2016

The Role of FoxO Transcription Factors in Alcohol-Induced Deficient Fracture Repair

Philip M. Roper
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

THE ROLE OF FOXO TRANSCRIPTION FACTORS IN ALCOHOL-INDUCED DEFICIENT FRACTURE REPAIR

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN INTEGRATIVE CELL BIOLOGY

BY

PHILIP M ROPER

CHICAGO, ILLINOIS

AUGUST 2016

ACKNOWLEDGEMENTS

I would like to thank everyone whose help has made this accomplishment possible. My family and friends have been a never-ending source of emotional support, specifically my mother who has always believed in me, Taylor who has given me more love and positivity than anyone has the right to ask for, and my Dad who made this all possible. I would also like to thank my Aunt Lynn, Aunt Laura, and Uncle Brian who have all taken an interest in my success and given me the advocacy and aid to succeed. You all share in this achievement.

I want to thank my committee for all of their invaluable counsel and direction. Finally, I cannot thank my mentor, John, enough. His guidance and patience as a boss, mentor, and friend have been paramount for my successful training and development.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AGE	Advanced Glycation End product
ANOVA	Analysis of Variance
APC	Adenomatous Polyposis Coli
BAC	Blood Alcohol Content
BCA	Bicinchoninic Acid assay
BMP	Bone Morphogenetic Protein
β2M	Beta 2 Microglobulin
cDNA	Complementary Deoxyribonucleic Acid
CFU	Colony Forming Unit
CK1α	Casein Kinase 1 Alpha
D-MEM	Dulbecco's Modified Eagle Medium
dL	Deciliter
Dsh	Dishevelled
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EtOH	Ethanol
FBS	Fetal Bovine Serum
FoxO	Forkhead Box O family of transcription factors
Fz	Frizzled

Gadd45	Growth Arrest and DNA Damage 45
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GSK3β	Glycogen Synthase Kinase 3 Beta
HSC	Hematopoietic Stem Cell
H&E	Hematoxylin and Eosin
IL	Interleukin
IGF	Insulin-like Growth Factor
IP	Intraperitoneal
JNK	c-Jun N-terminal Kinase
kD	Kilodalton
LEF	Lymphoid Enhancer Factor
LRP	Lipoprotein Receptor-related Protein
M-CSF	Macrophage Colony Stimulating Factor
MSC	Mesenchymal Stem Cell
MST1	Macrophage Stimulating 1
NAC	N-Acetyl-Cysteine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOX	NADPH Oxidase
OPG	Osteoprotegerin
OPN	Osteopontin
PBS	Phosphate-Buffered Saline
PGK1	Phosphoglycerate Kinase 1

PI3K	Phosphoinositide 3-Kinase
PP2A	Protein Phosphatase 2A
PVDF	Polyvinylidene Fluoride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RANKL	Receptor Activator of Nuclear Factor Kappa-B Ligand
RIPA	Radioimmunoprecipitation Assay buffer
ROS	Reactive Oxygen Species
RT	Reverse Transcription
Runx	Runt-related transcription factor 1
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
Sirt1	Sirtuin 1
SOD	Superoxide Dismutase
Sox	Sex determining region Y Box
TBS	Tris-Buffered Saline
TCF	T-Cell Factor
TGFβ	Transforming Growth Factor Beta
VEGF	Vascular Endothelial Growth Factor
Wnt	Wingless/Int signaling family or Wingless-related Integration site

ABSTRACT

Proper and complete repair of a bone fracture is essential in quality of life maintenance, but poor healing and fracture malunion are still medically and socially relevant problems. Alcohol abuse impairs normal fracture healing, leading to delayed or incomplete union. This dissertation aims to clarify mechanisms behind this alcohol-induced impaired healing, thereby elucidating potential methods of intervention.

Alcohol-induced oxidative stress has been linked to many morbidities associated with alcohol abuse. This dissertation elucidates a potential mechanism through which alcohol inhibits fracture healing by increasing oxidative stress. Using a rodent model, I found that alcohol exposure decreases fracture callus formation and endochondral ossification, and these changes are associated with markers of activation of FoxO transcription factors. FoxO transcription factors are known to be activated by oxidative stress and inhibit proper mesenchymal stem cell differentiation, which is crucial in callus formation. These deleterious effects of alcohol were prevented with the administration of an antioxidant. These results begin to illuminate how alcohol abuse can negatively affect fracture healing and bone health in general, while characterizing aspects of skeletal biology that are applicable beyond alcohol-associated pathologies.

CHAPTER 1

INTRODUCTION

Bone fracture healing is the end result of a complex orchestration of molecular and cellular events and processes. Proper and complete repair of a fracture is essential in quality of life maintenance, but poor healing and fracture malunion are still medically and socially relevant problems. Alcohol abuse is known to impair normal fracture healing, leading to delayed or incomplete bone union. This dissertation aims to clarify mechanisms behind this alcohol-induced impaired healing, thereby hopefully elucidating potential methods of intervention.

Alcohol abuse has been linked to many disease morbidities, and alcohol abusers have a significantly increased risk of developing osteopenia (1, 2). Alcohol abuse is also associated with a high percentage of traumatic orthopedic injury and an increase in perturbed healing, with binge drinking being the most problematic (3, 4, 5). Data from our laboratory and others have shown alcohol administration inhibits the normal healing process in rodents (9, 10, 21). Yet, there is still little known about the underlying mechanism of alcohol-induced deficient fracture repair.

Our *long-term goal* is to understand the mechanisms behind the effects alcohol exhibits on bone and fracture healing. The *objective of this dissertation* is to examine and elucidate how alcohol may be affecting a key oxidative stress

signaling pathway during indispensable regulatory steps of callus formation and fracture healing. Our ***Central Hypothesis is that alcohol-induced oxidative stress inhibits fracture repair through activation of FoxO transcription factors within the callus.*** This hypothesis is based on the following data. *First*, Wnt signaling and β-catenin activation are important for bone cells, especially the differentiation of mesenchymal stem cells (MSCs) and fracture healing. *Second*, our laboratory has demonstrated that alcohol perturbs Wnt signaling and downstream gene expression. *Third*, alcohol increases oxidative stress in many tissues, including bone, and data from our laboratory has shown that oxidative stress due to alcohol consumption plays a pivotal role in mediating the detrimental effects alcohol has on fracture repair. *Finally*, oxidative stress is known to activate FoxO transcription factors, and FoxOs need to bind β-catenin as a co-factor in order to upregulate their own target genes. Since β-catenin is the end effector molecule of the Wnt pathway, and there is only a limited pool of β-catenin that exists inside the cell. Any competition for β-catenin can lead to disruption of normal Wnt signaling. This disruption can have devastating consequences during times of fracture repair when Wnt signaling is of vital importance for proper differentiation of cells, and thus proper healing. The integration of all this information can provide a valid and novel molecular mechanism explaining the effects alcohol exhibits on fracture healing and bone homeostasis in general.

In order to test this hypothesis, two specific aims have been proposed:

Specific Aim 1: To Determine if Alcohol-Induced Oxidative Stress Antagonizes Normal Fracture Healing Through Activation of FoxOs in the Fracture Callus.

Working Hypothesis: Alcohol administration will lead to increased oxidative stress signaling in the fracture callus through FoxO activation, and this FoxO activation will be associated with perturbed fracture healing.

Experimental Approach: Aim 1 is an *in vivo* aim designed to test both the working hypothesis presented for Aim 1 as well as the central hypothesis for this research proposal. We utilized male C57BL/6 mice in order to investigate the ability of alcohol-induced oxidative stress to modulate intracellular signaling inside the fracture callus by examining protein expression, and we elucidated the effects of alcohol on specific callus components by examining the formation of the callus using histology. We also utilized an antioxidant to determine which of these effects are caused by alcohol-induced oxidative stress.

Specific Aim 2: To Elucidate Whether FoxO-Mediated Oxidative Stress Signaling Underlies Alcohol-Induced Perturbation of MSC Differentiation.

Working Hypothesis: EtOH-induced oxidative stress will be able to activate FoxO transcription factors within cultured MSCs, and this FoxO activation will inhibit the normal osteo-chondro lineage differentiation of MSCs.

Experimental Approach: Aim 2 utilized cultured MSCs to more closely examine the ability of alcohol to induce FoxO transcription factor activation specifically within MSCs. This helps to elucidate the impact alcohol and oxidative

stress are having on MSC commitment, inevitably leading to problems in fracture repair. To this end, we have cultured MSCs with EtOH and determined the ability of EtOH to induce FoxO expression, as well as its ability to increase FoxO activation, as measured by nuclear translocation, DNA binding, and FoxO-target gene expression.

CHAPTER 2

REVIEW OF THE RELEVANT LITERATURE

Pertinent Bone Biology

The human skeleton begins with 270 bones at infancy, but this number decreases to a total of 206 in the adult skeleton through fusion of some bones during development. Despite its apparent placidity, the skeleton is a complex, dynamic, and active system that is affected by, and has a wide range of effects on, many other systems in the body. The skeleton is also constantly in a state of turnover, with old bone being removed, or resorbed, and new bone being deposited. While ostensibly the main purpose of the skeletal system is to simply provide protection for vital organs within the body and essential support for muscles, tendons, and ligaments during locomotion, it acts as the body's main repository for minerals, is a vibrant niche for stem cells involved in blood and immunological support, buffers blood pH, and is even involved in the body's overall metabolic state.

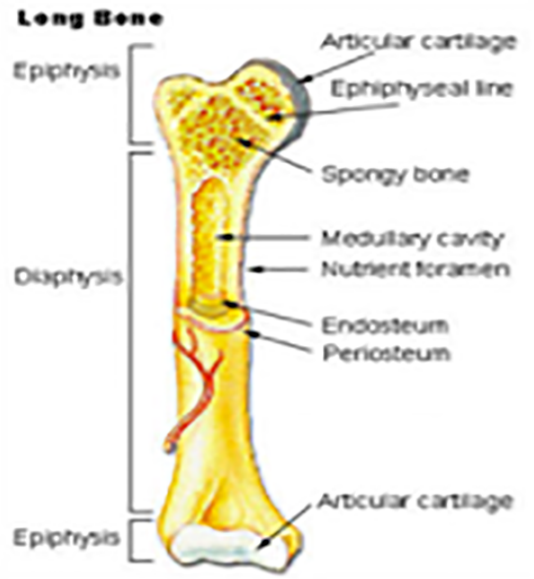
The skeletal system is divided into the axial skeleton, formed by the vertebral column, the rib cage, and the skull, and the appendicular skeleton, formed by the pectoral girdles, the upper limbs, the pelvis, and the lower limbs. Essentially, anatomists distinguish between two types of bones in the skeleton: flat bones, like the bones that comprise the skull and mandible, and long bones,

such as the bones of the limbs, the femur and humerous for example. These two bones are distinguished by their anatomy and their methods of formation. Flat bones, as their name would suggest, appear wide and flat and are formed by a process known as intramembranous ossification, in which bone mineral is directly deposited by osteoblasts without the use of a cartilaginous intermediary (42, 43). Long bones, conversely, are formed by endochondral ossification, which involves chondrocytes forming a cartilaginous matrix first that osteoblasts can later use to deposit mineralized bone (42, 43). Also, long bones are long, comprised of a hollow shaft, encapsulating the marrow cavity, known as a diaphysis and two cone-shaped ends known as metaphyses (42, 43) (Figure 1).

The skeleton is also comprised of two different types of bone. Cortical bone is very dense and comprises the diaphysis of bones, whereas trabecular bone (also known as cancellous bone or spongy bone), while still hard and supportive, appears honeycomb-like and makes up the metaphyses of bones (42, 43). Bones have an outer fibrous sheath called the periosteum and an inner sheath called the endosteum. The periosteum contains nerve endings and blood vessels. The endosteum lines the marrow cavity, in addition to lining the surface of trabecular bone (42, 43) (Figures 1 and 2).

At the microscopic level, bone itself is made up of structural units known as osteons (42, 43). Osteons, also known as Haversian systems, are comprised of concentric sheaths of bone circling a canal in the center. At this level, bone also contains canals for blood vessels to traverse known as Volkman’s canals.

Endosteum also covers the internal surfaces of these canals (42, 43) (Figures 2 and 3).



.

Figure 1. The anatomy of the long bone.

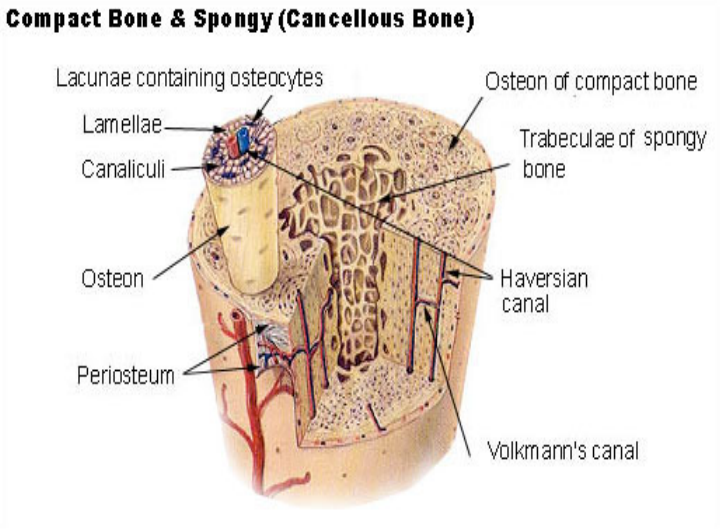


Figure 2. The structure and microanatomy of the long bone.

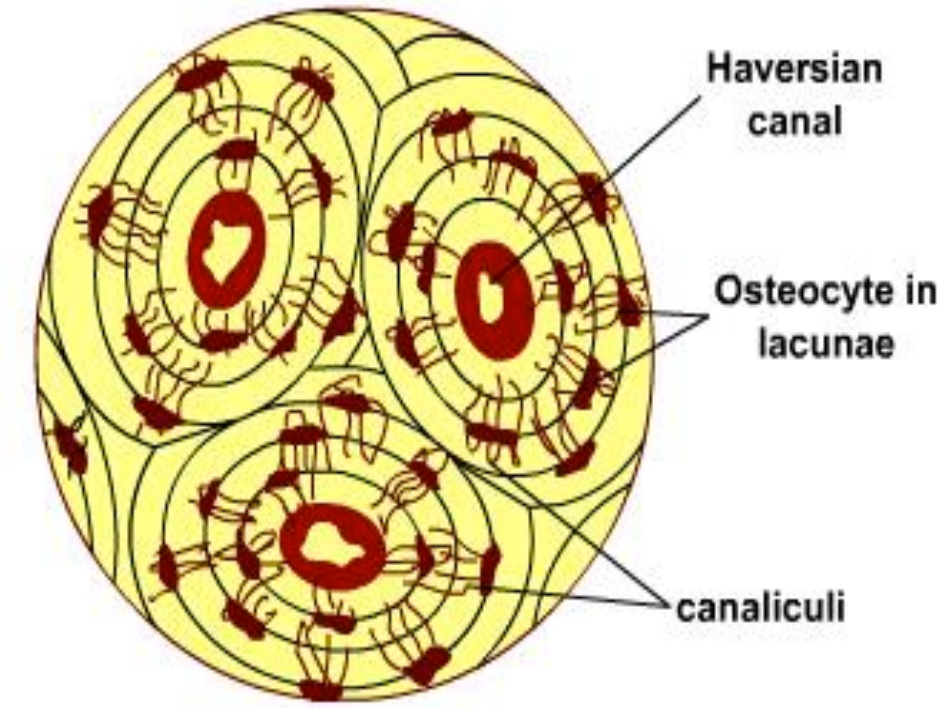


Figure 3. Illustration of osteocytes in Haversian systems within bone.

There are primarily four cells involved in bone formation and homeostasis. These are osteoblasts, osteocytes, chondrocytes, and osteoclasts. Osteoblasts are the cells primarily involved in creating bone by depositing a matrix known as osteoid, which is mostly made of type I collagen. This collagenous matrix will then be mineralized by the deposition of inorganic salts composed mostly of Calcium and phosphates. This mineral-phosphate component is stored in a form known as hydroxyapatite, [Ca10(PO4)6(OH)2], and gives bone its rigidity and strength. Type I collagen provides bone with its elasticity and flexibility (63). Both combine to ensure bone is strong enough to bear great weight, yet flexible

enough to not be brittle and easily broken. The detection of alkaline phosphatase enzyme activity is one of the earliest markers of the osteoblast phenotype, and is used to determine osteoblast activity and bone formation histologically as well as clinically (45). Osteocalcin is also produced by osteoblasts and similarly used as a marker of bone formation (63).

Osteocytes are terminally differentiated osteoblasts that become trapped within the osteoid matrix they secrete (45, 47). Once trapped, the osteocytes begin to form cellular extensions of filapodia processes that extend through the bone matrix in order to make connections with other osteocytes throughout the surrounding bone. The spaces within the bone matrix occupied by osteocytes and their processes are termed lacunae and canaliculi, respectfully (45). These connections ensure osteocyte viability and communication. Communication is especially important for osteocytes as they function to control bone remodeling and turnover through signaling with osteoblasts and osteoclasts (47). Osteocytes are mechanosensor cells that can sense bone loading and transmit activating signals to osteoblasts on the bone surface. Osteocytes also produce sclerostin, a secreted protein that acts to inhibit bone formation by binding to LRP5/6 coreceptors and blunting the canonical Wnt signaling pathway (will be discussed) (47) (Figures 2 and 3).

Chondrocytes are essential for endochondral ossification, and are thus essential for the formation of a majority of the skeleton and the repair of fractures. These cells undergo a very specific spatial and temporal pattern of

gene expression in order to coordinate the various cellular processes needed to give rise to new bone (40, 41). During endochondral bone development chondrocytes undergo ordered phases of proliferation, hypertrophic maturation, and eventual death. It is during proliferation that chondrocytes produce type II collagen. Once chondrocytes mature into hypertrophic chondrocytes they begin producing type X collagen (40, 41, 49). It is immediately following this stage that the hypertrophic chondrocytes begin to undergo apoptosis allowing for the invasion of osteoblasts to begin ossifying the deposited matrix of collagen X (40, 41, 48, 49).

Osteoclasts are unique because they are derived from hematopoietic stem cells (HSCs), specifically the fusion of macrophages, unlike osteoblasts and chondrocytes that are of mesenchymal stem cell (MSC) origin, leaving osteoclasts with characteristic multiple nuclei (51). The cytokines macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) are necessary and sufficient to drive osteoclastogenesis (50). Osteoclasts are exclusively tasked with the process of bone resorption. They act to resorb bone by using integrin proteins on their cell surface to form a microenvironment with the bone matrix. Once this isolated environment is achieved, osteoclasts use a proton pump to acidify this space, which causes the mineralized component of bone to mobilize and leave the organic matrix vulnerable to enzymatic degradation by the osteoclast (50). RANKL not only drives osteoclast development, but also acts to activate their bone resorption.

Osteoprotegrin (OPG) is the physiological inhibitor of RANKL, acting to essentially increase bone formation (50).

New bone formation, known as ossification, can be divided into two different mechanisms. Intermembranous ossification involves osteoblasts developing directly from mesenchymal stem cell condensations without needing a cartilaginous intermediate. This is primarily used in the formation of the flat bones of the skeleton. Endochondral ossification requires chondrocyte development from the mesenchymal condensations. These chondrocytes then form an extracellular matrix template for osteoblasts to later ossify. The long bones of the skeleton are created through endochondral ossification (40, 41, 42, 43).

