Figure 19. Lacunar colonization of cortical bone by MRSA. Gram stained slide of cortical bone adjacent to fracture site with high-power view (inlet, right). Gram positive organisms (dark stain) are seen colonizing an area of lacunar bone. Lack of nuclei in lacunae indicate necrotic bone, where sequestrum is formed.

2. MRSA-infected nonunion is characterized by impaired chondrocyte and osteoblast differentiation and maturation and is associated with IL-1 and NF-KB activation.

Failure of chondrocyte proliferation and maturation marked the major morphological change observed in MRSA-infected fracture healing compared to sterile controls. In sterile fracture healing, abundant soft callus bridged the distal and proximal bone fragments at 14 days. The soft callus contained active chondrocytes and abundant cartilage matrix observed via Safranin O stain.
On day 28, no active chondrocytes were present, but residual cartilage matrix was observed on Safranin O (figure 20).

The major morphological change in MRSA-infected fracture healing was reduced chondrocyte proliferation and maturation. MRSA infection did not preclude chondrocyte proliferation, as chondrocytes were observed at days 14 and 28. However, chondrocytes failed to form a soft callus bridge between the distal and proximal bone fragments at day 14, and only scant chondrocyte populations were observed. Proliferating chondrocytes were observed at the edges of the involucrum, and in rare cases distal and proximal involucrum were bridged by chondrocytes. If involucrum bridging occurred, it was only observed across one cortex. In contrast to the remnant cartilage matrix seen on day 28 in sterile fracture cases, no mature cartilage matrix (matrix without active chondrocytes) was present on Safranin O stain in MRSA-infected cases.

SOX9 and RUNX2 are key transcription factors for differentiation of chondrocytes, and RUNX2 is also involved in osteoblast differentiation. Immunohistochemistry performed at 14 days demonstrated SOX9 expression in the areas of soft callus in both sterile and MRSA-infected controls, however staining was significantly stronger with more positive cells among the sterile fracture group. RUNX2 immunostaining demonstrated a similar trend, with abundant RUNX2 positive cells at the fracture callus at 14 days versus scant cells with weak staining in MRSA-infected cases (figure 21).
Figure 20. Safranin O staining demonstrates poor soft callus formation and incomplete cartilage maturation in MRSA-infected fracture. Safranin O stain obtained from representative sterile fracture and MRSA infection cases (n=6 in each group) after sacrifice at 14 and 28 days. Sterile fracture is marked by dense, well-organized bony callus, mature residual cartilage matrix (red/purple stain on Safranin O), and lack of bacteria on Gram stain (not shown). MRSA infection demonstrates poor callus formation with incomplete bridging. Immature cartilage is identified by pink/red stain on Safranin O, with active chondrocytes present that have not undergone the hypertrophy/apoptosis stage. Soft callus area was quantified using QuPath software. Mean and 95%CI shown.
Figure 21. Chondrocyte differentiation occurs in MRSA infection, but failed chondrocyte maturation is marked by absence of RUNX2 expression. Representative immunohistochemical staining (IHC) following sacrifice at 14 days in sterile and MRSA-infected fracture (n=3). Brown staining indicates positive cells. Despite reduced chondrocyte population in MRSA infection, IHC revealed the presence of active expression of SOX9, a key transcription factor for chondrocyte differentiation. Staining for RUNX2, however, demonstrated absent signaling in MRSA infection. RUNX2 is a key transcription factor for facilitating chondrocyte differentiation into the hypertrophic phase.

Further, immunohistochemistry demonstrates that IL-1B activation was associated with decreased chondrocyte proliferation in our MRSA-infected nonunion model (figure 22).
Interestingly, chondrocytes at the fracture site stained positive for IL-1B in both sterile and infected conditions at 14 days. IL-1B staining was strongly positive in association with the dense, leukocytic infiltrate observed in MRSA-infected healing. Positive stains for IL-1B were observed at areas of cortical bone resorption as well as the area between involucrum and cortical bone. Proliferating chondrocytes in MRSA-infected samples were absent in areas adjacent to IL-1B expression. Immunohistochemical staining for p-p65 (activated NF-KB) demonstrated NFKB activation in chondrocytes in both sterile fracture healing and MRSA-infected fracture (figure 23).

Figure 22. IL-1β is expressed by chondrocytes in normal fracture healing, but also is highly expressed in areas absent of chondrocytes in MRSA infection. Representative immunohistochemical staining (IHC) following sacrifice at 14 days in sterile and MRSA-infected fracture (n=3). Brown staining indicates positive cells. Chondrocytes positive for IL-1β were
identified in both infected and sterile conditions. Densely positive IL-1β staining adjacent to bone (arrows) was associated with areas absent of chondrocyte populations. Involucrum is indicated by *.

Figure 23. NFκB is activated in chondrocytes in normal and infected fracture healing. Representative immunohistochemical staining (IHC) for p-p65, the activated form of the signaling protein NFκB following sacrifice at 14 days in sterile and MRSA-infected fracture (n=3). NFκB is a key signaling protein involved in inflammation and non-specific innate immune defense. Chondrocytes in both sterile and infected fractures stained positive for p-p65, the activated form of NFκB.
3. A high-fat, high-sugar diet induces a type 2 diabetic phenotype characterized by obesity, impaired glucose metabolism, increased infection burden, and poor fracture healing characteristic of type 2 diabetes.