The process by which new bone is formed during development is called modeling. However, the process by which bone is undergoing constant turnover to promote normal bone health and homeostasis is called remodeling. Bone remodeling involves the osteoclasts removing old bone, and osteoblasts replacing that resorbed bone with new bone tissue. An average of 10% of the total bone volume of an adult skeleton is replaced every year. Bone remodeling is necessary as it replaces old bone and bone damaged by either fractures or naturally occurring microdamage (79). This process is tightly regulated. It is necessary to ensure healthy, strong, functional bone, but a disregulation of either side of the process can lead to metabolic bone diseases, creating a state of high bone mass or osteoporosis. Bone remodeling also acts to reshape bone and

responds to functional demands of mechanical loading and hormonal signals (79).

Bone formation and resorption play vital roles in maintaining the body's serum mineral levels and pH balance (81). Bone is the body's main mineral repository, namely for calcium, and thus the body needs the processes of bone deposition or resorption to store or release Calcium, respectively. Chief cells in the Parathyroid glands secrete parathyroid hormone, which acts on osteoblasts to increase production of RANKL and decrease production of OPG, an inhibitor of RANKL (80). RANKL then acts to promote osteoclastogenesis to promote bone resorption (80, 81). This process serves to regulate the calcium and phosphate balance in the blood by releasing Ca2+ and P04. The parafollicular cells of the thyroid gland act antagonistically by releasing calcitonin, which serves to promote Ca2+ storage by inhibiting osteoclasts, among other ways (82).

Mesenchymal Stem Cells and Wnt Signaling

Mesenchymal stem cells (MSCs) are a group of multipotent stem cells that differentiate into a variety of cell types including osteoblasts, chondrocytes, and adipocytes, making them indispensible in bone biology and fracture healing (42, 43, 44). MSCs were first identified as colony-forming unit-fibroblasts found in the bone marrow stromal cell compartment, leading to MSCs also being known as marrow stromal cells or multipotent stromal cells (83, 84). Now many more tissue sources and niches for MSCs are known. They have been found in blood, adipose tissue, trabecular bone, muscle, and dermis (85). MSCs have the classic

properties of stem cells in that they self-renew and are multipotent. They are adherent and fibroblast-like. Since initial characterization of these cells, there has been extensive work done to elucidate the full differentiation potential of these cells. Under the right conditions MSCs can differentiate into other lineages, including myoblasts, cardiomyoblasts, tenoblasts, and neuroblasts, with the differentiation of these cells being fairly plastic, at least in culture (19).

MSCs eventually give rise to osteoblasts and chondrocytes at the onset of skeletal development. Osteoblastic commitment, differentiation, and function are all controlled by various transcription factors that drive the expression of osteoblast phenotypic genes. Runx2 has long been known as the major osteoblastic transcription factor (46, 52). Runx2, a member of the Runt family of transcription factors, begins and controls the differentiation of MSCs throughout their development into mature osteoblasts (46, 52). Chondrogenic differentiation, however, is mainly controlled by the transcription factor Sox9, and Sox9 is expressed throughout chondrocyte maturation, beginning at development, through the proliferative phase, and into the hypertrophic phase (40, 41, 49). Runx2 also plays a role in chondrocyte hypertrophy. It begins being expressed at this phase and helps facilitate the eventual invasion and activation of osteoblasts, as well as the apoptosis of the hypertrophic chondrocytes themselves (40, 41, 48, 49, 78).

Canonical Wnt signaling is indispensable in the formation and upkeep of bone health as Wnt signaling drives MSCs toward an osteoblastic or

chondrogenic lineage, while suppressing adipogenesis (23, 24, 64, 78). The end effector molecule in the Wnt signaling pathway is the transcription factor β-catenin, which is normally regulated by an inhibitory complex that phosphorylates β-catenin in the cytosol of the cell targeting it for degredation. This complex is made up of the proteins Axin, adenomatous polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 β (GSK3 β), and casein kinase 1α (CK1α) (23, 24, 44). Canonical Wnt signaling relies on soluble Wnt ligands binding to the G protein-coupled, seven transmembrane receptor Frizzled (Fz) and the LRP5 or LRP6 coreceptor. Once Wnt binds to the Fz/LRP receptor complex, Fz activates the protein Dishevelled (Dsh) within the cytosol. Activated Dsh can then act to inhibit GSK3β and axin (44, 65). This disrupts the inhibitory complex and results in the accumulation of β-catenin. Once β-catenin is free to accumulate inside the cytosol of the cell, it can translocate into the nucleus, bind to the cofactor TCF/LEF, and then upregulate specific target genes, which in the case of osteoblastogenesis or chondroblastogenesis can be Runx2 or Sox9, respectively (44, 65) (Figure 4).

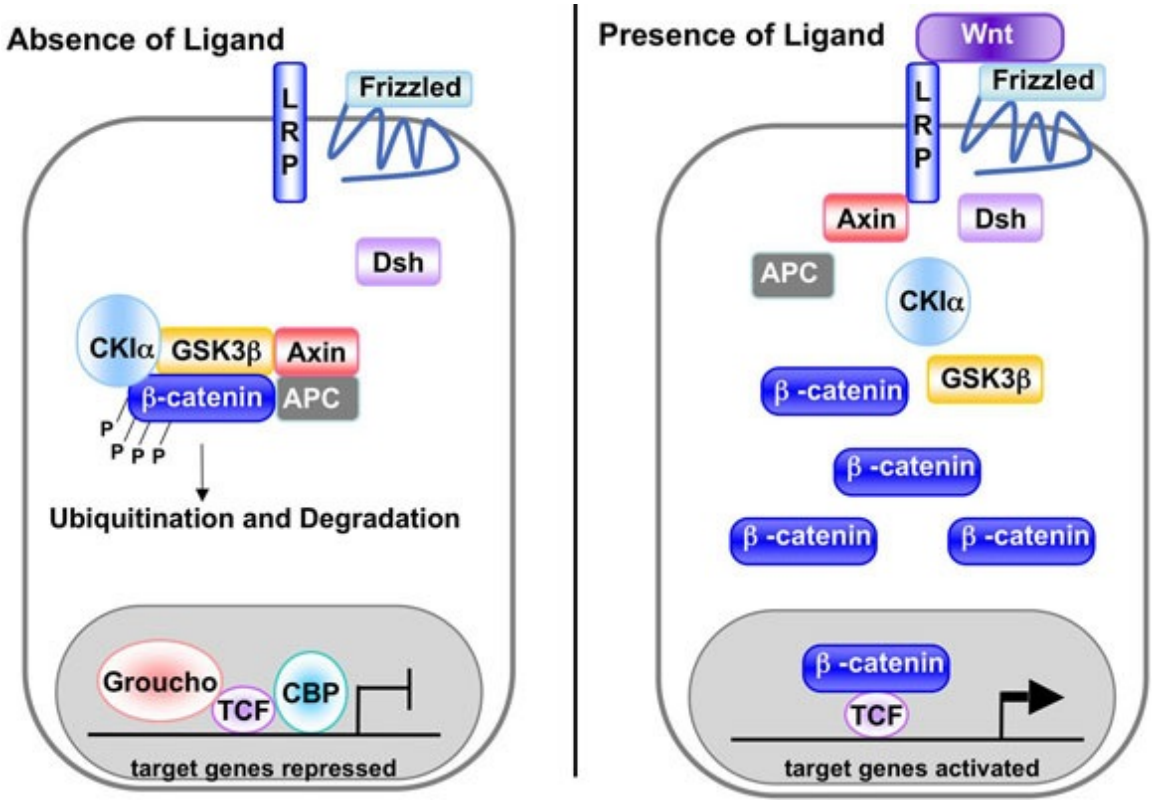


Figure 4. The canonical Wnt signaling pathway. Wnt ligand binds to the LRP/Frizzled coreceptors to initiate a signaling cascade that disrupts the degredation complex, leading to β-catenin accumulation and translocation into the nucleus.

Fracture Healing

Fracture healing is a very intricate and complex series of molecular and cellular events coordinating to create a unique and specialized form of wound healing. It is one of only a few forms of wound repair that leaves no scar. Fracture healing is also a process that somewhat recapitulates the formation of the developing skeleton embryologically. Thus, two methods of fracture healing occur, endochondral ossification, and intramembranous ossification. Semi-

stabilized, through the use of a cast, splint, or intramedullary fixation, fractures heal predominantly through endochondral ossification with some contribution from intramembranous ossification (19, 66). Semi-stabilized fractures have a small amount of movement at the fracture site, known as micromotion. This leads to the periosteum, bone marrow, and surrounding soft tissues becoming importantly involved in the wound healing (19, 66).

Fracture healing can be essentially broken down into four steps: hematoma formation, inflammation, callus formation, and bone remodeling (19). Hematoma formation occurs almost instantly once blood vessels in and around the bone are ruptured. This creates a minor amount of stabilization to the fracture site and leads into the inflammatory state. Inflammation is marked by the infiltration of immune regulators, namely macrophages, leukocytes, and lymphocytes that secrete proinflammatory cytokines. A complex cascade is initiated, involving inflammation effectors (IL-1, IL-6), mitogens (TGF-β, IGF), bone morphogenic proteins (BMPs), and angiogenic factors (VEGF) to coordinate angiogenesis and healing within the callus (19, 66). These mitogenic and morphogenic proteins have been shown to be expressed in the fracture callus within 24 hours of fracture, and can stay upregulated in the callus for anywhere between 7 and 28 days (66).

Another important family of proteins involved in fracture healing is the Wnt family of glycoproteins discussed earlier (64, 67, 68, 13, 86). Wnt proteins can be found upregulated in the callus as soon as 3 days post-fracture, and can remain

upregulated as long as 21 days post-fracture (70, 71, 13, 59). As discussed earlier, Wnt proteins, and Wnt/ β-catenin signaling, are indispensible in MSC differentiation towards and osteo-chondroprogenitor lineage, which makes Wnt signaling vitally important in callus formation during fracture healing. MSCs reside in a number of niches, namely, when pertaining to fracture healing, in the periosteum and the bone marrow (85, 19, 66). Under the direction of BMPs and Wnt proteins, these MSCs can differentiate. MSCs in the periosteum preferentially develop into osteoblasts that begin forming a section of the callus known as the periosteal callus, which is primarily formed by osteoblasts through intramembranous ossification (58, 59, 66, 69). MSCs within the bone marrow preferentially develop into chondrocytes that form the external callus portion of the fracture callus. The external callus makes up the majority of the callus volume and is composed of a cartilaginous matrix deposited by chondrocytes that will eventually become ossified through endochondral ossification (58, 59, 66, 69).

The time course of fracture healing in the rodent is on the order of days, with cartilage formation peaking around 9 days post-fracture (59, 66, 69, 72). The external callus has reached its maximum volume around day 14 after fracture and has undergone mineralization (59, 66, 69, 72). The fracture callus volume begins decreasing after two weeks, and the fracture finally unifies and reaches normal stiffness at 4 weeks post-fracture (69, 72). It is after this point when the

bone undergoes extensive remodeling to restore the normal bone architecture and morphology.

As previously stated, the family of Wnt proteins have been shown to be upregulated and expressed in the fracture callus during healing (64, 70, 71, 13, 59). Canonical Wnt signaling has also been shown to be vital to proper callus formation and eventual fracture healing. Wnt signaling inhibition coincides with inhibition of proper callus histology, volume, and strength. This is followed by overall delays in healing and fracture malunion (13, 59, 64, 67). Conversely, Wnt/β-catenin stabilization or enhancement acts to augment fracture healing, even mitigating the inhibition of healing caused by some factors (9, 62, 67, 68).

Alcohol Abuse and Bone

The number of annual fracture injuries occurring in the US is approximately 5 to 6 million (12). Alcoholics have an increased risk of developing osteopenia and a fracture rate four times higher than that of non-abusers (2, 10, 11, 56). Additionally, 25-40% of orthopedic trauma patients have a positive blood alcohol content at the time of hospital admission. Alcohol abuse has been shown to decrease overall bone mineral density, and alcohol consumption significantly raises the risk of fracture malunion and non-union (3, 56). Patients with alcohol on board at the time of injury also have a significantly longer fracture healing time as compared to patients with no blood alcohol content (56).

Alcohol is thought to be the most harmful drug when the harm to both users and others is taken into consideration (55). There are myriad methods of

alcohol abuse, and many of the alcohol-related maladies are not limited to the chronic alcohol abuse of alcoholic as traditionally thought (75, 76, 77). A rising number of alcohol abusers report binge drinking, and it appears to be a more significant cause of maladies than chronic drinking (54). 50% of the alcohol consumed by adults and 90% consumed by youths is consumed by binge drinking (57). Also, up to 78% of trauma patients report binge drinking (54). Binge drinkers are an especially at risk group because they tend to normally drink very little except during a binge episode. It is during a binge episode, defined by at least 4 drinks for a woman or 5 drinks for a man per hour, when normal alcohol tolerance is greatly exceeded that these drinkers significantly increase their chance of injury (75, 76, 77).

In addition to the clinical evidence, there is basic science data demonstrating that alcohol has a deleterious effect on bone health and disrupts the normal process of fracture healing (2, 6, 9, 10, 21). Both chronic and acute methods of alcohol exposure in rodents lead to increases in markers of osteopenia and bone resorption, leading to a decrease in overall bone strength and bone mineral density (8, 9, 10). Also, binge alcohol exposure has been shown to differentially decrease the expression of genes related to bone formation and osteoblast activity while increasing the expression of genes related to osteoclast activity (8, 9). Some of these alcohol-related decreases in bone mass and strength, as well as changes in the transcriptome, remain after periods of abstinence (7, 8, 9). There is also evidence that alcohol negatively affects the

osteogenic and chondrogenic potential of MSCs, even promoting their adipogenic differentiation in culture. MSCs that have been isolated from alcoholics show a decreased ability to differentiate into osteoblasts compared to MSCs from normal patients (93). Concurrently, MSCs isolated from non-alcohol-abusing patients but cultured with ethanol *ex vivo* show a perturbed differentiation potential, with osteogenic differentiation suppressed and adipogenic differentiation promoted even in the presence of an osteogenic medium (33).

In rodent models of binge alcohol administration fracture healing is similarly perturbed. Our laboratory has shown that mice and rats treated with alcohol show significant impairment of fracture healing as compared to their saline-treated cohorts. This is exemplified by perturbed callus histology and morphology, along with decreased callus volume and strength (9, 58, 59, 61, 62). Binge alcohol treatment is also shown to disrupt canonical Wnt signaling by decreasing the levels of β-catenin within the callus, as well as the expression of Wnt target genes (59). Our laboratory has also shown that the deleterious effects alcohol exhibits on fracture healing could be mitigated by treatment with antioxidants (9), the addition of MSCs systemically (58), or the activation of the canonical Wnt signaling pathway (62).

Yet the mechanism underlying this alcohol-related disruption in fracture repair is incompletely understood. Lately, much of the pathogenesis of alcohol abuse has been attributed to alcohol's systemic pro-oxidative quality (16).

Alcohol's ability to induce oxidative stress systemically comes from the metabolism of EtOH and its metabolites by alcohol dehydrogenase, microsomal ethanol oxidation system, or catalase, leading to the production of reactive oxygen species (ROS) (16). ROS can also be produced from EtOH through the induction of intracellular NADPH oxidase (NOX) activity (18). These data suggest that alcohol can increase oxidative stress inside distinct cells *in vivo* (22). Also, a recent study has shown that inhibiting NOX activity *in vivo* curbs ethanol induced bone loss in a rat model (20), suggesting that oxidative stress created from NOX activation impairs bone homeostasis contributing to bone loss. As previously mentioned, the potent antioxidant N-acetyl cysteine (NAC) has been shown by our laboratory to reverse the damaging effects of alcohol on fracture repair in a rat model (9), further validating the idea that alcohol-induced oxidative stress is a principal contributor to decreased callus size, strength, and endochondral ossification. Our laboratory also has found elevated markers of oxidative stress in the serum of animals receiving the binge alcohol treatment paradigm (data not published).

Based on the evidence from our laboratory, as well as others, alcohol is having an effect on canonical Wnt signaling in bone apparently through systemic oxidative stress induction (9). The FoxO family of transcription factors has now proven to be of great interest to our laboratory because FoxOs responds to elevated oxidative stress (26, 87).

There are three predominant members of the FoxO (forkhead box O) family of transcription factors, FoxO1, FoxO3, and FoxO4 (26, 89, 90). FoxOs have been shown to be activated by oxidative stress through GTPase ral-JNK and MST1 signaling, and inhibited by insulin signaling through PI3K-Akt activity (87, 88, 91, 92). Akt phosphorylate FoxOs on specific residues leading to FoxOs being bound to the 14-3-3 chaperone protein and sequestered in the cytosol of the cell (26, 88, 89). JNK and MST1 phosphorylate FoxOs on separate residues causing a disruption of the FoxO-14-3-3 protein complex, allowing FoxOs to translocate into the nucleus (87, 89, 90, 91). Once FoxOs localize within the nucleus, they bind the transcription factor β-catenin in order to upregulate the expression of target genes, including catalase, super oxide dismutase (SOD), and Gadd45 (87, 89, 91, 92) (Figures 5 and 6).

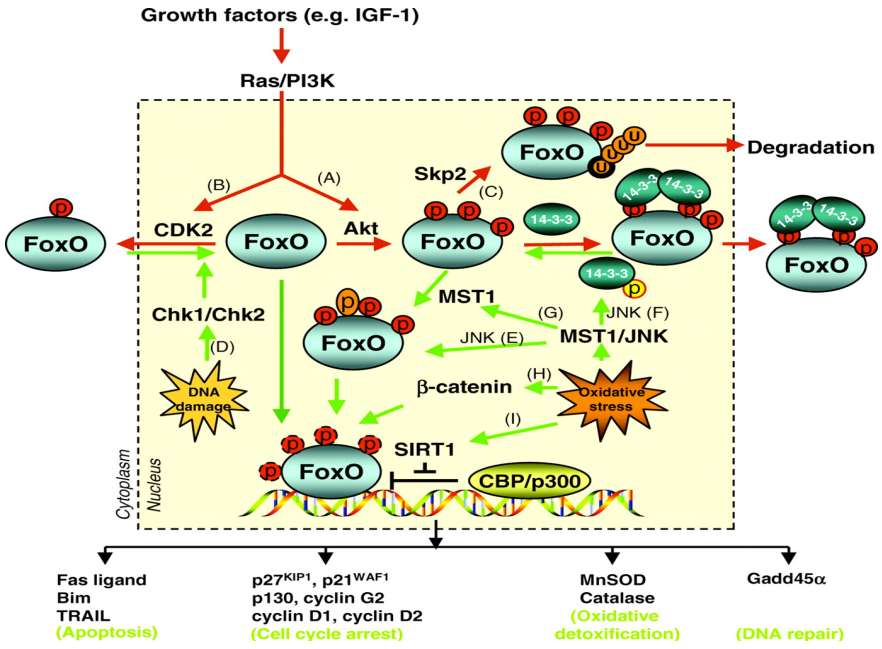


Figure 5. FoxO transcription factor activation. Associated protein interactions and activation included.

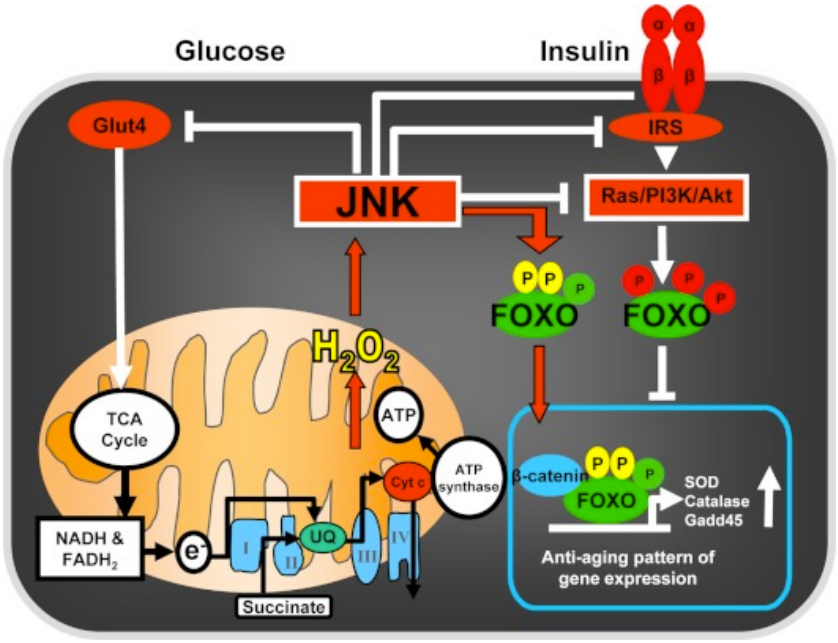


Figure 6. FoxO transcription factor post-translational modification modulation. The FoxOs translocation to the nucleus, binding β-catenin as a cofactor, and upregulating specific target genes included.

Oxidative stress is shown to activate FoxOs (26, 87), and once activated FoxOs bind β-catenin as a cofactor in order to upregulate specific target genes (29). This ability of oxidative stress to activate FoxO has also been shown to lead to a decrease in Wnt/β-catenin signaling by diverting β-catenin away from TCF/LEF in cells (15, 29, 35, 36, 73). Also, antioxidant treatment has been shown to augment Wnt signaling and osteoblastogenesis in MSCs (36, 37) and, as previously mentioned, antioxidants have been shown to reverse alcohol-induced deficient fracture healing (9). Additionally, it has recently been shown that targeted deletion of FoxOs in osteogenic progenitors in mice leads to increased bone mass and decreased adipogenesis in bone throughout life (74).

We believe that alcohol is able to mediate its effects on fracture healing by activating FoxOs through its induction of oxidative stress. The alcohol-induced increase in oxidative stress activates FoxOs, and once activated FoxOs compete with normal canonical Wnt signaling for a limited intracellular pool of the transcription factor β-catenin. This competition effectively suppresses Wnt signaling during important stages of MSC differentiation within the fracture callus, leading to perturbed fracture healing (Figure 7).

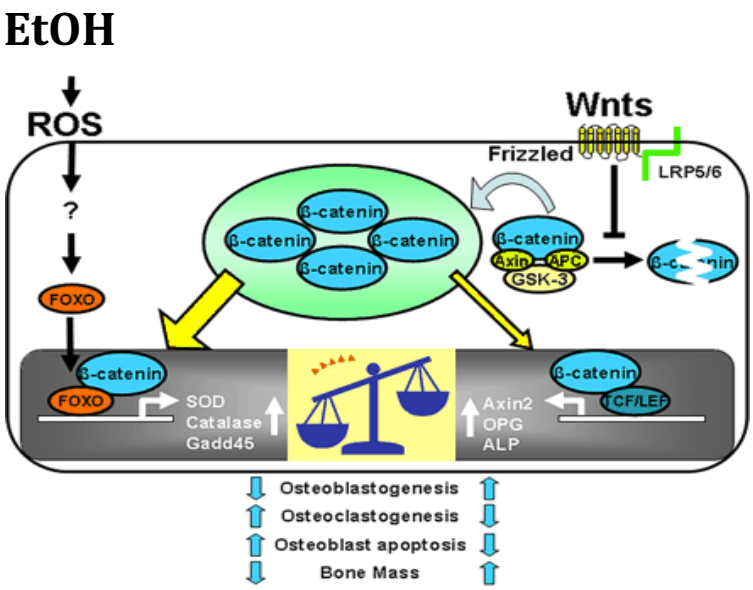


Figure 7. Schematic of functional antagonism of canonical Wnt signaling by FoxO transcription factor activation. Alcohol-induced oxidative stress can activate FoxOs. Once FoxOs are activated, they compete with Wnt signaling for the transcription factor β-catenin, which is the end effector molecule of the Wnt pathway.

Conclusions

Existing research into deficient fracture repair caused by alcohol exposure has focused on alcohol's deregulation of the inflammatory phase of fracture repair, without providing a proper mechanistic explanation of observed pathologies within the fracture callus. Our laboratory has focused on critical intracellular signaling and has identified canonical Wnt signaling as a target of alcohol consumption. We have also shown that the detrimental effects alcohol exhibits on fracture healing are attenuated with antioxidant treatment, implicating alcohol-induced oxidative stress as a causative agent. This research proposes to elucidate a mechanism linking alcohol-induced oxidative stress, perturbed Wnt

signaling, and poor fracture healing by examining the effects alcohol has on oxidative stress signaling and MSC differentiation. We will use both *in vivo* and *in vitro* models of alcohol administration and employ novel techniques in order to accurately assess the process by which alcohol is mediating these deleterious effects. Through this we will clarify the role of FoxO-mediated oxidative-stress signaling in this process, ultimately leading to a more complete understanding of the pathology of alcohol-induced fracture healing.

The proper orchestration of the fracture healing process is highly complicated and convoluted. Thus, this work understandably does not expect to completely explain every influence in fracture repair perturbation, but simply proposes to gleam some further insight into some of the myriad pathways and their interactions. And hopefully this greater understanding will help elucidate innovative therapies to ameliorate impaired fracture healing by modulating FoxO or Wnt/β-catenin signaling activity during repair.

CHAPTER 3

ALCOHOL-RELATED DEFICIENT FRACTURE HEALING IS ASSOCIATED WITH ACTIVATION OF FOXO TRANSCRIPTION FACTORS IN MICE

Abstract

The process of fracture healing is complex, and poor or incomplete healing remains a significant health problem. Proper fracture healing relies upon resident mesenchymal stem cell (MSC) differentiation into chondrocytes and osteoblasts, which are necessary for callus formation and ossification. Alcohol abuse is a leading contributor to poor fracture healing. Although the mechanism behind this action is unknown, excessive alcohol consumption is known to promote systemic oxidative stress. The family of FoxO transcription factors is activated by oxidative stress, and FoxO activation antagonizes Wnt signaling, which regulates mesenchymal stem cell differentiation. We hypothesize that alcohol exposure increases oxidative stress leading to deficient fracture repair by activating FoxO transcription factors within the fracture callus which disrupts chondrogenesis of mesenchymal stem cells. Our laboratory has developed an experimental model of delayed fracture union in mice using ethanol administration. We have found that ethanol administration significantly decreases external, cartilaginous callus formation and hallmarks of endochondral ossification, and these changes are concomitant with increases in FoxO

expression and markers of activation in fracture callus tissue of these mice. We were able to prevent these alcohol-induced effects with the administration of the antioxidant n-acetyl cysteine (NAC), suggesting that alcohol-induced oxidative stress produces the perturbed endochondral ossification and FoxO expression.

Introduction

Bone fracture healing is the end result of a complex orchestration of molecular and cellular processes. Although most fractures heal normally, fracture nonunion is a significant clinical issue, with approximately 10% of long bone fractures, and up to 19% of tibia fractures, failing to heal normally (96). Therefore, it is critical to gain a better understanding of the processes leading to abnormal fracture repair in order to find ways to bolster proper healing. A leading contributor to delayed and incomplete fracture union is alcohol abuse, which is also a well-known public health problem (10, 55). Alcohol abusers have an increased risk of developing osteopenia, and have a fracture rate four times higher than that of non-abusers (2, 10, 11, 56). Up to 40% of orthopedic trauma patients present with a positive blood alcohol content at the time of hospital admission, and alcohol consumption significantly raises the risk of healing complications, leading to nonunion and increased fracture healing times. Additionally, many trauma patients continue drinking after sustaining an injury (3, 5, 56). Basic science research using rodents has also shown that both acute and chronic alcohol administration have deleterious effects on bone health, as well as the ability to disrupt healing (2, 6, 8, 9, 11, 21). Our laboratory has developed an

experimental model of delayed fracture union in mice using a model of repeated episodic ethanol administration, as binge drinking, defined as reaching a blood alcohol concentration of 0.08 g/dL, has recently been shown to be more prevalent than chronic alcohol consumption, has highly significant deleterious health and social effects, and has a greater association with traumatic injury than chronic consumption (5).

Long bone fracture injuries that are semi-rigidly stabilized tend to heal through both intramembranous and endochondral ossification, processes that rely on mesenchymal stem cells at the fracture to differentiate into chondrocytes and osteoblasts (97). At the initiation of endochondral ossification, chondrocytes are needed to produce cartilage and form the robust cartilaginous callus around the fracture site. This supplies the matrix that osteoblasts will eventually ossify, leading to a complete union. This whole process completely hinges on complex intracellular signaling cascades within resident MSCs to differentiate properly during the initiation of healing, and any perturbation of these pathways could have significant effects downstream.

Previous research into the effects of alcohol abuse on fracture healing has predominantly used models of osteotomies, focusing on late-stage mineralization or early changes in systemic and local inflammatory markers (6, 11, 98, 99, 100, 101, 102). While important, these studies fail to examine the effects of alcohol on a clinically-relevant model of fracture healing, leaving much to be elucidated

about how alcohol affects early stages of healing, and the mechanisms behind these effects.

The metabolism of alcohol is known to contribute to increased systemic oxidative stress and reactive oxygen species (ROS) accumulation (16). Any increase in oxidative stress or shift in the redox state of the intracellular milieu can perturb key cellular mechanisms. One molecular responder to oxidative stress is the family of FoxO (forkhead box O) transcription factors, in which there are three predominant members, FoxO1, FoxO3, and FoxO4 (26, 89, 90). Oxidative stress is shown to activate FoxOs, and once activated FoxOs regulate the expression of genes related to oxidative stress resistance and cell cycle inhibition (26, 87). Increased oxidative stress, and the subsequent increase in FoxO activation, has been shown to be detrimental to overall bone health (15, 74). A reduction in FoxO expression lead to accelerated skin wound healing (103), however, the role of FoxO activation in fracture healing remains less well elucidated. To this point, our laboratory has devised a study to determine the effects alcohol has on specific callus components during fracture healing, and if alcohol has the ability to influence FoxO activity at any time during this process.

FoxO activation is especially intriguing to investigate during fracture healing because MSC differentiation towards chondrocytes and osteoblasts is controlled in large part by canonical Wnt signaling (23, 24, 64, 78). Activation of canonical Wnt signaling leads to the translocation of the transcription factor β-catenin into the nucleus where it can regulate specific target genes related to

chondrocyte or osteoblast differentiation (23, 24). Activated FoxOs bind β-catenin as a necessary cofactor in order to up regulate specific target genes related to oxidative stress resistance (29). Also, the ability of oxidative stress to activate FoxOs has been shown to lead to a decrease in Wnt/β-catenin signaling, and preventing the accumulation of ROS has been shown to augment Wnt signaling and osteoblastogenesis in MSCs (15, 29, 35, 36, 37, 73).

Our laboratory has shown that binge alcohol administration in rodents suppresses canonical Wnt signaling and the expression of Wnt target genes within the fracture callus, all while suppressing the formation of the external, cartilaginous callus (7, 8, 9, 59), yet a discrete mechanism behind these deleterious effects has yet to be elucidated. Here we hypothesize that episodic ethanol administration leads to deficient fracture repair by activating FoxO transcription factors within the fracture callus, which suppresses chondrogenesis and subsequent cartilaginous callus formation. We also hypothesize that administering an antioxidant, n-acetyl-cysteine (NAC), during healing to curb the accumulation of ROS would attenuate the deficiencies of fracture healing caused by alcohol.

Methods

Alcohol Administration:

Male C57Bl/6 mice 6–7 weeks of age were obtained from Harlan Laboratories (Indianapolis, IN) and housed in a facility approved by the Institutional Animal Care and Use Committee at Loyola University Medical

Center. The mice were allowed to acclimate to the environment for 1 week prior to initiation of the experimental procedures. Animals were randomly assigned to one of four treatment groups: Saline Only Group, Alcohol Only Group, Saline+NAC, Alcohol+NAC. The repeated binge model of alcohol administration consisted of a daily intraperitoneal (I.P.) injection of a 20% (v/v) ethanol/saline solution made from 100% molecular grade absolute ethanol (Sigma-Aldrich, St. Louis, MO) and sterile isotonic saline. Mice were administered the ethanol at a dose of 2 g/kg once per day for 3 consecutive days, then allowed to rest for 4 consecutive days. After the 4 days of rest, the binge ethanol administration protocol was repeated, one I.P. injection daily for three consecutive days. One hour after the sixth and final injection, all groups received the stabilized, mid-shaft tibia fracture surgery, as described. All animals were weighed daily prior to injection to ensure correct dosage. Mice in the saline control groups were administered sterile isotonic saline only. Blood alcohol levels averaged approximately 200 mg/dl at the time of fracture (1 hour post-injection). As post-injury drinking is a clinically relevant problem (5), and in order to ensure alcohol-induced oxidative stress would be present during healing, animals continued to receive group-specific injections once daily until they were humanely euthanized at either 3, 6, or 9 days post-fracture, with the Saline Only Group receiving saline, the Alcohol Only Group receiving ethanol at 2 g/kg, the Saline+NAC Group receiving saline along with NAC at 100 mg/kg, and the Alcohol+NAC Group receiving ethanol at 2 g/kg along with NAC at 100 mg/kg.

Stabilized Tibia Fracture Model:

Our fracture model is a semi-rigidly stabilized, surgical mid-shaft tibia fracture model based on the model used by Chen, et al (13), and has been previously used and validated by our laboratory (59). We chose semi-rigid fixation because of its clinical relevancy, as semi-rigid fixation allows healing through both endochondral and intramembranous ossification (13, 59, 96). One hour after administration of the final alcohol or saline injection, mice were given an induction dose of anesthesia (0.5–0.75 mg/kg ketamine and 0.06–0.08 mg/kg xylazine) to facilitate hair removal from the left hind limb of the animal. Mice were given 5 mg/kg prophylactic gentamicin subcutaneously and anesthetized completely with isofluorane for the duration of the procedure. Under sterile conditions, the surgery site was swabbed with povidone-iodine solution followed by 70% ethanol. A small incision was made to expose the patellar tendon and a 27-gauge needle was used to ream a hole into the medullary cavity at the proximal aspect of the tibia. A stainless-steel insect pin 0.25 mm in diameter (Fine Science Tools, Inc., Foster City, CA) was inserted into the reamed hole to stabilize the tibia. A pair of angled bone scissors (Fine Science Tools) was used to surgically create a mid-diaphyseal tibial fracture. The insect pin was cut flush with the bone and the wound was sutured closed. Mice were then placed in clean cages on heating pads with free access to food and water. All animals received post-operative buprenex subcutaneously (0.05 mg/kg/q8) for pain control for 24 hours post injury.

N-Acetyl-L-Cysteine Administration:

Sigma grade n-acetyl-cysteine (NAC) (≥99%, Sigma-Aldrich, St. Louis, MO) was diluted into solution in sterile isotonic saline and administered by once daily I.P. injections at 100mg/kg to the animals in both the Saline+NAC Group and the Alcohol+NAC group during the fracture healing process until the animals were humanely euthanized at either 3, 6, or 9 days post-fracture.

Fracture Callus Histology:

Injured and contralateral tibias were harvested from the mice 3, 6, and 9 days post-fracture and placed in 10% neutral buffered formalin for 48 hours. The tibias were decalcified in 10% EDTA with agitation for 5 days, processed through a graded series of alcohol solutions and xylene, and infiltrated overnight with melted paraffin at 56–58°C. The tibias were oriented identically during paraffin embedding in order to identify mid-callus sections. Five-μm longitudinal sections were taken at the middle of the callus and placed onto Superfrost©Plus slides (Fisher Scientific, Pittsburgh, PA) and baked on a 60°C slide warmer overnight. Sections from each group were stained routinely with hematoxylin and eosin. Slides were scanned and uploaded to high-resolution TIFF image files. ImageJ software (Public Domain, courtesy of the National Institutes of Health, Bethesda, MD) was used to quantify areas of the image files.

Western Blot Analysis:

Fractured tibias were harvested from mice 3, 6, and 9 days post-fracture and snap-frozen in liquid nitrogen. Fracture callus tissue was isolated using a

Dremel tool (Dremel Inc., Racine, WI) while frozen, and pulverized in RIPA lysis buffer using a freezer mill (SPEX CertiPrep Inc., Metuchen, NJ). Total protein was measured using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., Rockford, IL). Twenty μg of total protein from each sample was resolved on 4–20% SDS-PAGE, electro-transferred to PVDF membranes, and probed with rabbit anti-mouse total FoxO (Abcam, Cambridge, MA), FoxO P-S207 (Cell Signaling, Danvers, MA), or FoxO P-S253 (Abcam, Cambridge, MA). The FoxO P-S207 antibody was only used at Day 6 post-fracture because the antibody has been unavailable recently, and Day 6 is the time point in which we find the most drastic changes in callus histology. Our previous experiments indicate that the expression of typical housekeeping genes (GAPDH, actin, PGK-1, β-tubulin) changes throughout the course of fracture repair, and the expression of each is significantly altered by alcohol exposure. Therefore, to ensure equal loading of protein, the transferred membranes were Coomassie-stained following protein detection, and values were normalized to a 60 kD band on the stained membrane, as previously demonstrated (59). Densitometric analysis was carried out utilizing Image Lab software (Bio-Rad Inc., Hercules, CA) and western blot data were presented as the densitometric ratio of target protein:Coomassie-stained band.

Statistics:

Data are expressed as mean ± standard error. Statistical differences between the saline and alcohol groups for western blot analysis were calculated

using Student's t-test. Statistical differences in callus areas between all four treatment groups were calculated by one-way ANOVA with Tukey's post-hoc testing. A p-value below 0.05 was considered significant.

Results

Alcohol Administration Inhibited Cartilaginous Callus Formation

Figure 8A shows representative images of the fracture callus histology with hematoxylin and eosin (H&E) staining from all four treatment groups at 6 days post fracture; Saline, Alcohol, Saline+NAC, and Alcohol+NAC. Callus histology of saline-treated mice displays the normal patterns of fracture healing through endochondral ossification, including a large external, cartilaginous callus initially formed around the fracture, with cartilaginous callus formation peaking around this time point (Figure 8A). Additional hallmarks of endochondral ossification in the saline-treated animals include robust cartilage matrix deposition and abundant mature, hypertrophic chondrocytes within the cartilaginous callus. Illustrative examples of areas of hypertrophic chondrocytes have been denoted with arrows (Figure 8A, arrows). These characteristics are still prevalent in the histology at 9 days post-fracture (Figure 9A, arrows), however there is more evidence of ossification occurring and new, woven bone being formed within the callus.

Fracture callus histology of alcohol treated animals displayed decreased formation of the cartilaginous callus, as well as suppressed hallmarks of

endochondral ossification, cartilage matrix deposition and mature, hypertrophic chondrocytes (Figure 8B).

Computer imaging software was used to quantify different callus areas. At 6 days post-fracture, alcohol significantly inhibited the formation of both the total cartilaginous callus, as shown by the decrease in the cartilaginous area (Figure 8B, p=0.0254), and chondrocyte maturation, as shown by the decrease in the hypertrophic chondrocyte area (Figure 8C, p=0.0004). Even when the area of hypertrophic chondrocytes is expressed as a percentage of the total cartilaginous area, to control for the decrease in overall area available for the forming hypertrophic chondrocytes, there was still a significant decrease in the callus tissue of alcohol-treated animals as compared to saline-treated animals (Figure 8D, p=0.0003).