As discussed in the introduction, the pathogenesis of type 2 diabetes is dependent on several factors, but obesity poses the strongest correlation. After 3 months, mice fed a high fat, high sugar diet (HFHS) developed an obese phenotype compared to lean-fed controls, with copious intraperitoneal fat deposition and gross fat deposits in the liver (figure 24). HFHS mice had a mean body weight of 45.1 g (SD 5.6) compared to 28.3 g (SD 2.25) lean-fed controls (p<0.0001, figure 25). This indicates a 160% increase in mean body weight among the HFHS group. 79 lean-fed mice and 105 HFHS were used for experiments in this portion of this study.12

Figure 24. A high-fat, high sugar diet induces obesity in mice. A) Gross appearance of lean-fed mice and mice fed a high fat, high sugar diet (HFHS) on sacrifice after 6 months. B)

12 Fracture healing and histology results from the second “generation” of this experiment are pending, as noted throughout this section.
Photograph demonstrating copious intraperitoneal observed in the HFHS mice compared to lean controls. C) Normal appearance of gross liver specimen in lean-fed mice, and D) liver with gross enlargement and fatty deposition in HFHS mice.

Figure 25. A high-fat, high sugar diet induces obesity in mice by 3 months.

Mice maintained on a high fat, high sugar diet demonstrated a 160% increase in body weight by 3 months. Body weights with mean and 95%CI are shown. Difference assessed by unpaired T-test. ****p<0.0001.

The clinical diagnosis of diabetes can be made with a glucose tolerance test or increased fasting glucose. After 3 months on a high-fat, high-sugar diet, mice exhibited impaired glucose tolerance, with area under the curve (AUC) of 61,945 mg-min/dL (SD 9,228), compared to 47,128 mg-min/dL (SD10,862) among lean-fed controls (p<0.0001; figure 26). While there is no standardized
cutoff value for diagnosing diabetes among mice, the degree of elevation was similar to the Tallyho positive control, which exhibited AUC of 63,465 mg-min/dL (n=3, SD 6,198). After 6 months, fasting blood glucose levels were significantly higher in the HFHS mice (mean 171.4 mg/dL, SD 33.7, n=79) compared to lean controls (mean 129.8 mg/dL, SD 21.6, p<0.0001, n=105, **figure 27**).

**Figure 26.** Mice fed a high fat, high sugar diet develop impaired glucose tolerance by 3 months compared to lean-fed controls. Results of intraperitoneal glucose tolerance test after 3 months on a high fat, high sugar diet (HFHS) compared with lean (normal age-matched) controls and Tallyho mice (positive control). An intraperitoneal injection was given with glucose load 2g/kg. Blood glucose levels were measured at 15, 30, 60, and 120 minutes following injection. Geometric area under the curve (AUC) was calculated to quantify glucose tolerance. HFHS mice demonstrated significantly impaired glucose tolerance compared to lean-fed controls, with a degree of elevated AUC comparable to Tallyho. Mean and 95%CI are shown. AUC difference between HFHS and lean groups assessed by unpaired T test. ****p<0.0001.
Figure 27. Pre-operative blood glucose levels are elevated in mice fed a high fat, high sugar diet compared to lean-fed controls. Blood glucose levels were measured with a commercially available glucometer via tail vein sampling after 6 months. Blood glucose levels with mean and 95% CI are shown. Difference assessed by unpaired T-test. ****p<0.0001.

Diabetes is marked by poor fracture healing, and HFHS mice exhibited reduced radiographic fracture healing compared to lean-fed mice. 28 days after fracture, lean-fed mice exhibited a mean RUST score of 11 (n=3, SD 1) compared to HFHS fracture sites of 7.5 (n=4 SD 0.58), p<0.002 (figure 28). Micro-CT (n=4 per group) also demonstrated diminished micro-architecture of the diabetic fracture sites across all measured parameters (figure 29): bone density (bone volume/tissue volume), lean mean 48.8% vs HFHS mean 14.1%, p=0.0051; trabecular number, lean 6.4/mm vs HFHS 3.3/mm, p=0.0188; trabecular thickness, lean 0.11 mm vs HFHS 0.054 mm,

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13 These sample sizes are small; currently the author is waiting for access to the x-ray machine located in a collaborator’s lab to obtain measurements of more samples. Nevertheless, a significant difference was identified in RUST score.
p=0.0002; trabecular space, lean 0.19 mm vs HFHS 0.33, p=0.03; tissue mineral density, lean 960 g/cm³ vs HFHS 891.9 g/cm³, p=0.0053; and bone surface/bone volume, lean 29.32 mm²/mm³ vs HFHS 54.23 mm²/mm³, p=0.0009.

Figure 28. Mice maintained on a high fat, high sugar diet demonstrate poorer radiographic healing scores 28 days after fracture compared to lean-fed mice. Representative lateral (LAT) and antero-posterior (AP) radiographs from tibiae harvested from (A) lean and (B) high fat/high sugar fed (HFHS) mice 28 days after sterile fracture. (C) RUST scores demonstrating decreased healing among HFHS mice, n =3-4 per group. Mean with 95% CI shown. Differences were assessed by unpaired T test. **p<0.01.
Figure 29. Micro-CT images and analysis of fracture sites after 28 days reveal impaired callus formation in mice fed a high fat, high sugar diet compared to lean-fed controls. (a) Representative 3-dimmensional reconstructions of healed tibiae demonstrating lack of bony bridging and poor callus formation at the fracture site of mice fed a high fat, high sugar diet (HFHS). Quantitative analysis of callus B) bone density (BV/TV), C) trabecular number (Tb.N), D) trabecular thickness (Tb.Th), E) trabecular space (Tb.Sp), F) tissue mineral density (TMD), G) bone surface/bone volume (BS/BV), and H) connectivity density. n =4 per group. Mean with 95% CI. Differences assessed with unpaired T-test. *p<0.05, **p<0.01, ***p<0.001.