These trends were also apparent at 9 days post-fracture (Figure 9A). The callus tissue of alcohol-treated animals exhibited similar significant decreases in cartilaginous area (Figure 9B, p=0.0009) and hypertrophic chondrocyte area (Figure 9C, p=0.0146). However, the decreased hypertrophic chondrocyte area due to alcohol treatment was no longer statistically significant when expressed as a percentage of the total cartilaginous callus area (Figure 9D, p=0.1289).

Antioxidant Treatment Prevented Alcohol-Induced Callus Perturbation

In order to determine if the deleterious effects of alcohol on fracture formation could be attributed to alcohol's ability to increase oxidative stress, we administered n-acetyl-cysteine (NAC) during healing to prevent ROS

accumulation. First, we saw that NAC did not affect normal fracture healing in our saline treated animals (Figure 8A, 9A). The callus histology of animals treated with saline and NAC was normal at 6 and 9 days post-fracture, with the various callus components and all the indicative characteristics of endochondral ossification remaining similar to animals only treated with saline when viewed histologically (Figure 8A, 9A) and when quantified (Figure 8B, C, D and Figure 9B, C, D).

Figure 8
Day 6

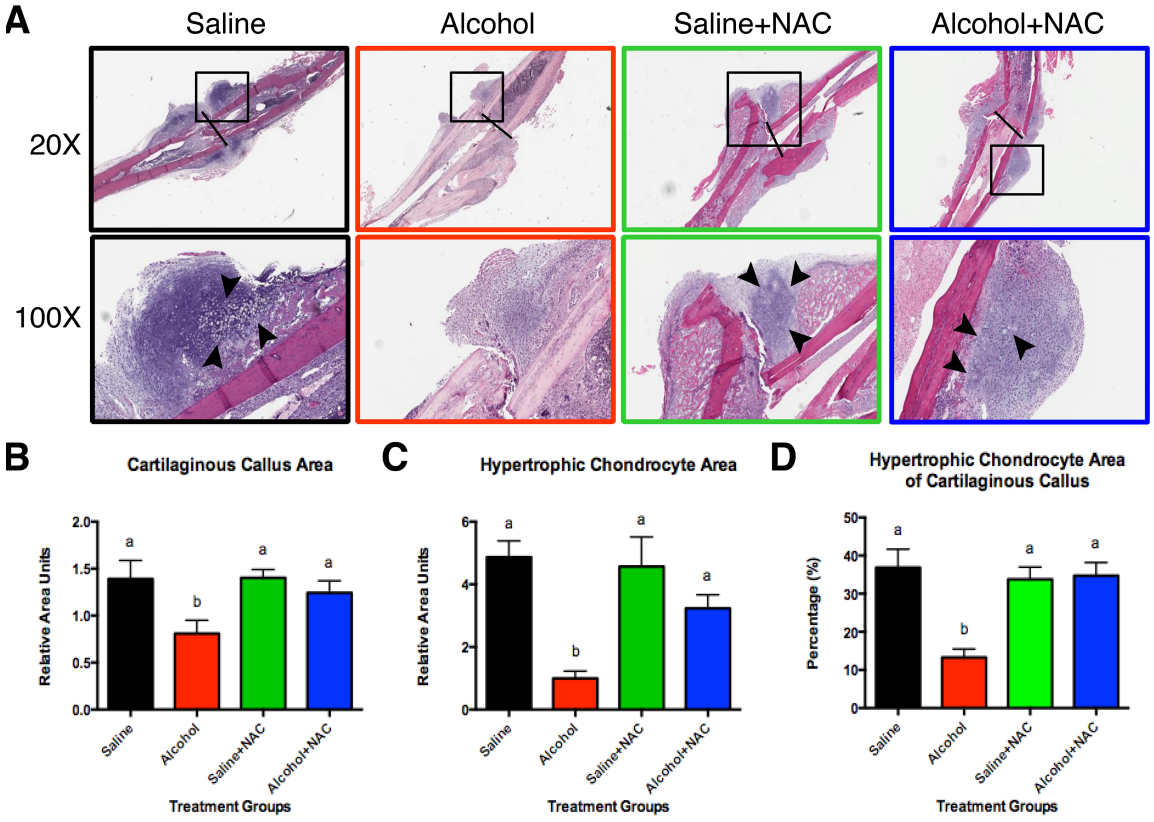


Figure 8. The effects of alcohol and antioxidant (NAC) treatment on callus components at Day 6 post-fracture. (A) Representative H&E images of fracture calluses from each treatment group (Saline, Alcohol, Saline+NAC, and Alcohol+NAC) 6 days after fracture at 20X and 100X magnification. Lines indicate transverse fracture site; boxes indicate site of magnification; arrowheads indicate sites of hypertrophic chondrocytes. (B, C, D) Quantifications of (B) the total area of the cartilaginous component of the callus, (C) the total area of hypertrophic chondrocytes within the callus, and (D) the percentage of the total area of the cartilaginous component of the callus that was composed of hypertrophic chondrocytes are shown comparing all four treatment groups. Different letters denote a statistical difference between groups compared by one-way ANOVA.

Figure 9
Day 9

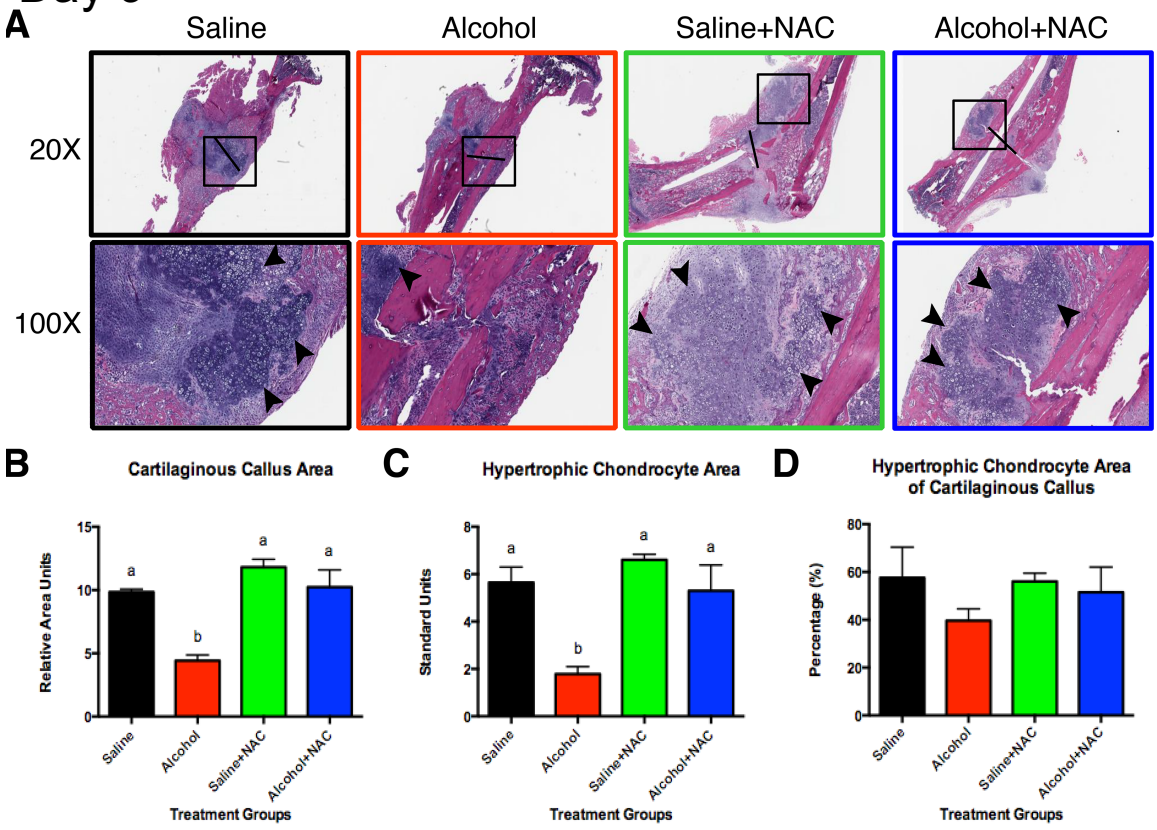


Figure 9. The effects of alcohol and antioxidant (NAC) treatment on callus components at Day 9 post-fracture. (A) Representative H&E images of fracture calluses from each treatment group (Saline, Alcohol, Saline+NAC, and Alcohol+NAC) 9 days after fracture at 20X and 100X magnification. Lines indicate transverse fracture site; boxes indicate site of magnification; arrowheads indicate sites of hypertrophic chondrocytes. (B, C, D) Quantifications of (B) the total area of the cartilaginous component of the callus, (C) the total area of hypertrophic chondrocytes within the callus, and (D) the percentage of the total area of the cartilaginous component of the callus that was composed of hypertrophic chondrocytes are shown comparing all four treatment groups. Different letters denote a statistical difference between groups compared by one-way ANOVA.

NAC was able to prevent the disruptions in normal callus formation seen with alcohol administration. NAC treatment largely recovered proper endochondral ossification and cartilaginous callus formation in alcohol treated animals, along with the recurrence of the characteristic hallmarks of cartilage matrix deposition and mature, hypertrophic chondrocytes at 6 days post-fracture (Figure 8A, arrows) and 9 days post-fracture (Figure 9A, arrows). Concurrent NAC treatment during healing restored the cartilaginous callus areas and the hypertrophic chondrocyte areas back to similar levels of animals treated with only saline or saline and NAC at days 6 and 9 post-fracture (Figure 8B, C, D and Figure 9B, C, D).

Alcohol Administration Increased FoxO Activation within the Fracture Callus

Next, our laboratory examined whether alcohol administration could modulate FoxO expression or activation in isolated callus tissue using western blot analysis. Total FoxO1 protein expression was increased in the fracture callus tissue of alcohol treated animals 6 days post-fracture (Figure 11A, $p=0.0045$). Alcohol-induced FoxO1 expression was not significant at 3 (Figure 10A, $p=0.3913$) or 9 (Figure 12A, $p=0.3487$) days post-fracture.

FoxO activity is primarily modulated through post-translational modifications. FoxO becomes inactivated when phosphorylated at Serine-253 and activated when phosphorylated at Serine-207 (60). By using antibodies specific for phosphorylation at these particular residues, we can approximate the activation status of FoxO. We found that alcohol increased the marker of FoxO

activation (P-S207) within callus tissue 6 days post-fracture (Figure 11B, p=0.0002), while alcohol concurrently decreased the marker of FoxO inactivation (P-S253) at days 3 (Figure 10B, p=0.0001) and 6 (Figure 11C, p=0.0185) post-fracture. The decrease in the marker of FoxO inactivation (P-S253) within callus tissue of alcohol-treated animals as compared to saline-treated animals at 9 days post-fracture failed to reach significance in this study (Figure 12B, p=0.0673).

Antioxidant Treatment Abolished Markers of Alcohol-Induced FoxO Activation

In order to validate that the change in phosphorylation status of FoxO transcription factors in our model of deficient fracture healing was being caused by alcohol-induced oxidative stress, we used western blotting to examine the expression and different phosphorylation markers of FoxO in callus tissue of animals treated with n-acetyl cysteine (NAC). In the presence of NAC, alcohol administration had no effect on FoxO1 expression at 3, 6, and 9 days post-fracture in the alcohol administration group as compared to their saline cohorts (Figure 10C, 11D, 12C). Likewise, there was no effect of alcohol on the marker of FoxO activation, phosphorylation at Serine-207, at Day 6 post-fracture with concurrent NAC administration (Figure 11E). In the presence of NAC there also was no alcohol-induced decrease of the marker of FoxO inactivation, phosphorylation at Serine-253, at each time point during healing (Figure 10D, 11F, 11G, 12D). It is also important to note that when all four treatment groups, Saline, Alcohol, Saline+NAC, and Alcohol+NAC, were examined together, only the alcohol-treated group was significantly different from all the other groups

(Figure 11G, p=0.0001). In animals receiving alcohol, NAC treatment was able to restore FoxO protein back to the levels of saline-treated animals.

Figure 10
Day 3

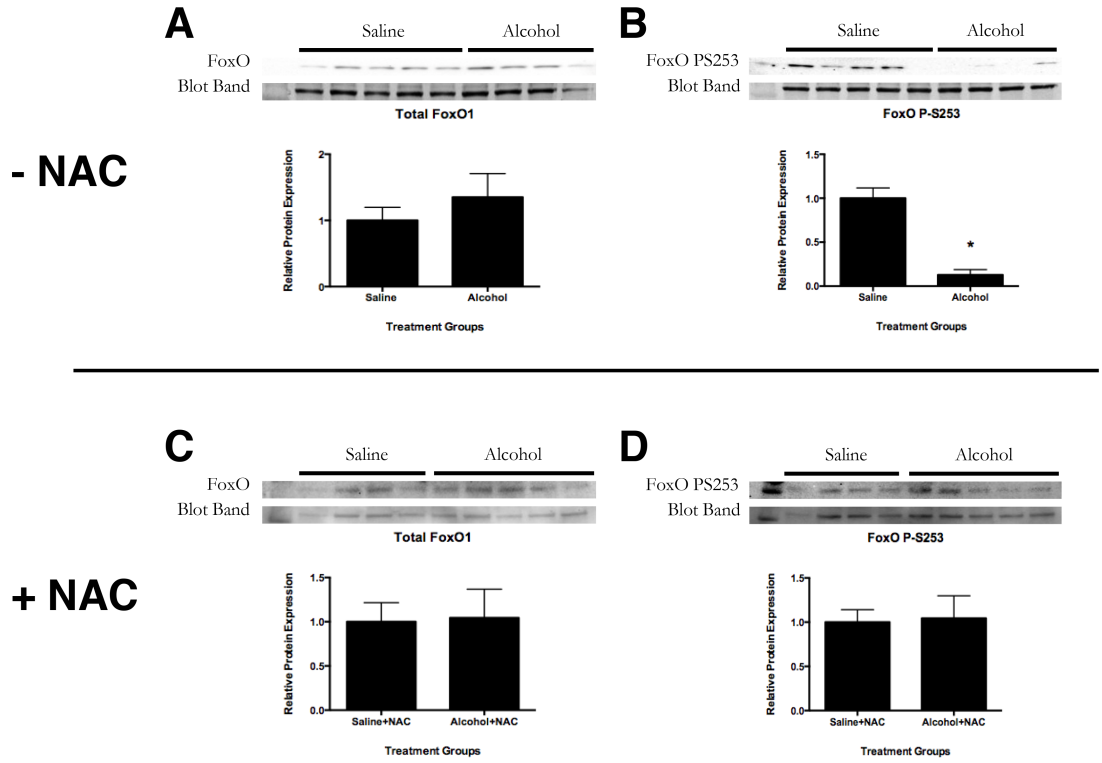


Figure 10. FoxO protein expression and markers of inactivation in callus tissue after Saline or Alcohol treatment with or without corresponding antioxidant (NAC) treatment at Day 3 post-fracture. Western blot of (A) Total FoxO1 without NAC; (B) FoxO protein phosphorylated at Serine 253 (P-S253) without NAC; (C) Total FoxO1 with NAC; and (D) FoxO protein phosphorylated at Serine 253 (P-S253) with NAC. Corresponding quantification is graphed underneath each blot; *$p<0.05$ by Student's t-test.

Figure 11
Day 6

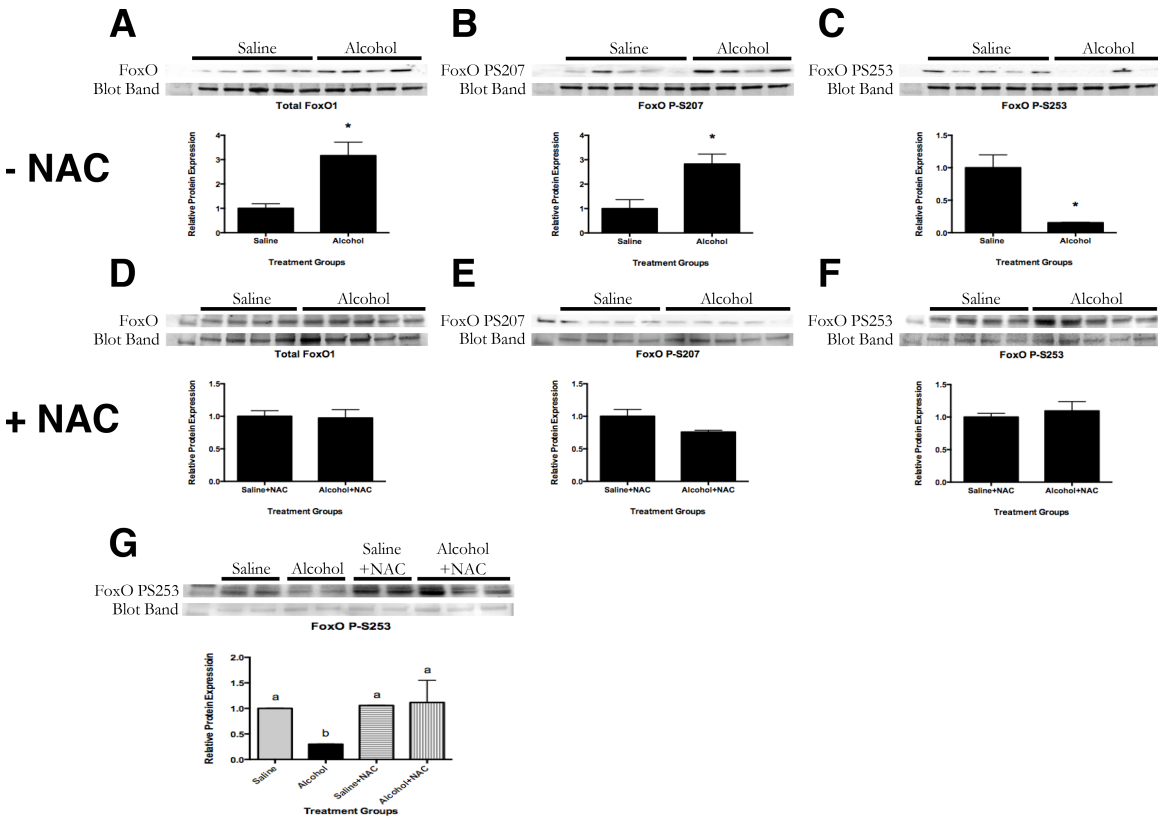


Figure 11. FoxO protein expression and markers of activation in callus tissue after Saline or Alcohol treatment with or without corresponding antioxidant (NAC) treatment at Day 6 post-fracture. Western blot of (A) Total FoxO1 without NAC; (B) FoxO protein phosphorylated at Serine 207 (P-S207) without NAC; (C) FoxO protein phosphorylated at Serine 253 (P-S253) without NAC; (D) Total FoxO1 with NAC; (E) FoxO protein phosphorylated at Serine 207 (P-S207) with NAC; (F) FoxO protein phosphorylated at Serine 253 (P-S253) with NAC; and (G) FoxO protein phosphorylated at Serine 253 (P-S253) in Saline and Alcohol groups with and without NAC. Corresponding quantification is graphed underneath each blot; different letters denote a statistical difference between groups compared by one-way ANOVA, and *p<0.05 by Student's t-test.