In addition to increased numbers for x-ray analysis, histologic analysis of the fracture sites of diabetic and normal fracture healing and biomechanical strength testing are in process and/or planned. These will be valuable in comparing fracture healing under diabetic and normal conditions.
4. **Local and systemic teriparatide improve radiographic healing in diabetic mice, but only systemic teriparatide improves micro-CT healing parameters.**

Teriparatide has been extensively described as an anabolic bone agent that has potential to improve fracture healing (see: pages 49-50). The author tested the utility of locally-applied teriparatide via bioactive hydrogel among diabetic and normal mice, as well as systemic teriparatide injection. After 28 days, HFHS mice treated with systemic teriparatide exhibited a mean RUST score of 10 (SD 0.89) compared to 7.5 (SD 0.56) among untreated fracture sites, \( p=0.0014 \) (**figure 30**). After 28 weeks, HFHS mice treated with local PTH exhibited a mean RUST score of 10.25 (SD 0.95), \( p=0.0013 \) compared to untreated fracture. Micro-CT \((n=4 \text{ per group})\) demonstrated improved micro-architecture of the HFHS fracture sites among groups treated with systemic teriparatide (PTH-S) compared to untreated fracture (FX) for bone density (bone volume/tissue volume), 30.41\% PTH-S vs 14.1 FX, \( p=0.004 \); trabecular number 6.51/mm PTH-S vs 3.28/mm FX, \( p=0.0013 \); trabecular space, 0.17 mm PTH-S vs 0.33 FX , \( p=0.0025 \); and connectivity density, 542.6/mm\(^3\) PTH-S vs 208.2/mm\(^3\) FX, \( p=0.004 \). No significant differences \((p>0.05)\) were identified between PTH-S and FX groups in HFHS mice for trabecular thickness, 0.069 mm PTH-S vs 0.054 mm FX; tissue mineral density, 865.2 g/cm\(^3\) PTH-S vs 891.9 g/cm\(^3\); and bone surface/bone volume, 44.81 mm\(^2\)/mm\(^3\) vs HFHS 54.23 mm\(^2\)/mm\(^3\). Local PTH treatment in HFHS mice resulted in no significant differences \((p>0.05)\) between the local teriparatide group (PTH-L) and FX for any micro-CT parameter including: bone density, 20.2\% PTH-L vs 14.1 FX; trabecular number 4.03/mm PTH-L vs 3.28/mm FX; trabecular space, 0.27 mm PTH-L vs 0.33 FX; connectivity density, 252.1/mm\(^3\) vs 208.2/mm\(^3\) FX; trabecular thickness, 0.060 mm PTH-L vs...
0.054 mm FX; tissue mineral density, 906.5 g/cm³ PTH-L vs 891.9 g/cm³; and bone surface/bone volume, 49.25 mm²/mm³ vs HFHS 54.23 mm²/mm³.

Figure 30. Local teriparatide improves radiographic healing only, while systemic teriparatide improves radiographic healing and micro-CT callus parameters in mice fed a high fat, high sugar diet. (a) RUST score demonstrates improved radiographic healing in mice fed a high fat, high sugar diet (HFHS) after 28 days when treated with teriparatide systemically over 28 days (PTH-S) and locally (PTH-L) as a single dose at the time of fracture. Quantitative analysis of callus B) bone density (BV/TV), C) trabecular number (Tb.N), D) trabecular thickness (Tb.Th), E) trabecular space (Tb.Sp), F) connectivity density, G) tissue mineral density (TMD),
and H) bone surface/bone volume (BS/BV). n = 4 per group. Mean with 95% CI. Differences assessed by ANOVA with multiple comparisons. *p<0.05, **p<0.01, ***p<0.001.

Further data is currently being collected and processed for this investigation including increased numbers for x-ray analysis, histology/immunohistochemistry, and biomechanical testing.

5. Systemic administration of parathyroid hormone, along with systemic antibiotics, improves fracture healing in MRSA-infected fracture.

Infection is a potentially devastating complication of fracture, and diabetic patients are at higher risk. These results demonstrate the effect of MRSA infection on a diabetic mouse model, and demonstrates the effectiveness of antibiotics alone and antibiotics with systemic teriparatide in improving fracture outcomes compared to sterile diabetic healing.

HFHS mice developed nonunion and profuse involucrum formation 28 days following fracture surgery and MRSA infection (figure 31). Mean RUST score of MRSA-infected fracture was 6 (SD 1.5), with zero out of 6 samples achieving radiographic union (figure 32). Micro-CT revealed “bone within bone” appearance of involucrum, or reactive bone, typical of chronic osteomyelitis (figure 31).

Treatment with systemic, combinatory rifampin and vancomycin resulted in restored fracture healing, with significant radiographic improvement from baseline compared to uninterrupted sterile HFHS healing (figure 31, 32). A RUST score of 10.25 (SD 0.96) was achieved in the MRSA infection group treated with antibiotics alone (MRSA+ABX, p=0.012) and a RUST score of 9.75 (SD 0.96) in the MRSA infection group treated with antibiotics and teriparatide (MRSA+ABX+PTH-S, p=0.038). Callus formation was grossly restored on imaging compared to
sterile and infected HFHS samples (figure 30). Micro-CT analysis ($n=4$ per group) resulted in significant improvement between MRSA+ABX compared sterile HFHS healing (FX) for trabecular thickness, 0.084 mm MRSA+ABX vs 0.054 FX (p=0.015), and nonsignificant (p>0.05) trends toward improved callus parameters for bone density (bone volume/tissue volume), 23.93% MRSA+ABX vs 14.1% FX; trabecular number 4.06/mm MRSA+ABX vs 3.28/mm FX; trabecular space, 0.26 mm MRSA+ABX vs 0.33 FX; and connectivity density, 290.2/mm$^3$ MRSA+ABX vs 208.2/mm$^3$ FX. MRSA+ABX+PTH-S demonstrated significant improvement of callus parameters compared to FX for bone density (28.06%, p=0.03), trabecular number (6.53/mm, p=0.0003), and trabecular space (0.1607 mm, p=0.0004), and connectivity density (452.4/mm$^3$, p=0.016). With multiple comparison’s test, MRSA+ABX+PTH-S demonstrated significant differences with improved healing over MRSA+ABX for trabecular number (p=0.0035) and trabecular space (p=0.0112).

More data is currently being collected and processed for this investigation including increased numbers for x-ray analysis, histology/immunohistochemistry, and biomechanical testing.
Figure 3. 3-dimmmensional reconstructions of tibiae 28 days after fracture in sterile and infected fracture healing treated with antibiotics and systemic teriparatide.

Samples were harvested 28 days following fracture surgery and underwent 3-dimmedensional micro-computed tomography (micro-CT) analysis. Sterile fracture (FX) in the high-fat, high sugar mouse model demonstrated poor healing and nearly absent callus formation. MRSA-infected fracture (MRSA) demonstrated profuse involucrum development, a hallmark of chronic osteomyelitis. Treatment of MRSA infection with combinatory vancomycin/rifampin antibiotic therapy (MRSA+ABX) for 3 days immediately following MRSA inoculation resulted in improved fracture healing compared to sterile fracture group. The addition of systemic teriparatide treatment (MRSA+ABX+PTH-S) further improved gross appearance of fracture healing.
Figure 31. Radiographic and micro-CT analysis of infected fracture sites treated with antibiotics and teriparatide demonstrate improved fracture healing after 28 days.

Radiographic and micro-CT measurement of fracture callus among HFHS mice 28 days following fracture. RUST score is given (A) with quantitative micro-CT analysis of callus including B) bone density (BV/TV), C) trabecular number (Tb.N), D) trabecular thickness (Tb.Th), E) trabecular space (Tb.Sp), F) connectivity density, G) tissue mineral density (TMD), and H) bone surface/bone volume (BS/BV). Surprisingly, the presence of MRSA inoculation at the time of fracture, when treated with combinatory antibiotics for 3 days, appeared to have a positive effect on fracture healing, with improved RUST score and improved trabecular thickness on micro-CT. The addition of systemic teriparatide treatment allowed for improvement in trabecular number and trabecular space measurements. n =4 per group. Mean with 95% CI. Differences assessed by ANOVA with multiple comparisons. *p<0.05, **p<0.01, ***p<0.001.
Discussion

1. Rifampin-loaded hydrogels reduce bacteria load and improve fracture healing in a MRSA-infected, open fracture mouse model.

Rates of surgical site infection following operative fixation has remained largely unchanged over the past 40 years [260]. New approaches are needed to combat infection after fracture. The issues of antibiotic resistance and microbiome disturbances increase the importance of infection prevention and limiting systemic antibiotic use. In this study, we present a novel local antibiotic delivery method that reduces bacterial burden, decreases implant colonization, and improves fracture healing in mouse model of MRSA-contaminated, infected nonunion.

Our results demonstrate that rifampin (300ug) hydrogel can suppress bacterial loads to nearly undetectable levels in the tissue fracture site after 3 days from infection. Rifampin (60ug) hydrogel were still able to reduce bacterial burden in our model by an average of 50% compared to non-treated MRSA infection. No bacteria were recovered from the implants after three days following high-dose local therapy. A wide range of bacterial cells were recovered from the implant (108–4201 CFU), likely due to the relatively short time for infection to establish biofilms. This variance resulted in limited statistical power in demonstrating an effect from the low-dose hydrogel, and improvements in sample number can be made in follow-up studies.

Rifampin hydrogels also supported fracture healing with improved RUST score compared to MRSA-infected models. Although high-dose rifampin hydrogels were effective in reducing bacterial load to a near-zero level at the fracture site, we still observed an infected non-union rate of 50%. In contrast, nearly all MRSA-infected samples treated with high-dose systemic antibiotics demonstrated radiographic union, with a minimum RUST score of 8. We postulate that resulting infection following initial hydrogel treatment could be due to bacterial seeding of the surgical limb
that was not in the area of the fracture site and applied hydrogel. Conversely, while bacterial counts were not reduced to zero with the low-dose rifampin hydrogel, this hydrogel was still able to support fracture healing and improve radiographic union.

Whereas previous studies have demonstrated efficacy of loading hydrogel with lysostaphin and gentamycin in animal models in combatting Staphylococcus aureus, our study is notable in that it is the first to our knowledge to study the efficacy of locally-applied, rifampin-loaded hydrogel to combat MRSA infection [114].

In addition to demonstrating treatment efficacy, a major strength of our study is our clinically-relevant mouse model of post-traumatic infection. We used a challenging infection model including higher infective bacterial load and use of a resistant organism. Additionally, we introduced infection 10 minutes before hydrogel treatment was introduced to the infection site, as opposed to delivering bacteria within the antibiotic-containing hydrogel as previous authors have done [19]. Our infection model is clinically meaningful as it invariably progressed to nonunion, with hallmarks of osteomyelitis including involucrum characterized by profuse reactive bone formation. In addition to further fracture healing studies, we believe that further investigation of biologic pathways in our model could potentially lead to therapeutic strategies for bone regeneration and healing.

This present study is not without limitations. First, it is recognized that rifampin should not be used as systemic monotherapy due to rapid resistance. We chose to use rifampin in our study due to its ability to target intracellular bacteria and biofilms and its efficacy against MRSA,

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14 A better understanding of elution kinetics of the rifampin-loaded hydrogel increases clinical usefulness of this data. Our lab has performed kinetic release experiments of rifampin from the hydrogel, however the author did not participate in any of these experiments and therefore has decided to not include the data here.
so as to be used as a potential adjunct to current antibiotic practice. Other rifampin-only local delivery methods, including mesh and powders, have been studied for local infection prevention strategies [261-262]. We believe our study adds to this existing body of literature by focusing on a delivery method that is readily applied and does not require surgical exposure. Rifampin has been demonstrated to be an effective local antibiotic and has been used in drug-eluting and antibiotic coated implants [263-264].

Another potential limitation of rifampin-loaded hydrogels is that in vitro studies have demonstrated rifampin toxicity to osteogenic cells [265], raising concern about high-dose local rifampin therapy in the setting of fracture healing. Our data show no difference in radiographic healing between sterile fracture and the highest-dose rifampin treatment group, and exposure to a low dose of rifampin is not as effective in eliminating bacteria and improving healing. Additionally, previous in vivo studies using more than three-fold our maximum local rifampin dose demonstrated no deleterious effect on bone healing [262], with one study suggesting that rifampin can actually improve bone regeneration [266].