Figure 12
Day 9

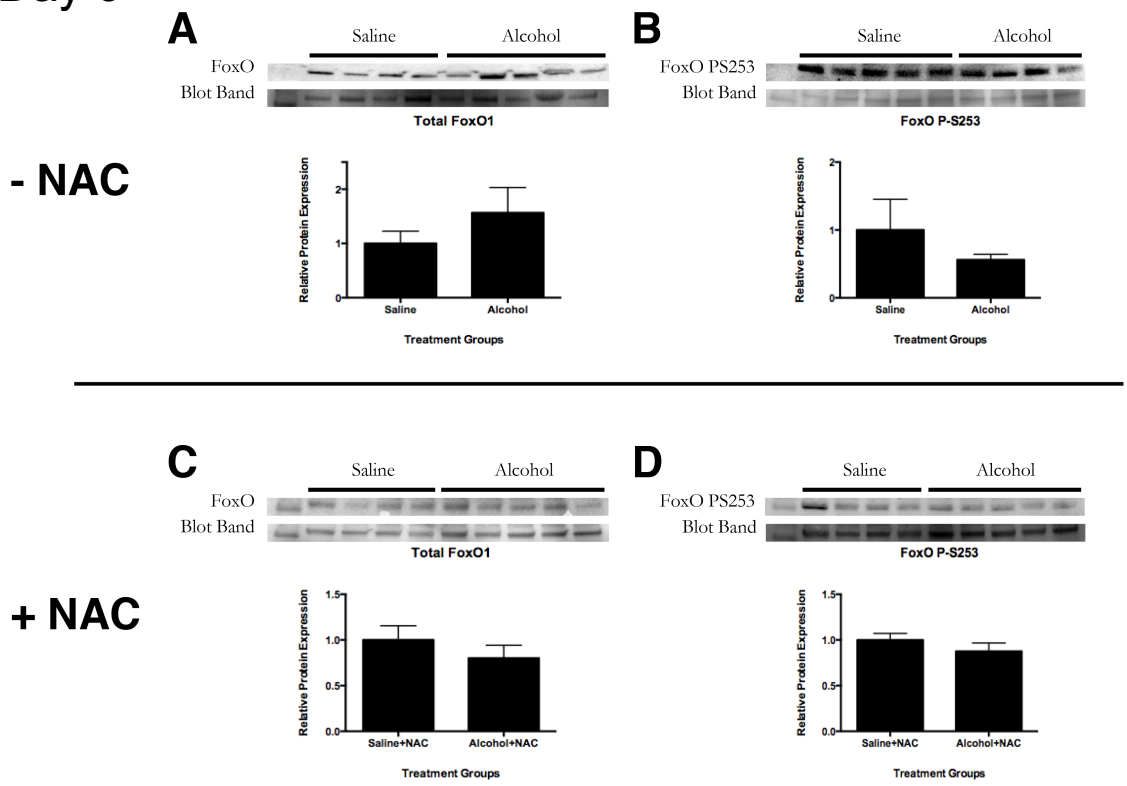


Figure 12. FoxO protein expression and markers of inactivation in callus tissue after Saline or Alcohol treatment with or without corresponding antioxidant (NAC) treatment at Day 9 post-fracture. Western blot of (A) Total FoxO1 without NAC; (B) FoxO protein phosphorylated at Serine 253 (P-S253) without NAC; (C) Total FoxO1 with NAC; and (D) FoxO protein phosphorylated at Serine 253 (P-S253) with NAC. Corresponding quantification is graphed underneath each blot; *$p<0.05$ by Student's t-test.

Summary

The results of the preceding studies show a definitive link between alcohol's ability to induce oxidative stress and alcohol's disruption of normal fracture healing. When alcohol is left to increase the oxidative load on tissues unfettered, proper callus formation is perturbed. Namely, callus components directly involved in endochondral ossification are detrimentally affected. There is a significant reduction in the external, cartilaginous callus, with concomitant decreases in cartilaginous matrix deposition and the formation of mature, hypertrophic chondrocytes. These findings are associated with an increase in FoxO protein and markers of activation within the fracture callus. However, when alcohol-induced oxidative stress is abated with an antioxidant during healing, the deleterious effects of alcohol on callus formation are prevented, as are the associated increases of FoxO expression and markers of activation. Altogether, these data support the hypothesis that alcohol-induced oxidative stress is having a substantial negative impact on fracture healing by suppressing specific aspects of callus formation, and furthermore, the association of increased FoxO expression and markers of activation within the callus suggest a possible mechanism in which FoxO transcription factors actively inhibit MSC differentiation, as FoxOs are known to competitively inhibit Wnt signaling.

CHAPTER 4

ALCHOL EXPOSURE INCREASES THE ACTIVITY OF FOXO TRANSCRIPTION FACTORS IN CULTURED MESENCHYMAL STEM CELLS

Abstract

The process of fracture healing is complex, and poor or incomplete healing remains a significant health problem. Normally, fractures heal largely through endochondral ossification. This is a process by which resident mesenchymal stem cells (MSCs) differentiate first into chondrocytes, to form the cartilaginous callus, and then osteoblasts, to ossify the collagen matrix. Previous data from our laboratory have shown that alcohol administration leads to a decrease in cartilage formation and subsequent endochondral ossification within the fracture callus of rodents during healing. These decreases are also concurrent with FoxO transcription factor activation within the callus. Importantly, activation of FoxO transcription factors has been shown to inhibit the canonical Wnt signaling pathway, which is a necessary pathway in driving proper MSC differentiation towards chondrocytes and osteoblasts. These findings have led to our hypothesis that alcohol exposure decreases chondrogenesis in MSCs through the activation of FoxO transcription factors. In order to examine our hypothesis, we harvested MSCs from the bone marrow of long bones in 6-7 week old lewis rats. These MSCs were then exposed to ethanol in culture in order to examine the effects on FoxO activation. Ethanol exposure increased the

expression of FoxOs at both the mRNA and protein levels. This was accompanied by an increase in FoxO nuclear translocation, DNA binding, and target gene expression. These data show that ethanol has the ability to activate FoxO transcription factors within MSCs, and this elucidates a possible mechanism behind alcohol-induced MSC differentiation deficiencies, and inevitably alcohol-induced deficient fracture healing.

Introduction

Bone fracture healing is the result of an elaborate orchestration of many different cell types and signaling pathways in and around the fracture. Typically, most fractures heal without complication, with only about 10% of long bone fractures failing to heal correctly (96). However, there are several conditions and co-morbidities that contribute to improper, delayed, or incomplete healing. These include conditions that are mostly uncontrollable, like aging or diabetes, but these also include more controllable behavioral habits such as abusing alcohol (10, 55). Alcohol abuse has many detrimental effects across myriad tissues in the body, and the skeleton is key among them. Alcohol abusers have an increased risk of developing osteopenia, and have a fracture rate four times higher than that of non-abusers (2, 10, 11, 56). Up to 40% of orthopedic trauma patients present with a positive blood alcohol content at the time of hospital admission, and alcohol consumption significantly raises the risk of healing complications, leading to nonunion and increased fracture healing times. Additionally, many trauma patients continue drinking after sustaining an injury (3, 5, 56). And while it

is true that alcohol abusers may have contributing factors that could be detrimental to bone health and augment the likelihood of sustaining a fracture, such as poor nutrition, lack of exercise, or engaging in risky behavior, these factors alone do not fully explain the effects alcohol exposure has on the skeleton and fracture healing. This is evident in the basic science research using rodents which has shown that both acute and chronic alcohol administration have deleterious effects on bone health, as well as the ability to disrupt healing (2, 6, 8, 9, 11, 21).

Mesenchymal stem cells (MSCs) are indispensably important to both the homeostatic health of bone and proper fracture healing. MSCs are a group of multipotent stem cells that differentiate into a variety of cell types including osteoblasts, chondrocytes, and adipocytes (42, 43, 44). Continual differentiation of MSCs into osteoblasts is necessary to maintain the coupled balance of bone formation and resorption. When MSCs are depleted from the bone marrow, bone resorption outpaces bone formation leaving bones weak, brittle, and prone to fracture (109, 110). Proper differentiation of MSCs is also critical for normal fracture healing. After a fracture is sustained and during the repair process, MSCs in the periosteum preferentially develop into osteoblasts that begin forming a section of the callus known as the periosteal callus, which is primarily formed by osteoblasts through intramembranous ossification (58, 59, 66, 69). MSCs within the bone marrow preferentially develop into chondrocytes that form the external callus portion of the fracture callus. The external callus makes up the

majority of the callus volume and is composed of a cartilaginous matrix deposited by chondrocytes that will eventually become ossified through endochondral ossification (58, 59, 66, 69).

There is evidence that alcohol negatively affects the osteogenic and chondrogenic potential of MSCs, even promoting their adipogenic differentiation in culture. MSCs that have been isolated from alcoholics show a decreased ability to differentiate into osteoblasts compared to MSCs from normal patients (93). Concurrently, MSCs isolated from non-alcohol-abusing patients but cultured with ethanol *ex vivo* show a perturbed differentiation potential, with osteogenic differentiation suppressed and adipogenic differentiation promoted even in the presence of an osteogenic medium (33).

One of the most important signaling pathways involved in directing MSC differentiation is canonical Wnt signaling because activation of Wnt signaling drives MSCs toward an osteoblastic or chondrogenic lineage, while suppressing adipogenesis, by mobilizing the transcription factor β-catenin (23, 24, 64, 78). Our laboratory has previously shown that binge alcohol treatment in mice disrupts canonical Wnt signaling and the expression of Wnt target genes within bone and the fracture callus (59). We have also shown that binge alcohol exposure leads to an increase in expression and markers of activation of FoxO transcription factors within the fracture callus during healing, and these increases are associated with an inhibition of endochondral ossification and are prevented by the administration of an antioxidant during healing (111).

Altogether, these findings begin to delineate a potential mechanism behind alcohol-induced deficient fracture repair because FoxOs, once activated by oxidative stress, directly inhibit Wnt signaling by diverting β-catenin away from pro-osteochondrogenic gene transcription (15, 29, 35, 36, 73).

Previously, we have not shown that alcohol was inhibiting fracture healing by directly affecting MSCs. Therefore, we presently hypothesize that alcohol is able to induce activation of FoxOs in cultured MSCs. By showing an alcohol-induced activation of FoxOs in MSCs, we stand to further elucidate a mechanism of action behind alcohol-induced bone loss and deficient fracture healing.

Methods

Rat MSC Isolation

Mesenchymal stem cells were isolated from 6-7 week old male Lewis rats. Briefly, animals were humanely euthanized, and both tibiae and femurs were harvested. The proximal and distal ends of each bone were cut off with bone snips. The marrow of each bone was flushed with D-MEM supplemented with 20% FBS using an 18-gauge needle and 10mL syringe. The resulting marrow cell suspension was filtered through a 70 uM filter to remove any contaminating bone, muscle, or cell clumps. This cell suspension was centrifuged at 450g for 5 minutes to pellet the cells, and then the pellet was resuspended in 5mL of D-MEM containing 20% FBS and transferred to a T-25, 25 cm^2 culture flask. The culture medium was changed the following day and then every 3-4 days, as needed. The cells were subcultured before colonies became multilayered. After

one passage for expansion, the cells were then harvested and aliquoted in freezing medium at 2 million cells/mL and stored in the vapor phase of liquid nitrogen to be used as needed.

MSC Alcohol Exposure

Rat mesenchymal stem cells were cultured according to proper ascetic cell culture techniques. D-MEM supplemented with 10% FBS was used as the growth medium. Cells were grown in T-75, 75 cm^2 culture flasks until approximately 80% confluence was reached. MSCs were then harvested using Trypsin-EDTA (0.25%) and split into the appropriate number of T-25, 25 cm^2 culture flasks at approximately 750,000 cells per flask. Cells were allowed to grow for 2-3 days before treatment. EtOH and H2O2 treatments were made in D-MEM without FBS supplementation using molecular grade 100% EtOH and 30% (w/w) H2O2 (Sigma-Aldrich, St. Louis, MO). 5mL of the treatments were directly applied to cultured cells. Additionally, culture flasks treated with EtOH were incubated in a sealed system at standard culture conditions (5% CO2, 37 degrees celsius, humidified) with excess EtOH at the same concentration as the treatment in order to mitigate EtOH loss by evaporation.

RNA Isolation and RT-PCR

3mL of TRI reagent was applied directly to the cells on each flask, and incubated for 5 minutes in order to harvest the cell lysates. RNA was isolated and purified from each group using the Ambion RNA Purification Kit (Thero Fisher Scientific, Waltham, MA) following manufacturer's specifications. RNA was

quantified using a NanoDrop ND-1000 spectrophotometer. RNA quality was checked using the Agilent 2100 Bioanalyzer according to manufacturer's specification. RNA that met the appropriate quality and quantity was used in reverse transcription reactions to create a cDNA library using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). These cDNA libraries were subjected to real-time PCR analysis using the 7500 Fast RT-PCR System, and the resulting data was analyzed by the delta-delta Ct method. Compatible FAM primer probes specific for FoxO3a, Catalase, and β2M, the endogenous control, were obtained from Thermo Fisher Scientific.

Protein Isolation, Western Blotting, and DNA Binding Assay

Cells from each treatment group were harvested and pelleted. The cell pellet was either lysed in RIPA lysis buffer for total protein or lysed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific) to isolate the proteins specific for the nuclear and cytoplasmic fractions, respectively. Total protein concentration was measured using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., Rockford, IL). Twenty µg of total protein from each sample was resolved on 4–20% SDS-PAGE, electro-transferred to PVDF membranes, and probed with rabbit anti-mouse total FoxO (Abcam, Cambridge, MA). To ensure equal loading of protein, PGK1 was used as the loading control. Densitometric analysis was carried out utilizing Image Lab software (Bio-Rad Inc., Hercules, CA) and western blot data were presented as the densitometric ratio of target protein:loading protein (PGK1) bands. Nuclear

fractions were used with the TransAM FKHR (FoxO1) kit (Active Motif, Carlsbad, CA) to evaluate FoxO-DNA binding.

Statistics

Data are expressed as mean ± standard error. Statistical differences between treatment groups were calculated by either one-way or two-way ANOVA as appropriate as described. A p-value below 0.05 was considered significant.

Results

Alcohol Exposure Increases FoxO Expression within Cultured MSCs

In order to determine if direct exposure to EtOH would increase FoxO expression within MSCs, we cultured primary MSCs harvested from the bone marrow of long bones of lewis rats in the presence of 50mM EtOH. We also examined if there was a time-course of FoxO expression by exposing the MSCs to EtOH for 3, 6, or 9 hours. EtOH exposure was able to increase FoxO mRNA expression within MSCs when compared to MSCs cultured in media alone, as shown by qRT-PCR (Figure 13). At 3 hours of exposure, FoxO mRNA expression reached an increase of over 2 times that of the expression seen with media alone, and FoxO mRNA remained significantly elevated throughout the course of treatment (Figure 13).

Next, we used western blotting to ascertain whether this increase in FoxO mRNA expression caused by EtOH exposure resulted in an increase in FoxO protein within cultured MSCs. MSCs cultured with EtOH had an increase in FoxO protein expression, reaching a peak of almost 2 times the FoxO protein

expression of MSCs cultured in media alone (Figure 14A and B). This peak occurred at 6 hours of EtOH exposure, following the FoxO mRNA peak at 3 hours, but remained elevated at 3 and 9 hours (Figure 13, Figure 14A and B). Hydrogen peroxide was also used as a positive control of inducing oxidative stress. MSCs exposed to hydrogen peroxide did have an increase in FoxO protein expression at 3 hours, however, this elevation did not remain over the course of exposure (Figure 14A and B).

Figure 13

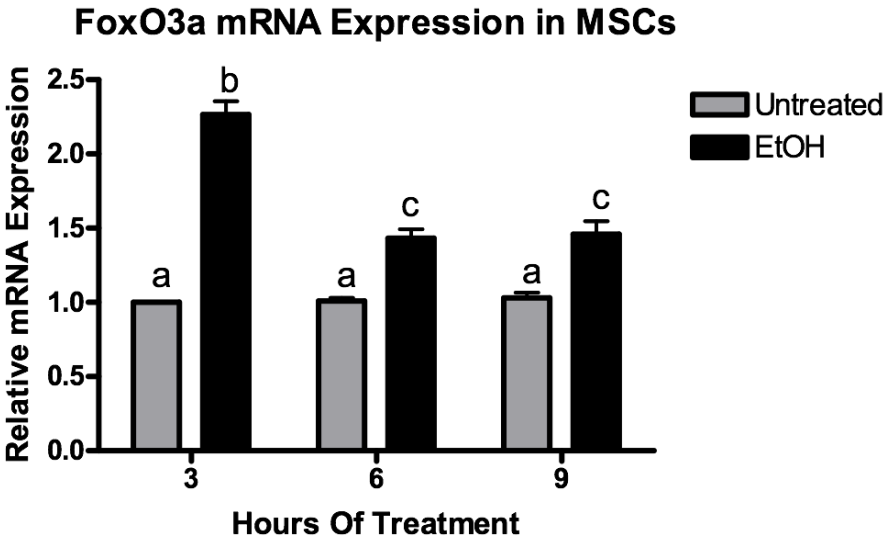
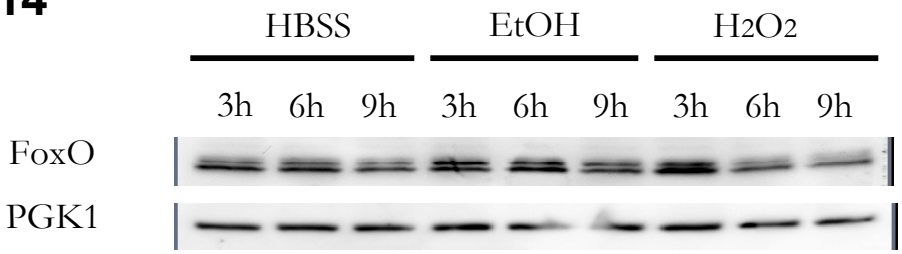


Figure 13. Time-course of FoxO mRNA expression within cultured MSCs with EtOH exposure. Primary rat MSCs cultured with either 0 or 50mM EtOH for 3, 6, or 9 hours. Relative FoxO3a mRNA expression given as found by qRT-PCR. n=3 separate experiments per group, groups sharing the same letter are statistically similar, $p<0.05$ by two-way ANOVA.

Figure 14

A



B

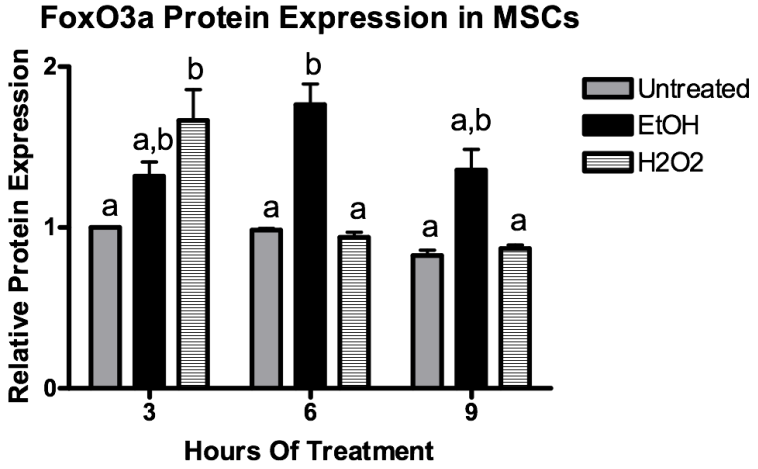


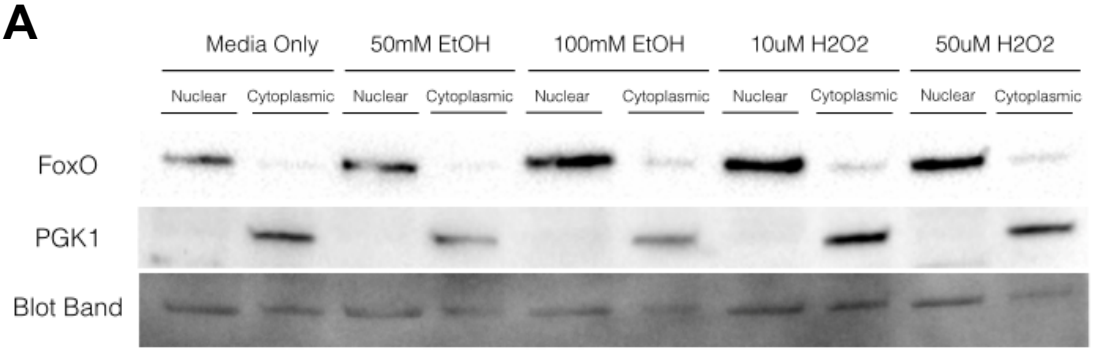
Figure 14. Time-course of FoxO protein expression within cultured MSCs with EtOH exposure. Primary rat MSCs cultured with either media alone, 50mM EtOH, or 50uM H2O2 for 3, 6, or 9 hours. Relative FoxO protein expression given as found by representative western blot (A) and quantification (B). n=3 separate experiments per group, groups sharing the same letter are statistically similar, $p<0.05$ by two-way ANOVA.