In summary, our study demonstrates that a rifampin-loaded hydrogel may be a useful therapy to compliment systemic antibiotic treatment after open fracture. Hydrogel can be readily applied without surgical exposure, demonstrating their potential especially in treating low-grade open injuries that do not require surgical debridement. A rifampin-loaded hydrogel may also help reduce the need for systemic antibiotic treatment, limiting microbiome disruption. Further studies are needed to investigate whether antibiotic-loaded hydrogel application can reduce infection rates and improve outcomes in the clinical setting.

2. MRSA-infected fracture is marked by poor chondrocyte proliferation and maturation as well as IL-1 and NFKB inflammatory signaling
Histologic analysis and immunostaining revealed failure of chondrocyte proliferation and maturation in infected fracture, but chondrocyte differentiation was preserved. At 14 days, the sterile fracture sites demonstrated robust chondrocyte proliferation and bridging cartilage, whereas only scant chondrocytes were observed in the MRSA-infected cases. At 28 days, the chondrocyte/cartilage scaffold had largely been replaced by mineralized tissue, with only remnants of cartilage matrix apparent on Safranin O staining. Sox9 immunostaining was positive in both sterile fracture and infected samples, indicating chondrocyte differentiation in both conditions. However, RUNX2 staining was significantly diminished in infected healing. As RUNX2 transcription is necessary for progression to the hypertrophic phase of chondrocyte differentiation, failure of RUNX2 transcription in the MRSA cases supports the conclusion that chondrocytes fail to mature in the setting of infection.

The histological and immunohistochemical analysis highlights the both necessary and detrimental role of inflammation in the fracture healing process. For one, chondrocyte IL-1B and NF-KB expression are evident in sterile fracture healing, leading to the postulation that these inflammatory-related pathways are necessary for successful fracture healing. This finding upsets the idea of a strict demarcation of an “inflammatory” phase of fracture healing; even after the inflammatory phase is complete and soft callus bridging has occurred, inflammatory signaling via IL-1B and NF-KB is still observed. Nevertheless, overexpression of IL-B at the infected fracture site is associated with absence of proliferating cartilage and involucrum formation. Although the results presented here are limited in that causation cannot be proven from histologic analysis alone, they do strongly suggest the role of IL-1B in perpetuation of infected fracture non-union by failing to allow for the proliferation and maturation of chondrocytes at the fracture site.
This hypothesis that IL-1B is a major factor in preventing chondrocyte proliferation and maturation in infected fracture healing is supported by a large body of evidence that IL-1B is a major driver of cartilage damage in osteoarthritis (OA). While OA is traditionally thought of as a mechanical disease, inflammatory mediators including IL-1, tissue necrosis factor alpha, and toll-like receptors have been identified as playing key roles in OA pathogenesis. The role of IL-1 as a key mediator of cartilage destruction in osteoarthritis was described by the early 1990s [267-268], and recent studies have focused on IL-1 and the IL-1 receptor as targets for inhibiting and preventing IL-1 related damage to cartilage and tissues [269-270]. A 2017 study used CRISPR to generate tissues resistant to inflammatory changes via targeted deletion of the IL-1 receptor [271].

The observation that the inflammatory IL-1B and NF-KB pathways are highly activated in sterile fracture healing raises a major clinical challenge in targeting infected nonunion. While antibiotics and bacterial elimination are the gold standard treatment for osteomyelitis, development of targeted therapeutics for promoting chondrocyte maturation in the setting of inflammatory fracture healing may be challenging. For one, our data, put in context of a large body of literature regarding IL-1 signaling and chondrocyte death, points to the possibility of targeting the IL-1B pathway to improve chondrocyte proliferation and maturation, and hopefully improve fracture healing in the setting of infection. Throughout this dissertation, both in literature review and with our original data, the importance of inflammatory pathways in fracture healing has been highlighted. Although infection results in an altered inflammatory environment, controlled inflammation and related signaling pathways are necessary for sterile fracture healing, beyond the initial hematoma/inflammatory phase. Therefore, inhibiting this process likely would impair the normal chondrocyte development and fracture healing. Therefore, the challenge of such an approach would depend on selectively targeting extracellular IL-1B and other inflammatory
mediators at the fracture site, while not interfering with intracellular signaling processes, or at least those that occur within the chondrocyte. This would potentially require an advanced delivery method or a molecular compound that could be transported to the fracture site without penetrating cartilage cells.

Alternatively, in developing potential therapeutics for infected nonunion beyond antibiotics, these considerations highlight the potential role of anabolic agents as a potentially promising therapeutic strategy. We have demonstrated that, although chondrocyte proliferation is markedly reduced in our model, differentiation is still taking place; stimulating these cells to further proliferate despite an altered inflammatory environment may be a potentially valuable path of future investigation.

While our immunostaining results provide insight into the molecular changes that occur at the callus site in infected fracture healing, our results do have limitations that need to be addressed. First, while our data present quite dramatic morphologic differences in chondrocyte differentiation and transcription factor expression, quantification of positive-staining cells and callus area would improve the quality and reproducibility of our results. Additionally, obtaining histology at various time points (7 days, 14 days, 21 days, 28 days) would help better qualify cellular development that occurs at the fracture site. Third, as previously mentioned, histologic study cannot definitively prove causation in biologic pathways; more advanced in vivo investigational techniques are required to draw more definite conclusions.

In conclusion, although this study is relatively simple and has several limitations, it is helpful in providing a basic groundwork for future studies for therapies targeting infected nonunion. The results described point therapeutic efforts, in addition to antibiotic therapy, away from inflammation reduction and towards promoting anabolism and chondrocyte proliferation in the
infected fracture site. To this end, the next step that the author would like to pursue is investigating whether teriparatide can be used to enhance fracture healing in an infected fracture model. A limited investigation focused on diabetic mice is discussed later in this section.

3. High-fat, high-sugar diet induces a mouse model of type 2 diabetes.

This investigation into diabetic fracture healing and infection relied on a mouse model that was achieved by feeding a high-fat, high sugar diet for several months in order to obtain a diabetic phenotype. Mice exhibited important hallmarks of type 2 diabetes. First, they developed obesity, marked by a 160% increase in body fat compared to lean-fed, age-matched controls. As discussed in the introduction, obesity is considered to be the major risk factor for developing type 2 diabetes, although genetics also plays a role [115]. Our mouse model also demonstrates significantly impaired glucose tolerance, with glucose tolerance test results similar to those exhibited by Tallyho mice, a genetically-induced diabetic strain. The results of fasting blood glucose levels, however, were more modest. Although significant differences were identified between lean-fed and HFHS mice, the mean blood glucose levels in our study was approximately 180 mg/dL and mice rarely had levels greater than 200 mg/dL, even with a long-term feeding period of greater than 6 months. “Diabetic” mice are typically considered to have blood glucose levels in the 200-250 mg/dL range, although there are no widely-agreed upon cutoff values [272]. This is similar to previous author’s reports of the phenotype in diet-induced diabetic models achieving only a modest elevation in blood glucose level.

Although our diabetic mouse model presented with mild metabolic phenotype, it nevertheless accurately reflected challenges faced in orthopaedic surgical practice with diabetic patients. This raises the possibility that the cited range of greater than 250 mg/dL as being
First, this study demonstrates that the chronic, diet-induced diabetic model has increased susceptibility to MRSA infection, with significantly more bacteria recovered from fracture site tissues and implants three days following initial surgery and infection. Additionally, and perhaps most importantly, the present model demonstrates clearly impaired fracture healing, with decreased RUST scores and decreased micro-CT parameters after 28 days compared to lean-fed, age-matched control mice.

Our diabetic mouse model holds distinct advantages over other models commonly used in the orthopaedic literature. A common means of modeling diabetes in mice is via streptozotocin injection (SZT), a chemical that preferentially targets and kills islet cells of the pancreas. Depending on dosing, SZT can be used to achieve a type 1 diabetic phenotype (high-dose) or a type 2 diabetic phenotype (low dose) [272]. Although widely used, this model has several distinct drawbacks, especially for modeling type 2 diabetes. First, this method does not incorporate obesity, a key component of diabetes pathogenesis. As discussed in the introduction, marrow fat and adipose cells have been found to play important regulatory roles in bone metabolism and signaling, many of which are still being discovered [26]. Thus, the SZT model is unable to provide insight into the role of adipose tissue in fracture healing and musculoskeletal disease. Additionally, it does not model insulin resistance, as target tissues are not subjected to chronic over-exposure to insulin. Third, this model tends to produce extreme phenotypes; even in modeling type 2 diabetes, investigators have reported mean blood glucose levels over 350 mg/dL [273]15. Such an extreme phenotype may limit clinical relevance of conclusions drawn from that model.

Genetic models of diabetes are the other most common means of studying diabetic bone disease. The Tallyho and Db/Db mouse strains are most prevalent. Tallyho is a multigenic diabetic

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strain. Tallyho mice demonstrate elevated fasting blood glucose levels, impaired glucose tolerance. Db/Db mice are leptin-receptor deficient, and diabetes pathogenesis is caused by constant overeating. Db/Db mice exhibit extreme obesity, high fasting blood glucose levels, and impaired glucose tolerance. While Db/Db mice display poor fracture healing and are widely used in fracture healing studies, they are (based on the author’s experience) difficult to work with because they are prone to infection and die spontaneously after sterile surgical procedures at a very high rate. Considering their high cost, these mice are likely not ideal for infection studies that require a long time of follow-up because of an anticipated high mortality and low data yield.

In summary, our mouse model of diabetic fracture healing, like all other mouse models, has important strengths and limitations. Phenotypically, it exhibits key elements of type 2 diabetes, including obesity, impaired glucose tolerance, in increased blood glucose levels. As type 2 diabetes is typically a diet-associated disease, the pathogenesis is arguably more accurate than chemically-induced and genetically-altered mouse models. It is a model that is prone to infection and, perhaps most importantly, exhibits poor fracture healing. The major drawback of our model, however, is that the elevation of blood glucose level is only modest compared to other models, resulting in a potentially less dramatic phenotype of diabetic bone disease. Additionally, our model is of chronic diabetes in aged mice, which improves the clinical relevance for fracture healing but means that our model is expensive and time-intensive; research using this model cannot happen quickly and must be done carefully and with careful planning.

4. Fracture healing is improved by systemic and hydrogel-delivered teriparatide treatment in diabetic mice.
Our data demonstrates that intermittent, systemically administered teriparatide improved fracture healing in both HFHS and lean-fed mice. Systemic injection of teriparatide resulted in improved RUST score and micro-CT parameters in HFHS mice. Among lean-fed mice, RUST score did not significantly improve, as baseline RUST scores were already high with all samples achieving union with an average RUST of 11. Micro-CT parameters likewise were not significantly improved.

While teriparatide is a powerful anabolic agent, systemic injection carries major drawbacks. First, teriparatide is an expensive agent and prohibitive for wide-spread use; although there have been no cost-benefit analyses to date, it would be infeasible to prescribe a month-long course of teriparatide to all diabetic fracture patients. As a protein, teriparatide cannot be administered orally and must be injected, contributing to patient inconvenience. Additionally, there is a theoretical concern that systemic injection of teriparatide may increase the risk of osteosarcoma due to its stimulation of pre-osteoblastic cells [274]. Thus, while a promising anabolic agent, different approaches to teriparatide administration may be needed to derive most cost-effective and patient-centered benefit.