Alcohol Exposure Increases FoxO Nuclear Localization

Since the activity of FoxO transcription factors is modulated by its shuffling into or out of the nucleus, we tested if EtOH exposure led to its translocation into the nucleus. Cultured rat MSCs were incubated with either 50mM or 100mM EtOH, 10uM or 50uM hydrogen peroxide, or media alone for 24 hours, to determine any dose-dependency, and then subjected to nuclear and cytoplasmic fractionation in order to isolate proteins that were located in either compartment. 50mM and 100mM EtOH exposure lead to 2 and 4 times the amount of FoxO being found within the nucleus when compared to media alone, respectively (Figure 15A and B). There was also an approximately 4-fold increase in nuclear FoxO with 10uM and 50uM hydrogen peroxide exposure (Figure 15A and B).

Figure 15

A



B

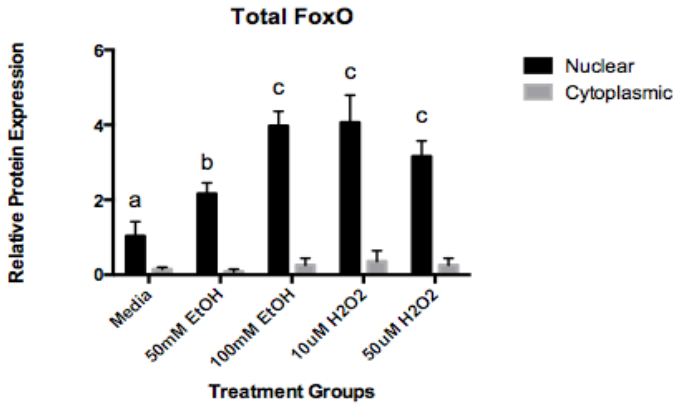


Figure 15. FoxO protein nuclear localization within cultured MSCs with EtOH exposure. Primary rat MSCs cultured with either media alone, 50mM or 100mM EtOH, or 10uM or 50uM H2O2 for 24 hours. Relative FoxO protein expression given as found by representative western blot (A) and quantification (B). n=3 separate experiments per group, groups sharing the same letter are statistically similar, $p<0.05$ by one-way ANOVA.

Figure 16

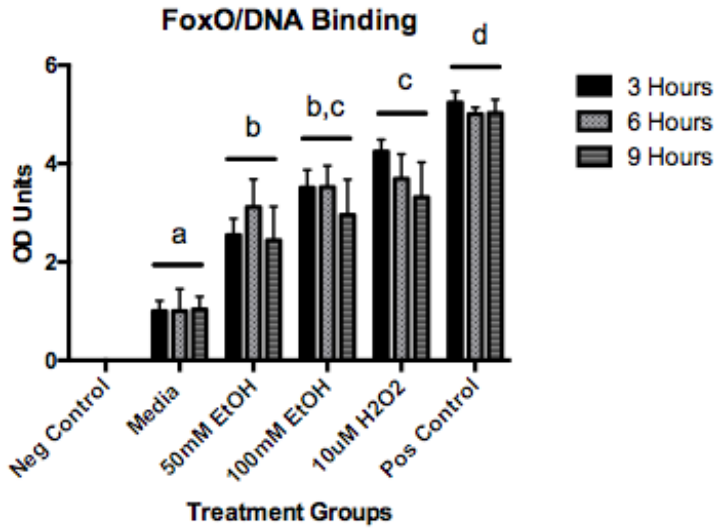


Figure 16. The effects of EtOH exposure on the ability of FoxO to bind to DNA. Primary rat MSCs cultured with either 50mM EtOH, 100mM EtOH, 10uM H2O2, or media alone for 3, 6, or 9 hours. Quantification of the FoxO bound to DNA using the TransAM FKHR ELISA expressed as units of optical density (OD). n=3 separate experiments per group, groups sharing the same letter are statistically similar, $p<0.05$ by two-way ANOVA.

Alcohol Exposure Increases the Binding of FoxO to DNA

Next, we wanted to verify that the alcohol-induced nuclear FoxO was able to bind to DNA as an indication of FoxO activity as a transcription factor. Cultured MSCs were exposed to either 50mM EtOH, 100mM EtOH, 10uM hydrogen peroxide, or media alone for either 3, 6, or 9 hours. The nuclear FoxO of each different treatment group was assayed for DNA binding ability using a specialized ELISA containing double-stranded oligonucleotides specific to activated FoxO. The ability of FoxO to bind to DNA increases with increasing doses of EtOH and

peaks with hydrogen peroxide exposure at 4 times that of media alone (Figure 16). There were no time-dependent differences between groups within the same EtOH or hydrogen peroxide dose (Figure 16).

Figure 17

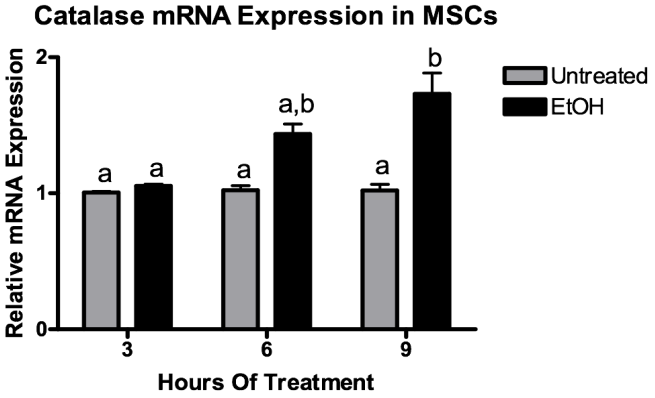


Figure 17. Time-course of Catalase mRNA expression within cultured MSCs with EtOH exposure. Primary rat MSCs cultured with either 0 or 50mM EtOH for 3, 6, or 9 hours. Relative Catalase mRNA expression given as found by qRT-PCR. n=3 separate experiments per group, groups sharing the same letter are statistically similar, $p<0.05$ by two-way ANOVA.

<u>Alcohol Promotes FoxO-Target Gene Expression</u>

In order to further elucidate if EtOH exposure was increasing FoxO activation and activity, the expression of the mRNA transcript of the FoxO-target gene Catalase within the cultured MSCs was assayed at 3, 6, or 9 hours of exposure. 50mM EtOH exposure increased Catalase mRNA expression, reaching peak expression at 9 hours (Figure 17).

Summary

The results of the preceding studies show that alcohol has the ability to directly modulate the activity of FoxO transcription factors within MSCs. Alcohol

exposure increased the expression of both FoxO mRNA and protein. There was also more nuclear FoxO in MSCs cultured in the presence of alcohol, and this nuclear FoxO had a greater ability to bind to DNA. All this coincided with an alcohol-induced increase in the mRNA expression of the FoxO-target gene Catalase. Altogether, these data support the hypothesis that alcohol is able to induce activation of FoxOs in MSCs, further elucidating a possible mechanism through which alcohol can decrease bone formation and fracture healing by directly inhibiting normal MSC differentiation.

CHAPTER 5

SUPPLEMENTAL DATA

Alcohol Increases FoxO mRNA Expression in the Early Callus

As part of our efforts to examine if alcohol exposure influenced FoxO expression in the fracture callus, we harvested fracture callus tissue at Day 2 post-fracture from animals exposed to ethanol or saline, as described previously, and extracted total mRNA using the method described in the appendix (Callus RNA Isolation). We found that FoxO mRNA was increased by two-fold in the callus tissue of animals treated with ethanol as compared to saline treated animals (Figure 18). This shows that alcohol has the ability to increase FoxO expression at very early time points during healing.

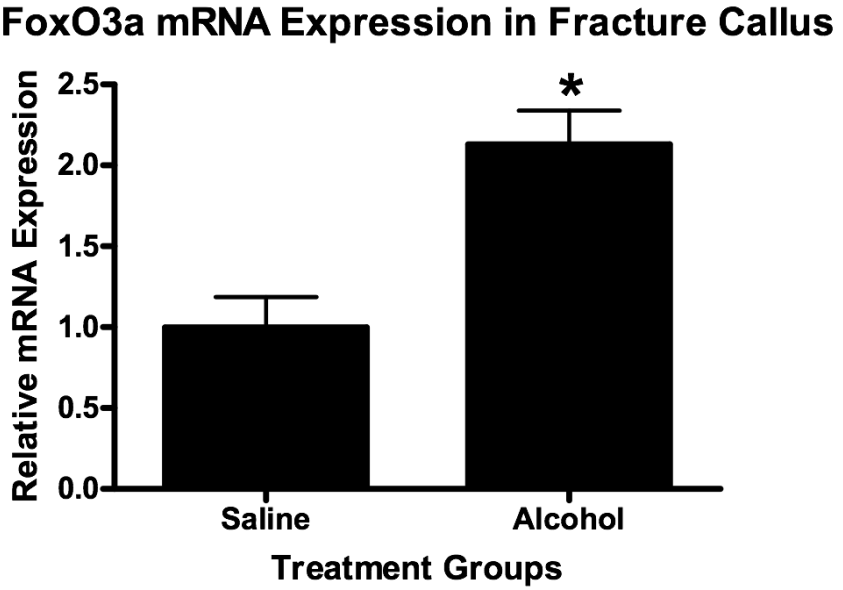


Figure 18. The effects of alcohol exposure on FoxO mRNA expression in callus tissue at Day 2 post-fracture. n=5-6/group, *p<0.05 Student's t-test.

Alcohol Increases Catalase mRNA Expression in the Early Callus

In addition to determining the effects of alcohol on FoxO expression within the callus, we also assayed whether alcohol administration influenced the expression of the FoxO-target gene, Catalase. Animals treated with alcohol had a two-fold increase in Catalase mRNA expression in callus tissue as compared to saline treated animals (Figure 19). This data, along with our FoxO expression data, suggests that not only is alcohol leading to increased FoxO expression and markers of activation, but that this alcohol-induced FoxO expression is driving FoxO-mediated transcription.

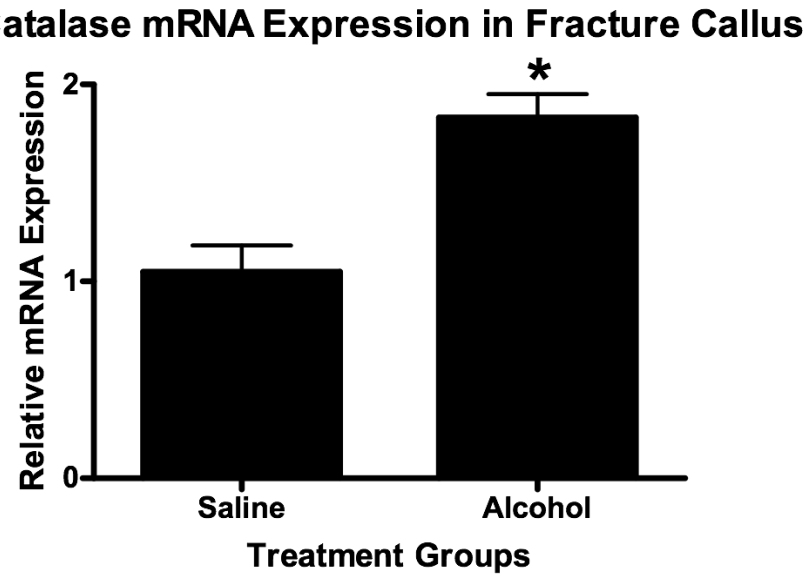


Figure 19. The effects of alcohol exposure on Catalase mRNA expression in callus tissue at Day 2 post-fracture. n=5-6/group, *p<0.05 Student's t-test.

Alcohol Decreases External Callus Formation at Day 3 Post-Injury

We examined the effects of alcohol treatment on callus histology at Day 3 post-fracture in order to assess how alcohol affects the early formation of the

fracture callus. At this stage in normal healing, the callus is so immature that it is impossible to delineate the distinct callus components. Therefore, we could not measure the cartilaginous callus area and the area of hypertrophic chondrocytes. Instead we could only measure the total callus, an area defined as all of the tissue areas combined (the internal callus, external callus, and bone), and the external callus, an area defined as all the callus tissue surrounding the fracture site on the outside or periosteal side of the bone. We found that alcohol did not significantly decrease the total callus area, but alcohol did significantly decrease the external callus area (Figure 20). This deleterious effect on the formation of the external callus could perpetuate throughout healing, leading to the decreased cartilaginous callus areas observed at Day 6 and Day 9 post-fracture.

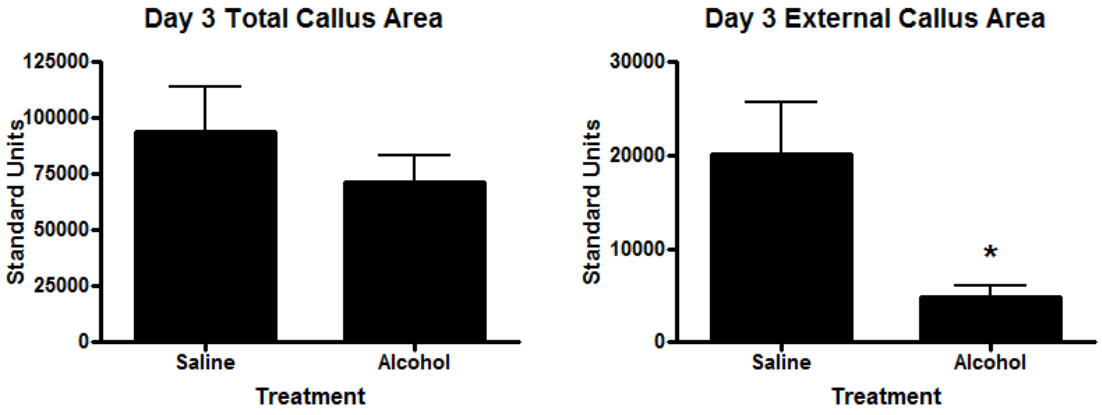


Figure 20. The effects of alcohol treatment on external callus formation at Day 3 post-fracture. n=3-4/group, *p<0.05 Student's t-test.

Alcohol Exposure Has No Effect on Apoptosis within the Callus

In order to more assuredly confirm our hypothesis that alcohol exposure was affecting differentiation in the callus, we had to exclude alcohol-induced apoptosis as a possible explanation for the diminished callus formation in our

alcohol treated animals. When the levels of caspase 3 were measured in fracture callus tissue, there were no differences in the levels of cleaved caspase 3 or the ratio of cleaved caspase 3 to procaspase 3 between the alcohol and saline treated groups of animals at either Day 3 or Day 6 post-fracture (Figure 21A, B, C, and D). These data help to belie the notion that alcohol-induced apoptosis is significantly contributing to the pathomorphologies exemplified in our experimental model, further elucidating alcohol-induced perturbed differentiation within the fracture site and callus as the main mechanism.

MSCs Pre-Loaded with Oxidative Stress Have Diminished Wnt-Induced β-catenin Activation

In addition to examining how EtOH exposure was modulating FoxO expression within cultured MSCs, we tested if oxidative stress affected canonical Wnt signaling in these cells. We preloaded MSCs with oxidative stress by exposing them to 50uM hydrogen peroxide for 30 minutes and then incubated them with only 50nM Wnt3a for 24 hours to activate canonical Wnt signaling. After 24 hours, an active form of β-catenin was measured by western blotting, as previously described, using a primary antibody specific for the dephosphorylated (active) form of β-catenin (Millipore, Cat #: 05-665). We discovered that the cells preloaded with oxidative stress had diminished β-catenin activation by Wnt3a induction when compared to cells that were not preloaded with oxidative stress (Figure 22). This illustrates that the negative effects of alcohol could be multifaceted. Not only could the alcohol-induced oxidative stress be causing an increase in FoxO activation that leads to a sequestration of β-catenin within the

cell, but this oxidative stress could also be decreasing the overall amount of active β-catenin available within the cell.

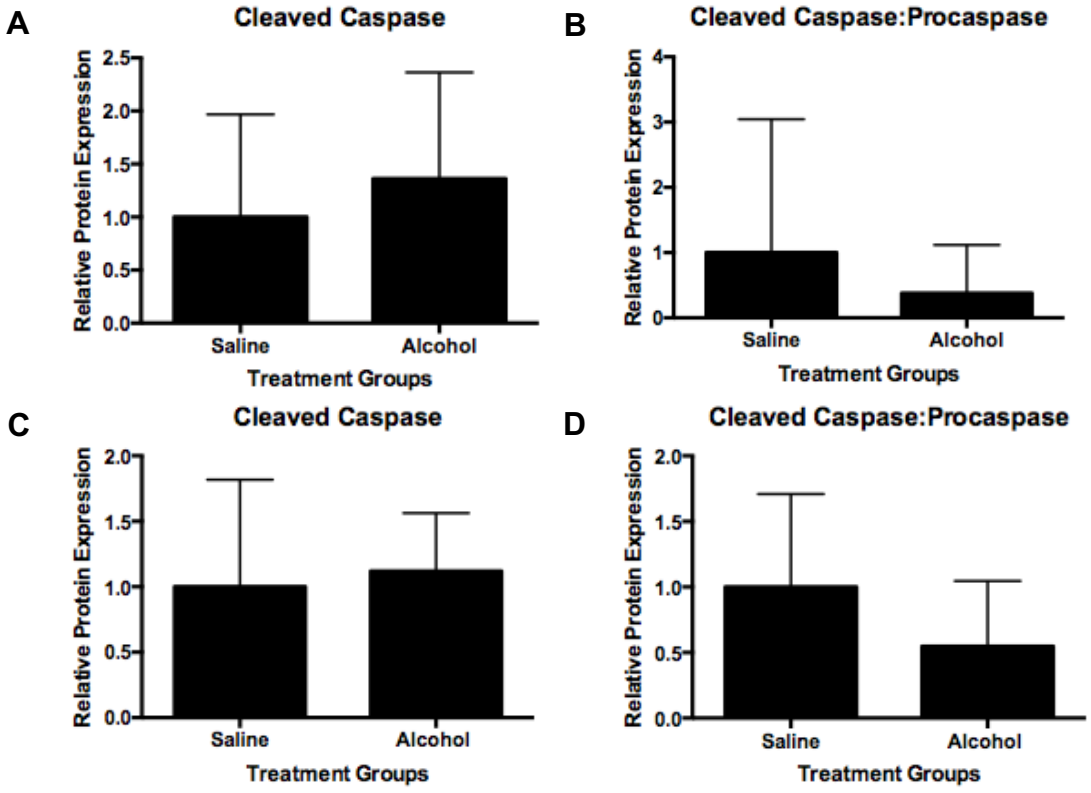


Figure 21. The effects of alcohol exposure on markers of apoptosis within fracture callus tissue. n=5-6/group, *p<0.05 Student's t-test.