Therefore, testing a locally-applied, single dose administration of teriparatide-containing hydrogel is an important step in improving the clinical utility of teriparatide as a means of supporting fracture healing among patients at risk for non-union. Our data demonstrated that locally-applied teriparatide improved radiographic union score among HFHS mice. Although radiographic score demonstrated this benefit, micro-CT parameters were similar to the fracture-only group.

Overall, the data suggests that the anabolic activity of locally-applied teriparatide is not as potent as repeated systemic injection but may have clinical utility. While the idea of a one-time
dose of the anabolic agent at time of surgery to improve fracture healing is appealing, it is perhaps unsurprising that repeated dosing over the course of 28 days had greater effect on fracture healing. Our lab has tested *in-vitro* release kinetics of the teriparatide-containing hydrogel and demonstrated that virtually all of the teriparatide was eluted from the hydrogel after a few days.\(^\text{16}\)

Although a period of long-term release was demonstrated, it is therefore reasonable to assume that the effect of pre-osteoblast stimulation is relatively short lived in this model compared to repeated systemic injection. Another weakness of this experiment that should be addressed is that in this experiment there was no group of mice that received only the hydrogel without teriparatide. Since the hydrogel may act as a scaffold on which immune and progenitor cells may more efficiently bind and interact at the fracture site, the modest effect of improved fracture healing may be attributed to the presence of the hydrogel at the fracture site, with the teriparatide playing only a minor role, if any. To test this hypothesis, another group of HFHS mice would have been needed to undergo fracture surgery and treatment with hydrogel only. As only a limited number of HFHS mice were available for this phase of the study, this experiment should be performed in the next phase of follow-up studies to close this knowledge gap.

**5. Use of teriparatide to improve fracture healing in a MRSA-infected open fracture model in diabetic and normal mice.**

This is the first study, to the author’s knowledge, to investigate infected fracture healing in using a diabetic mouse model. This has clinical importance, as diabetes is a major risk factor for post-traumatic infection as well as diminished fracture healing., as discussed in the introduction of this dissertation. It is the author’s hope that this work may lay the foundation for further investigation into treatment strategies in the setting of diabetic infected fracture.

\(^\text{16}\) The author did not participate in these experiments, therefore this data was not included in this dissertation.
This section of this dissertation was predicated on the hypothesis that introducing MRSA-infection and treating with antibiotics would result in diminished fracture healing in diabetic mice. In the first section of this discussion, “Rifampin-containing hydrogels reduce bacteria load and improve fracture healing in a MRSA-infected, open fracture mouse model”, the observation was made that treated and cleared MRSA infection resulted in reduced fracture healing that healing parameters. This observation was made in other similar studies [264].

Interestingly, however, this was not the observed result in diabetic mice. Following acute infection treatment with systemic antibiotics, RUST scores and micro-CT parameters improved; this result is being replicated in a second generation of experiments. This result is particularly surprising because, in contrast to our hypothesis, the presence and subsequent clearance of MRSA had an independent, positive effect on diabetic fracture healing. The author postulates two reasons for this observation: first, a potential osteogenic property of rifampin independent of its antibiotic effect, and second, altered inflammatory environment due to the presence of bacteria at the fracture site.

As discussed earlier in this section (page 96), rifampin has several effects at the cellular and molecular level, which may result in improved fracture healing in diabetic mice. It is an interesting bioactive compound because of its ability to enter cells and bind to RNA polymerase [103,105]. Rifampin’s potential role in diminishing or augmenting fracture healing has been debated with conflicting evidence, with some authors demonstrating rifampin’s detrimental effect on mesenchymal progenitor cells [265] while others show a positive or neutral effect on bone healing, potentially through BMP signaling [266]. Therefore, injecting HFHS mice with high doses of rifampin over three days may have enhanced molecular and cellular signaling at the fracture site, resulting in improved fracture healing outcomes. Of note, vancomycin was also co-delivered
with rifampin. However, vancomycin is an unlikely candidate for modulating the fracture healing process as has been shown to have only neutral properties on mesenchymal stem cells in previous studies [265], and does not target transcription/translation pathways.

To this end, a follow-up study was recently performed by injecting a series of diabetic mice with 3 days of rifampin (25mg/kg dose) following sterile fracture healing. Tissues have been harvested but x-ray and micro-CT results are pending. Although more follow-up studies are needed to evaluate histologic characteristics and biomechanics of the fracture site, this investigation poses a potentially exciting new avenue for improving fracture healing among diabetic patients using a drug that is already FDA approved and widely available.

The other potential mechanism that could account for the improvement in fracture healing in the case of treated acute infection is an altered inflammatory environment at the fracture site due to the presence of bacteria and killed bacteria particles. Despite the action of antibiotics which kill bacteria at the fracture site, killed bacteria debris would remain at the fracture site that would contribute to innate immune system signaling via PRRs and PAMPs (see introduction for a detailed discussion). This additional immune stimulation may potentially help recruit a more robust immune response without leading to the unchecked inflammatory response that is seen in the setting of infection. Further investigation into this effect would benefit from a set of experiments using killed bacteria to inoculate the diabetic fracture and observing resulting healing potential. The changes in the inflammatory milieu could by assessed by multiplex cytokine array. Further study of the effect of dead bacteria and inflammatory environment is warranted.

Nevertheless, addition of intermittent teriparatide injection over the course of 28 days, in addition to initial antibiotic therapy, improved micro-CT parameters compared to antibiotic treatment alone. This result is significant because, to our knowledge, it is the first time teriparatide
has been used to improve fracture healing in the setting of infection. However, the clinical relevance of this result is uncertain, as the teriparatide was administered with a powerful antibiotic combination that eliminated infection early in the healing process. The effect observed is likely similar to that of teriparatide in sterile fracture healing.