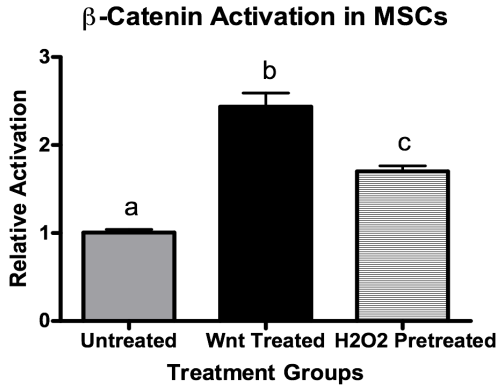


Figure 22. Wnt-Induced β-catenin activation in MSCs pre-loaded with oxidative stress. n=9/group, different letters denote groups that are statistically different, p<0.05 one-way ANOVA.

CHAPTER 6

DISCUSSION

There are approximately 16 million bone fractures that occur annually within the United States alone, yet, around 10% of those fractures fail to heal properly without extensive intervention contributing to a significant burden on the individual and the healthcare system as a whole (96). These facts underscore the significance of understanding the intricate factors that contribute to improper healing, as well as elucidating any potential clinical therapy.

The underlying factors contributing to nonunion are not well understood, but our laboratory has developed a clinically relevant model of delayed union using binge alcohol administration in rodents. Previous studies into the effects of alcohol on fracture healing have utilized osteotomies with immobilization, and have focused primarily on late-stage mineralization (6, 11, 101, 102). While important, these studies are limited to examining how alcohol affects osteoblast function and subsequent mineralization. Also, the studies that have investigated alcohol's effects on callus initiation and early formation concentrate on systemic and local inflammatory signaling (98, 99, 100).

In these studies, we have created a clinically-relevant model of fracture healing that recapitulates real world parameters, including age of animals, paradigm of alcohol administration, and timing of injury. We used force to

generate the fracture, and utilized intramedullary pinning to create semi-rigid fixation of the fracture. Semi-rigid fixation is especially important because a small amount of motion at the fracture site during healing is necessary to drive cartilaginous callus formation and subsequent endochondral ossification. Rigid fixation of the fracture produces healing through intramembranous ossification, and therefore would not be an appropriate model to examine effects of treatment on external, cartilaginous callus formation. Thus, our model makes it possible to study the process through which most long bone fractures heal in humans, both at early and late stages of healing.

We have shown that episodic binge alcohol exposure in mice before and after fracture impairs the process of endochondral ossification. We see significant reductions in the formation of the cartilaginous callus and the maturation of chondrocytes in our alcohol-treated animals. Interestingly, alcohol does not seem to negatively affect the formation of the periosteal callus, which forms from mature osteoblasts embedded within the periosteum directly creating new bone through intramembranous ossification. This could be because the mature osteoblasts are not as susceptible to the effects of alcohol as immature cells, like MSCs, may be. Mature osteoblasts seemingly could simultaneously combat excessive oxidative stress and deposit osteoid. However, maybe MSCs tasked with the substantial undertaking of differentiation can not simultaneously shift intracellular signaling to fight oxidative stress and differentiate. Both the reductions in cartilaginous callus formation as well as the unaffected periosteal

callus formation may speak to an impairment in MSC differentiation and chondrogenesis. Previous data from our lab have shown that alcohol disrupts gene expression regulated by canonical Wnt signaling within the fracture callus (59, 60). The canonical Wnt signaling pathway is important during healing as it drives the differentiation of MSCs towards osteoblasts and chondrocytes that are indispensable in proper healing. To this end, Wnt signaling relies on the transcription factor β-catenin to bind to the cofactor TCF/LEF in order to up regulate genes related to chondrogenesis and osteogenesis. The family of FoxO transcription factors, however, binds to β-catenin, leading to the antagonism of Wnt-related gene induction. FoxOs are known to be activated by oxidative stress, and alcohol-abuse is a known contributor to oxidative stress in the body (16). Therefore, we hypothesized that episodic ethanol administration would lead to deficient fracture repair by activating FoxO transcription factors within the fracture callus, suppressing chondrogenesis and subsequent cartilaginous callus formation. In these studies, not only did we demonstrate alcohol's suppression of fracture healing, namely through impairments in cartilaginous callus formation, but we have shown that alcohol-administration in mice was able to produce a pattern of post-translational modifications indicative of FoxO signaling activation during the healing process. We also went on to delineate that these findings were the result of alcohol-induced oxidative stress by using an antioxidant n-acetyl-cysteine (NAC) to prevent the aforementioned perturbations in callus component formation, as well as the increase in FoxO expression and markers of activation

within the callus of alcohol-treated animals. This is the first report to quantify alcohol's inhibition of cartilaginous callus formation early in the healing process, as well as the first to associate these effects with FoxO activation induced by systemic oxidative stress.

We have also begun elucidating the underpinnings of alcohol's effects directly in MSCs. By showing that EtOH exposure can activate FoxO signaling within MSCs, we have contributed to our overall understanding of how alcohol affects the health of the skeleton and fracture healing. All the data taken together cohesively supports our overall hypothesis that alcohol is negatively affecting fracture healing by acting on MSCs to increase FoxO activation which can then go on to competitively inhibit canonical Wnt signaling and thus inhibit osteochondroblastic differentiation. Normally, abating excessive oxidative stress accumulation is ultimately beneficially to overall cell survival and, by extension, tissue health. However, during times when proper and timely differentiation is necessary, as with fracture healing, deviation of differentiation machinery can prove ultimately detrimental. Furthermore, this may also come to explain how excessive alcohol consumption over a longer duration, like with chronic binge drinking or alcoholism, leads to poor bone health, as alcohol can suppress the MSC-mediated replenishment of osteoblasts, even though alcohol may not have a great impact on already mature and functioning osteoblasts.

Our findings in a fracture injury model support the work of other groups, which have found that activation of FoxO signaling in skeletal cells *in vivo* and *in*

vitro leads to a decrease in Wnt signaling, osteoblast formation, and overall bone health (74). Interestingly, there appears to be a link between aging and an increased systemic oxidative load, leading to an activation of FoxO signaling and subsequent skeletal involution (27, 104), which serves to further highlight the importance of our work as alcohol abuse seems to be recapitulating a proposed mechanism of skeletal involution related to aging.

Alcohol consumption may have similar effects on fracture healing as diabetes, another pro-oxidative condition. Diabetes, much like alcohol abuse, has associated maladies like osteopenia, decreased bone mineral density, and impaired fracture healing (107, 108, 112, 113). Animal studies have elucidated many possible explanations for these effects of diabetes on the skeleton. Increased inflammation, oxidative stress, and advanced glycation end product (AGEs) formation are shown to lead to an increase in osteoclastogenesis and osteoclast activity, a decrease in osteoblastogenesis, an increase in adipogenesis, and an overall increase in stem cell egression from their bone marrow niches (112, 113, 114). One study has found that this increased adiposity also occurs during fracture healing in diabetic mice (112).

Interestingly, FoxO transcription factors have been implicated in the pathobiology of diabetes-induced diminished bone health, with an increase in oxidative stress caused by diabetes leading to an increase in FoxO expression and activity (107, 108, 113, 114, 116, 117). This supports the premise that the two seemingly disparate conditions of alcohol abuse and diabetes could be

acting through a shared mechanism in FoxO activation to elicit such similar deleterious effects on bone health. More to this point, Sirtuin 1 (Sirt1) activation has become an attractive target for osteoprotective treatments for diabetic patients (116). Sirt1 deacetylates FoxO, and this action prevents FoxOs from sequestering β-catenin, thereby promoting Wnt signaling (117). So, not only could the bone-specific pathologies of aging, diabetes, and alcohol abuse originate through a shared mechanism, the treatment of each could be the same in using Sirt1 activators as therapeutics.

One proposed mechanism for the perturbed healing in diabetics is through FoxO1 stimulating chondrocyte apoptosis (107, 108). In our study, however, we did not observe an increase in markers of apoptosis in our day 3 and day 6 post-injury fracture callus samples. The possible explanations for these varying results include the differences in time points following injury examined between the two studies, as well as the differences between the chronic nature of diabetes-related stress responses and the more acute stress of alcohol exposure, with their associated differences in signaling upstream of FoxO1 connected to either chronic diabetic hyperglycemia or transient alcoholic oxidative stress. This means that activation of FoxO could happen through different means in each model, with diabetic activation happening through suppression of Akt signaling and alcohol-associated activation happening through MST1 or JNK signaling. These findings just serve to highlight the varied roles that FoxO signaling may play based upon the sum of different intracellular cues. Perhaps in our acute

alcohol-based model, FoxO1 activation disrupts MSC differentiation pathways away from chondrogenesis, rather than inducing apoptosis in differentiated chondrocytes.

Some unexpected results of these studies are that we did not witness significant changes in FoxO expression or markers of activation at day 9 post-fracture, and we had some conflicting results using hydrogen peroxide as a positive control of oxidative stress induction. As of yet, we do not completely understand why alcohol is failing to elicit much of a FoxO response at this time point. This could possibly be due to compensatory cellular mechanisms taking place after the extended alcohol treatment, or could be more likely due to the gross changes in the callus at this time. The callus by day 9 has become a lot less cellular than previous time points and is now beginning to ossify. So at day 9 there is more mineral deposition, less active chondrocytes, and less chondrocyte maturation taking place. All of this, paired with the marked decrease in MSC differentiation at day 9, may explain why our alcohol treatment failed to elicit much of a change in FoxO compared to the saline treatment.

Using hydrogen peroxide as an inducer of oxidative stress proved problematic, at least at some of the doses used in these studies. We found promising and expected results when using lower doses of hydrogen peroxide or a shorter exposure time. This could be because our higher and longer doses proved too stressful for our cells, resulting in cell quiescence or death, ultimately confounding our results.

Since alcohol appears to predominantly affect cartilaginous callus formation, maybe alcohol disproportionately affects chondrogenesis and chondrocyte maturation and function. Also, it is possible that already-formed and mature, resident osteoblasts within the bone lining can function relatively undisturbed by alcohol exposure. Maybe it is only the MSCs and chrondoblastic or osteoblastic precursors that bear the brunt of alcohol's deleterious effects as the cells cannot properly balance fighting an increased oxidative load while concomitantly attempting to differentiate. If alcohol is disrupting MSC differentiation during early callus formation, then it could have long lasting effects on late-stage callus formation and eventual fracture healing.

Ultimately this study leaves more work to be done to directly connect alcohol-induced oxidative stress and FoxO signaling with decreased canonical Wnt signaling and perturbed differentiation potential of MSCs. The purpose of this study was to merely determine if alcohol-induced oxidative stress was a leading contributor to poor fracture healing in our model and if this process could involve FoxO signaling. There are many different cell types within the fracture callus. Our study is unable to differentiate between these cell types. We next need to show alcohol's ability to increase oxidative stress in MSCs, promote the interaction between FoxOs and β-catenin, disrupt Wnt driven transcription and increase FoxO driven transcription, and disrupt normal differentiation all in MSCs. These are all necessary to prove that this is indeed a mechanism through which alcohol is having its effects, and that these overall effects of alcohol on fracture

healing act through MSCs and the modulation of differentiation, and not through other, more indirect means.

For now, the findings of these studies may be limited to the rodent model, as fracture healing in mice is accelerated compared to humans and rodents have growth plates that persist throughout aging, and an *in vitro* model of EtOH exposure. However, we have shown that episodic or binge drinking can lead to significant delays in fracture healing, which could eventually impact the treatment of patients presenting with a high blood alcohol content. Also, this work has begun to elucidate a link between alcohol-induced oxidative stress and perturbed fracture healing. Certain basal levels of ROS are important for intracellular signaling at different stages of fracture healing, so a blanket suppression of ROS during healing may not be advantageous (28, 105). However, antioxidant therapies stand to be potentially beneficial for individuals at risk for a pathologically elevated oxidative load, such as alcohol abusers. This may not only be true for fracture repair, but could extend to a myriad of MSC therapies, as excessive oxidative stress could impair MSC function and differentiation. And, as previously mentioned, Sirt1 activators are also interesting potential therapies for all of these different bone maladies.

However, the findings from our culture studies show particular promise, in so much as our results have a nicely fitting time course. With EtOH exposure, we first see the highest levels of FoxO mRNA being transcribed at 3 hours. This is followed by a peak in FoxO protein translation at 6 hours. And finally, we find the

highest levels of FoxO-target gene expression, in Catalase, at 9 hours. So even though attenuating ROS during healing may prove a difficult endeavor to properly titrate clinically, mitigating excessive ROS accumulation may prove beneficial for normal bone homeostasis. This may be especially true for chronic pro-oxidative conditions, such as aging and diabetes, as it is often the excessive accumulation of oxidative stress that is implicated in the myriad deleterious pathologies associated with these conditions.

APPENDIX

DETAILED METHODS DESCRIPTIONS

Mouse Fracture Surgery

Administer alcohol/saline 1 hour prior to fracture, Buprenorphine approximately 35 min prior to surgery, Ket/Xyl cocktail 5 minutes prior to surgery, gentamicin immediately prior to surgery. See drug makeup sheet for dosages.

1. Prepare animal prep, surgical, and recovery areas.
    a. Animal prep: Put down clean lab diaper, lay out gauze, cotton tip applicators, nair, saline, all animal drugs.
    b. Surgical area: Put down heating pad with clean lab diaper. Tape down isoflurane tube.
        i. Set up bead sterilizer, cup of 70% ethanol, betadine/iodine, gauze, alcohol swabs and a light overhead if needed
        ii. Prepare sterile gloves, 27 G needle for reaming, size 000 autoclaved stainless steel insect pins, 6-0 prolene sutures
        iii. Have sterilized instruments: pair iris scissors, angled bone cutting scissors, fine and blunt forceps, adson pickups, needle driver
        iv. Warm sterile saline for resuscitation
    c. Recovery Area: heating pads, clean cages with food and water. Add some food pellets to the bottom of the cage for easy access to food.
2. In Prep area: After Ket/Xyl cocktail induction, scruff mouse at nape of neck (similar to IP injection). Immobilize animal to apply nair using a cotton tip applicator to entire leg. Allow to set for 1 minute before removing with saline soaked gauze. Dry animal with clean gauze. Administer antibiotic (gentamicin) via subcutaneous injection.
3. In Surgical area: Place animal on clean drape on warm heating pad. Allow animal to anesthetize completely under isoflurane. Check animal for responsiveness.
    a. Open 27 G needle and put on surgical gloves
    b. Clean naired tibia with betadine/iodine and gauze. Wipe clean with alcohol swab.
    c. Using pickups and iris scissors, make a 2-3 mm incision over lower knee cap
    d. Find the white patellar tendon and use a 27 G needle to find the patellar tendon insertion point on the tibia. Using slight pressure, insert the 27 G needle into the tendon insertion and into the tibia until resistance is met.
    e. Remove the needle and insert the insect pin. Ensure insect pin is fully down to the ankle. Double check placement by comparing with another insect pin.
    f. At about the midshaft, place the scissors so that one shear is above the tibia and the other between the tibia and fibula and cut to fracture

just the tibia. The insect pin should be low enough to hold the fractured bones together still.

g. Cut the insect pin so it is flush with the tibia at the tendon insertion point, leaving just the portion of the pin that stabilizes the fractured bones. Close the skin with continuous suture stitches. Clean the stitching with alcohol. Give the animal 1 ml warm saline ip.

4. In Recovery area: Place the animal on its stomach in a clean, warm cage on a heating pad. Place some food pellets on the floor of the cage and be sure the water bottle is not leaking. Administer bup 8-10 hours post surgery and again 8-10 hours after that.

Quick Guide:

1. Alcohol/saline 1 hour prior to surgery (IP)
2. Buprenex 35 minutes prior to surgery (SC)
3. Ket/Xyl 5 minutes pre surgery (IP)
4. Nair leg/ wipe clean
5. Gentamicin (SC)
6. Fully anesthetize with isoflurane
7. Clean leg with povidone/iodine and wipe with alcohol swab
8. Create incision over knee
9. Insert 27 G needle into patellar tendon insertion point, into tibia, and remove
10. Insert insect pin into hole bore by 27 G needle, into tibia until resistance is met
11. Fracture tibia mid-shaft, taking care not to fracture fibula
12. Cut Insect pin flush
13. Suture wound and clean
14. Administer 1 ml warm saline IP

Supplies and Reagents:

- 20% Alcohol
    - Dilute 200 proof molecular grade EtOH to 20% (v/v) and filter
    - Ethanol density is 0.789 g/ml at 200 proof
    - Multiply body weight in kg by 0.158 g/ml by alcohol dose in g/kg body weight to get dose for each mouse

Callus Protein Isolation

Materials:

- Dry Ice
- Ice
- Dremel
- Forceps
- Liquid Nitrogen
- Freezer Mill
- Freezer Mill Tubes, Caps, and Metal Pulverizer
- RIPA Buffer
- 10X Protease and 10X Phosphatase Inhibitor
- P1000 and Pipette Tips
- 50mL Conical Tubes
- Microcentrifuge Tubes

Method:

1. Keep the snap frozen, harvested tibiae in their micro centrifuge tubes on dry ice until the sample is to be used.
2. Add appropriate amount of protease and phosphatase inhibitor into the needed working volume of RIPA buffer to reach 1X concentrations. Keep on ice.
3. Remove the insect pin from the bone
4. Isolate the callus by cutting off the proximal and distal ends of the tibia with the Dremel on a flat piece of dry ice.
5. All the following steps occur in liquid nitrogen as much as possible.
6. Add the isolated callus to a Freezer Mill tube with the bottom cap already affixed. Add 1mL of RIPA buffer. Add the metal pulverizer. Cap the top of the Freezer Mill tube.
7. Insert the assembled Freezer Mill tube into the Freezer Mill, close, and run cycle.

8. After cycle is complete, remove Freezer Mill tube and uncap using the provided Freezer Mill cap-removers. Carefully remove the metal pulverizer and then dump the contents into a pre-labeled 50mL conical. Make sure to get all of the lysate out of the Freezer Mill tube. Cap 50mL conical and keep on liquid nitrogen.
9. Clean Freezer Mill tube with warm water. Dry with paper towels and get ready to be reused.
10. After pulverizing all the samples with RIPA buffer, allow samples to thaw on ice.
11. Further disrupt and agitate samples by passing the sample through a pipette tip using a P1000. Transfer samples to 1mL microcentrifuge tubes, and centrifuge at 1,000rpm for 10 minutes and then collect the supernatant.
12. Aliquot supernatant into clean microcentrifuge tubes to be stored at -80 degrees celsius to be used as desired.

Callus RNA Isolation

Materials:

- Dry Ice
- Wet Ice
- Dremel
- Forceps
- Liquid Nitrogen
- Freezer Mill
- Freezer Mill Tubes, Caps, and Metal Pulverizer
- TRI Reagent
- P1000 and Pipette Tips
- 50mL Conical Tubes
- Microcentrifuge Tubes
- Chloroform
- EtOH
- RNAase
- RNA Spin Filter/Collection Kit

Method:

1. For cells grown in monolayer: Remove media, add 3mL of TRI Reagent per T75 flask. Incubate for 5min at room temperature. Do not wash the cells before adding the TRI Reagent. For fracture callus tissue: add 1mL of TRI Reagent into Freezer Mill tube with the isolated callus. Run through Freezer Mill cycle. All steps to be preformed in liquid nitrogen. After pulverization, allow lysate to thaw in a tube on ice. (Similar to Callus Protein Isolation procedure)
2. Transfer the cell suspension in a plastic 14mL Polypropylene Round-Bottom Tube.
3. Add 300uL of chloroform (w/o isoamyl alcohol) or bromocloropropane (BCP).
4. Cap each tube thightly and vortex at maximum speed for 15sec.
5. Incubate the mixture at room temperature for 5min.