To improve clinical relevance of our investigation of teriparatide in infected fracture healing, the author proposes an infection model with delayed treatment. For this experiment, fracture surgery would take place, with MRSA infection occurring at the time of surgery. Treatment would begin 5-7 days later, with systemic antibiotics to reduce the infective burden. Daily antibiotic administration would occur for 21 days, with daily teriparatide injection. Animals would be euthanized 28 days after surgery, and the quality of fracture healing would be compared among sterile fracture, untreated MRSA infection, infection with antibiotics, and infection with antibiotics and teriparatide. The hypothesis would be that systemic teriparatide injection improves fracture healing as assessed by RUST score, micro-CT, and mechanical strength testing. This experiment could be extended to aged diabetic mice for greater clinical relevance.

The issue of infected fracture healing in diabetes is complex, with various inflammatory impetuses playing roles that are not fully understood. This very limited study demonstrates the potential for antibiotic and anabolic agents for improving fracture healing in case of infection. While more work needs to be done in the topic in order to draw more robust conclusions, there is potential for enhancing our clinical approaches to ultimately improve patient outcomes and move the field of fracture care forward.

5. Inflammatory fracture healing: summary, conclusions and future directions.

This dissertation provides fundamental data and methods for future studies in animal models and humans for improving outcomes in inflammatory fracture healing. This is an exciting
area of investigation because of its potential to improve patient outcomes and quality of life for many patients who receive orthopaedic care. That being said, this work is not without limitations and much more investigation is required.

Infection following open fracture is a devastating complication. Early antibiotic administration is the cornerstone of early fracture care, but does not eliminate infection in all cases. The results of antibiotic-loaded hydrogel experiments support a potential new approach to reducing infective burden and improving fracture healing. It was demonstrated that rifampin-loaded hydrogel reduces MRSA burden in an open fracture mouse model, the first study of its kind that the author is aware of in published literature to test antibiotic hydrogels on a resistant organism. In support of a recent paper submission, the author will be improving the clinical relevance of this data by testing a combinatory antibiotic hydrogel with rifampin and vancomycin.

Nonunion poses a burden on patient quality of life and our healthcare system, with infection being an important cause of failed fracture healing. While antibiotic therapy is the cornerstone of infection treatment, new approaches to enhancing healing in the setting of infection should be investigated. The histological data presented in this dissertation has identified a key issue in infected fracture healing, cartilaginous callus formation in an altered inflammatory environment. Therapies that support callus formation through proliferation and maturation of cartilage may be beneficial. Anabolic therapies such as teriparatide may also be effective. The author has proposed an experimental design of using teriparatide to enhance fracture healing in a chronic infection model, a project that will likely begin in the spring of 2020.

Diabetes is characterized by a pro-inflammatory state, with AGEs accumulation and activation of RAGE being key inflammatory factors contributing to diabetic bone disease. Diabetic patients are also more prone to infection. Currently, there are few therapies available for enhancing
fracture healing in diabetes that have strong clinical evidence. This dissertation has described a chronic, diet-induced diabetic mouse model that has been viable in studying diabetic fracture healing. This study attempted to use a local, teriparatide hydrogel to enhance fracture healing. Although the local teriparatide treatment improved radiographic healing, more investigation is required as to the effectiveness of this treatment and the role the hydrogel itself has as a biologic scaffold. Interestingly, the presence of MRSA infection treated by a systemic vancomycin-rifampin therapy significantly improved healing. Current investigation is being performed as to whether rifampin alone or the presence of dead bacteria mediate this effect.

In conclusion, this work, like most research projects, may have raised more questions than it answered. Despite this dissertation’s length, the author realizes its many limitations that require more data collection and further investigation. The author hopes that this work will allow some clinical benefit, large or small, by prompting either more basic science investigation or future clinical studies.
References


21. Chen H, Senda T, Kubo K: The osteocyte plays multiple roles in bone remodeling and


76. Bar-Shavit, Z. Taking a toll on the bones: regulation of bone metabolism by innate immune


[103] Sanchez CJ, Jr, Shiels SM, Tennent DJ, et al. Rifamycin derivatives are effective against
staphylococcal biofilms in vitro and elutable from PMMA. *Clin Orthop Relat Res* 2015;473(9):2874-2884.


start in post-teenage years in women with type 1 diabetes? *Diabetes care* 26:2365-2369.


[141] Chen FP, Kuo SF, Lin YC, Fan CM, Chen JF. Status of bone strength and factors
associated with vertebral fracture in postmenopausal women with type 2 diabetes.


[158] Yamamoto M, Yamaguchi T, Yamauchi M. Low serum level of the endogenous secretory


[167] Follak N, Kloting I, Merk H. Influence of diabetic metabolic state on fracture healing in


of periprosthetic joint infection following total joint arthroplasty?: A systematic review and meta-analysis. *Medicine (Baltimore).* 2017;96(51):e8805.


[236] Filion TM, Skelly JD, Huang H, et al. Impaired osteogenesis of T1DM bone marrow-derived
stromal cells and periosteum-derived cells and their differential in-vitro responses to

FGF-2 and BMP-2 genes in diaphyseal long bone radial defects in a diabetic rabbit model.

factor(rhPDGF-BB) and beta-Tricalcium Phosphate/Collagen Matrix Enhance Fracture

[239] Azad V, Breitbart E, Al-Zube L, et al. rhBMP-2 enhances the bone healing response in a


[250] Schindeler A, Morse A, Harry L, et al. Models of tibial fracture healing in normal and Nf1-

[251] Stavrakis AI, Niska JA, Shahbazian JH, et al. Combination prophylactic therapy with
rifampin increases efficacy against an experimental *Staphylococcus epidermidis*
subcutaneous implant-related infection. *Antimicrob Agents Chemother* 2014;58(4): 2377-
2386.

bacterial kill but not final outcome in a novel mouse model of *Staphylococcus aureus*

Has Enhanced Efficacy against an Experimental *Staphylococcus aureus* Prosthetic Joint