6. Centrifuge at 10,000rpm for 10min at 4°C to separate the mixture into a lower, red, organic phase; an interphase; and a colorless, upper, aqueous phase.
   NOTE*** Centrifugation at temperatures >8°C may cause some DNA to partition in the aqueous phase.
7. Transfer 400uL of the aqueous phase (top layer) to a new-labeled 1.5mL microcentrifuge tube. Use as many tubes as you need
   NOTE***You can discard the tube after removing the aqueous phase, or the other phases can be processed for protein and/or DNA isolation.
8. For the RNA purification, add 200uL of 100% ethanol to 400uL of aqueous phase.
9. Vortex immediately at maximum speed for 5sec to avoid RNA precipitation.
10. Now, pass the samples through a filter cartridge. For each sample, place a filter cartridge in one of the collection tubes supplied.
11. Transfer the sample to a filter cartridge-collection tube assembly and close the lid.
12. Centrifuge the assembly at 12,000xg for 30sec at room temperature or until all of the liquid is through the filter.
13. Discard the flow-through and return the filter cartridge to the same collection tube. The RNA is now attached in the Filter Cartridge.
14. Next, wash the filter twice by applying 500uL of Wash Solution to the filter cartridge-collection tube assembly, and close the lid.
15. Centrifuge for 30s at room temperature or until all of the liquid is through the filter.
16. Discard the flow-through and return the filter cartridge to the same collection tube.
17. Repeat steps 14-16 for the second wash.
18. Centrifuge for 30sec at room temperature to remove residual wash solution.
19. Now, transfer the filter cartridge to a new collection tube.
20. Elute the RNA by adding 100uL of Elution buffer to the filter column.
21. Incubate at room temperature for 2min.
22. Centrifuge for 30sec to elute the RNA from the filter. The RNA will be in the elute in the collection tube.
23. Store the recovered RNA at 4°C or, for long term storage, at -20°C.

Rat MSC Isolation

Isolation of Rodent Bone Marrow Derived Mesenchymal Stem Cells:

Materials:

- D-MEM supplemented with 1% Pen/Strep
- FBS
- HBBS supplemented with 1% Pen/Strep
- Clipper
- Ethanol 70%
- Povidone iodine
- Hypodermic needles: 18G, 23G and 25G
- 10 ml syringe
- Surgical Gloves, Scalpel Blade No 15
- 2 instrument packs

Method:

- In a laminar flow hood, pipette 30 ml of HBBS solution into a 50 ml centrifuge tube. Store the tube on ice.
- Sacrifice the rodent by asphyxiation with CO2
- Shave fur from the hind limbs, back, and belly with clipper
- Place the animals inside a 2-L plastic beaker and cover it with 70% ethanol for 5 minutes
- Prepare the working area by wiping the surface with 70% ethanol
- Remove the mouse from the beaker and place it on a sterile drape.
- With one gauze sponge, wipe the legs of the mouse with povidone iodine and then with ethanol.

- Make an incision around the perimeter of the hind limbs where they attach to the trunk and remove the skin by pulling toward the foot, which is cut at the anklebone

- Separate the femur and tibia from the animal and transfer them to the 50 ml HBBS-containing centrifuge tube

- Scrape off any remaining soft tissue from the bones and separate tibia and femur from the knee under the hood. Place all bones inside 100-mm dishes containing HBBS solution.

- Add 10 ml of serum-containing medium to a 100 mm dish.

- Carefully open the packs containing the syringe, needles, and one scalpel blade and drop their contents onto a sterile drape under the hood. Transfer the 60 mm medium-containing dishes onto the sterile drape

- Don a fresh pair of sterile gloves.

- Attach a 23-gauge needle for mice or 18-gauge needle for rats to the syringe, and use the needle to drill a hole in the distal end of the femur and the proximal end of the tibiae. Cut off the distal end of the tibiae and the proximal end of the femur with a rongeur and transfer them to a second dish

- Draw the medium into the 10-mL syringe; attach a fresh 23-gauge needle for mice or 18-gauge needle for rats.

- Insert the needle into the holes just prepared and inject a small volume of medium into the bone to flush the marrow into a second dish.

- When the marrow from all of the bones has been ejected, disrupt the marrow by passing it though 23-gauge and 25-gauge needle.

- Filter the cell suspension through a 70-μm filter mesh to remove any bone spicules or muscle and cell clumps. Determine the yield and viability of cells by Trypan blue exclusion and counting

- Centrifuge at 450g for 5 min.

- Remove the supernate and resuspend the cells in 5 ml of serum-containing medium and transfer it to a 25 cm^2 cell culture flask.

- Culture medium is changed after 3 hours and then the next day and the cells are subcultured before colonies become multilayered, usually around day 14.

REFERENCE LIST

1. McGinnis, J. and W. Foege (1999). Mortality and morbidity attributable to use of addictive substances in the United States. Proc Assoc Am Physicians. **111:** 109-18.

2. Bikle, D., H. Genant, C. Cann, R. Recker, B. Halloran, and G. Strewler. (1985). Bone disease in alcohol abuse. Ann Intern Med. **103:** 42-8.

3. Levy, R., C. Hebert, B. Munn, and R. Barrack. (1996). Drug and alcohol use in orthopedic trauma patients: a prospective study. Journal of orthopaedic trauma. **10:** 21-7.

4. Savola, O., O. Niemelä, and M. Hillbom. (2004). Blood alcohol is the best indicator of hazardous alcohol drinking in young adults and working-age patients with trauma. Alcohol Alcohol. **39:** 340-5.

5. Savola, O., O. Niemelä, and M. Hillbom. (2005). Alcohol intake and the pattern of trauma in young adults and working aged people admitted after trauma. Alcohol Alcohol. **40:** 269-73.

6. Jänicke-Lorenz, J. and R. Lorenz (1984). Alcoholism and fracture healing. A radiological study in the rat. Archives of orthopaedic and traumatic surgery Archiv für orthopädische und Unfall-Chirurgie. **103:** 286-9.

7. Lauing, K., R. Himes, M. Rachwalski, P. Strotman, and J. Callaci. (2008). Binge alcohol treatment of adolescent rats followed by alcohol abstinence is associated with site-specific differences in bone loss and incomplete recovery of bone mass and strength. Alcohol. **42:** 649-56.

8. Callaci, J., R. Himes, K. Lauing, F. Wezeman, and K. Brownson. (2009). Binge alcohol-induced bone damage is accompanied by differential expression of bone remodeling-related genes in rat vertebral bone. Calcif Tissue Int. **84:** 474-84.

9. Volkmer, D., B. Sears, K. Lauing, R. Nauer, P. Roper, S. Yong, M. Stover, and J. Callaci. (2011). Antioxidant therapy attenuates deficient bone fracture repair associated with binge alcohol exposure. Journal of orthopaedic trauma. **25:** 516-21.

10. Chakkalakal, D. (2005). Alcohol-induced bone loss and deficient bone repair. Alcohol Clin Exp Res. **29:** 2077-90.

11. Kristensson, H., A. Lundén, and B. Nilsson. (1980). Fracture incidence and diagnostic roentgen in alcoholics. Acta orthopaedica Scandinavica. **51:** 205-7.

12. Bishop, G. and T. Einhorn (2007). Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery. Int Orthop. **31:** 721-7.

13. Chen, Y., H. Whetstone, A. Lin, P. Nadesan, Q. Wei, R. Poon, and B. Alman. (2007). Beta-catenin signaling plays a disparate role in different phases of fracture repair: implications for therapy to improve bone healing. PLoS Med. **4:** e249.

14. Bennett, C., K. Longo, W. Wright, L. Suva, T. Lane, K. Hankenson, and O. MacDougald. (2005). Regulation of osteoblastogenesis and bone mass by Wnt10b. Proc Natl Acad Sci USA. **102:** 3324-9.

15. Almeida, M., L. Han, M. Martin-Millan, C. O'Brien, and S. Manolagas. (2007). Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box O-mediated transcription. J Biol Chem. **282:** 27298-305.

16. Das, S. and D. Vasudevan (2007). Alcohol-induced oxidative stress. Life Sci. **81:** 177-87.

17. Chakkalakal, D., J. Novak, E. Fritz, T. Mollner, D. McVicker, K. Garvin, M. McGuire, and T. Donohue. (2005). Inhibition of bone repair in a rat model for chronic and excessive alcohol consumption. Alcohol. **36:** 201-14.

18. Zima, T., L. Fialová, O. Mestek, M. Janebova, J. Crkovska, I. Malbohan, S. Stipek, L. Mikulikova, and P. Popov. (2001). Oxidative stress, metabolism of ethanol and alcohol-related diseases. J Biomed Sci. **8:** 59-70.

19. Bielby, R., E. Jones, and D. McGonagle. (2007). The role of mesenchymal stem cells in maintenance and repair of bone. Injury. **38 Suppl 1:** S26-32.

20. Chen, J.-R., O. Lazarenko, K. Shankar, M. Blackburn, C. Lumpkin, T. Badger, and M. Ronis. (2011). Inhibition of NADPH Oxidases Prevents Chronic Ethanol-Induced Bone Loss in Female Rats. J Pharmacol Exp Ther. **336:** 734-742.

21. Elmali, N., K. Ertem, S. Ozen, M. Inan, T. Baysal, G. Guner, and A. Bora. (2002). Fracture healing and bone mass in rats fed on liquid diet containing ethanol. Alcohol Clin Exp Res. **26:** 509-13.

22. Lecomte, E., B. Herbeth, P. Pirollet, Y. Chancerelle, J. Arnaud, N. Musse, F. Paille, G. Siest, and Y. Artur. (1994). Effect of alcohol consumption on blood antioxidant nutrients and oxidative stress indicators. Am J Clin Nutr. **60:** 255-61.

23. Glass, D. and G. Karsenty (2006). Canonical Wnt signaling in osteoblasts is required for osteoclast differentiation. Ann N Y Acad Sci. **1068:** 117-30.

24. Glass, D. and G. Karsenty (2007). In vivo analysis of Wnt signaling in bone. Endocrinology. **148:** 2630-4.

25. Ambrogini, E., M. Almeida, M. Martin-Millan, J. Paik, R. Depinho, L. Han, J. Goellner, R. Weinstein, R. Jilka, C. O'Brien, and S. Manolagas. (2010). FoxO-mediated defense against oxidative stress in osteoblasts is indispensable for skeletal homeostasis in mice. Cell metabolism. **11:** 136-146.

26. Sedding, D. (2008). FoxO transcription factors in oxidative stress response and ageing--a new fork on the way to longevity? Biol Chem. **389:** 279-83.

27. Hoogeboom, D. and B. Burgering (2009). Should I stay or should I go: beta-catenin decides under stress. Biochim Biophys Acta. **1796:** 63-74.

28. Manolagas, S. (2010). From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. Endocrine reviews. **31:** 266-300.

29. Hoogeboom, D., M. A. G. Essers, P. Polderman, E. Voets, L. Smits, and B. Burgering. (2008). Interaction of FOXO with beta-catenin inhibits beta-catenin/T cell factor activity. J Biol Chem. **283:** 9224-30.

30. Szabo, I. L., H. Kawanaka, M. Jones, R. Pai, B. Soreghan, D. Baatar, S. Husain, and A. Tarnawski. (2001). Activation of hypoxia inducible factor-1alpha in gastric mucosa in response to ethanol injury: a trigger for angiogenesis? Life Sci. **69:** 3035-44.

31. Li, L., S.-H. Chen, Y. Zhang, C. Yu, S. Li, and Y. Li. (2006). Is the hypoxia-inducible factor-1 alpha mRNA expression activated by ethanol-induced injury, the mechanism underlying alcoholic liver disease? HBPD INT. **5:** 560-3.

32. Gong, Z. and F. H. Wezeman (2004). Inhibitory effect of alcohol on osteogenic differentiation in human bone marrow-derived mesenchymal stem cells. Alcohol Clin Exp Res. **28:** 468-79.

33. Wezeman, F. H. and Z. Gong (2004). Adipogenic effect of alcohol on human bone marrow-derived mesenchymal stem cells. Alcohol Clin Exp Res. **28:** 1091-101.

34. Almeida, M., E. Ambrogini, L. Han, S. Manolagas, and R. Jilka. (2009). Increased lipid oxidation causes oxidative stress, increased PPAR{gamma} expression and diminished pro-osteogenic Wnt signaling in the skeleton. J Biol Chem. **284:** 27438-48.

35. Chen, J.-R., K. Shankar, S. Nagarajan, T. Badger, and M. Ronis. (2008). Protective effects of estradiol on ethanol-induced bone loss involve inhibition of reactive oxygen species generation in osteoblasts and downstream activation of the extracellular signal-regulated kinase/signal transducer and activator of transcription 3/receptor activator of nuclear factor-kappaB ligand signaling cascade. J Pharmacol Exp Ther. **324:** 50-9.

36. Chen, J.-R., O. P. Lazarenko, K. Shankar, M. Blackburn, T. Badger, and M. Ronis. (2010). A role for ethanol-induced oxidative stress in controlling lineage commitment of mesenchymal stromal cells through inhibition of Wnt/beta-catenin signaling. J Bone Miner Res. **25:** 1117-27.

37. Zhou, H., L. Shang, X. Li, X. Zhang, G. Gao, C. Guo, B. Chen, Q. Liu, Y. Gong, and C. Shao. (2009). Resveratrol augments the canonical Wnt signaling pathway in promoting osteoblastic differentiation of multipotent mesenchymal cells. Exp Cell Res. **315:** 2953-62.

38. Gimble, J. M., S. Zvonic, Z. Floyd, M. Kassem, and M. Nuttall. (2006). Playing with bone and fat. J Cell Biochem. **98:** 251-66.

39. Huff, N. K., N. D. Spencer, J. Gimble, G. Bagby, S. Nelson, and M. Lopez. (2011). Impaired expansion and multipotentiality of adult stromal cells in a rat chronic alcohol abuse model. Alcohol. **45:** 393-402.

40. Provot, S. and Schipani, E. (2005). Molecular mechanisms of endochondral bone development. Biochem Biophys Res Commun. **328:** 658-665.

41. de Crombrugghe B., V. Lefebvre, and K. Nakashima. (2001). Regulatory mechanisms in the pathways of cartilage and bone formation. Current Opinion in Cell Bio. **13:** 721-727.

42. Olsen, B. R. (2006). Chapter one: bone embryology. Am Society for Bone and Miner Res. 2-6.

43. Dempster, D. W. (2006). Chapter two: anatomy and functions of the adult skeleton. Am Society for Bone and Miner Res. 7-11.

44. Ling, L., V. Nurcombe, and S. Cool. (2009). Wnt signaling controls the fate of mesenchymal stem cells. Gene. **433:** 1-7.

45. Aubin, J. E., J. B. Lian, et al. (2006). Chapter four: bone formation: maturation and functional activities of osteoblast lineage cells. Am Society for Bone and Miner Res. 20-29.

46. Marie, P. J. (2008). Transcription factors controlling osteoblastogenesis. Arc of Biochem and Biophys. **473:** 98-105.

47. Pajevic, P. D. (2009). Regulation of bone resorption and mineral homeostasis by osteocytes. IBMS BoneKEy. **6:** 63-70.

48. Yu, D. J. Han, and B. Kim. (2012). Stimulation of chondrogenic differentiation of mesenchymal stem cells. Int J Stem Cells. **5:** 16-22.

49. Wallis, G. A. (1996). Bone growth: coordinating chondrocyte differentiation. Curr Bio. **6:** 1577-1580.

50. Ross, F. P. (2006). Chapter five: osteoclast biology and bone resorption. Am Society for Bone and Miner Res. 30-35.

51. Teitelbaum, S. L. (2000). Bone resorption by osteoclasts. Science. **289:** 1504-1508.

52. Nakashima, K. and B. de Crombrugghe. (2003). Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends in Genetics. **19:** 458-466.

53. Savola, O., O. Niemela, and M. Hillbom. (2004). Blood alcohol is the best indicator of hazardous alcohol drinking in young adults and working-age patients with trauma. Alcohol and Alcoholism. **39:** 340-345.

54. Savola, O., O. Niemela, and M. Hillbom. (2005). Alcohol intake and the pattern of trauma in young adults and working aged people admitted after trauma. Alcohol and Alcoholism. **40:** 269-273.

55. Soffer, D., O. Zmora, J. Klausner, O. Szold, A. Givon, P. Halpern, C. Schulman, and K. Peleg. (2006). Alcohol use among trauma victims admitted to a level 1 trauma center in Israel. IMAJ. **8:** 98-102.

56. Nyquist, F., M. Berglund, B. Nilsson, and K. Obrant. (1997). Nature and healing of tibial shaft fractures in alcohol abusers. Alcohol and Alcoholism. **32:** 91-95.

57. Kanny, D., Y. Liu, and R. Brewer. (2012). Vital signs: binge drinking prevalence, frequency, and intensity among adults-United States, 2010. Cent Dis Cont Prev: Morb Mort Week Rep. **61**.

58. Obermeyer, T. S., D. Yonick, K. Lauing, S. Stock, R. Nauer, P. Strotman, R. Shankar, R. Gamelli, M. Stover, and J. Callaci. (2012). Mesenchymal stem cells facilitate fracture repair in an alcohol-induced impaired healing model. J Orthop Trauma. **26:** 712-718.

59. Lauing, K. L., P. M. Roper, R. K. Nauer, and J. J. Callaci. (2012). Acute alcohol exposure impairs fracture healing and deregulates β-catenin signaling in the fracture callus. Alcohol Clin Exp Res. **36:** 2095-2103.

60. Callaci, J. J., R. Himes, K. Lauing, and P. Roper. (2010). Long-term modulations in the vertebral transcriptome of adolescent-stage rats exposed to binge alcohol. Alcohol and Alcoholism. **45:** 332-346.

61. Elmali, N., K. Ertem, S. Ozen, M. Inan, T. Baysal, G. Guner, and A. Bora. (2002). Fracture healing and bone mass in rats fed on liquid diet containing ethanol. Alcoholism: Clin and Exp Res. **26:** 509-513.

62. Lauing, K. L., S. Sundaramurthy, R. K. Nauer, and J. J. Callaci. (2014). Exogenous activation of Wnt/β-catenin signaling attenuates binge alcohol-induced deficient bone fracture healing. Alcohol and Alcoholism. Advance Access doi: 10.1093/alcalc/agu006.

63. Robey, P. G. and A. L. Boskey. (2006). Chapter three: extracellular matrix and biomineralization of bone. Am Society for Bone and Miner Res. 12-19.

64. Monroe, D. G., M. E. McGee-Lawrence, M. J. Oursler, and J. J. Westendorf. (2012). Update on Wnt signaling in bone cell biology and bone disease. Gene. **492:** 1-18.

65. Li, L., H. Yuan, C. D. Weaver, J. Mao, G. H. Farr, D. J. Sussman, J. Jonkers, D. Kimelman, and D. Wu. (1999). Axin and Frat1 interact with Dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. Eur Mol Bio Org J. **18:** 4233-4240.

66. Einhorn, T. A. (2005). The science of fracture healing. J Orthop Trauma. **19**(Suppl.)**:** S4-S6.

67. Secreto, F. J., L. J. Hoeppner, and J. J. Westendorf. (2009). Wnt signaling during fracture repair. Curr Osteoporos Rep. **7:** 64-69.

68. Komatsu, D. E., M. N. Mary, R. J. Schroeder, A. G. Robling, C. H. Turner, and S. J. Warden. (2010). Modulation of Wnt signaling influences fracture repair. J Orthop Res. **28:** 928-936.

69. Hiltunen, A., E. Vuorio, and H. T. Aro. (1993). A standardized experimental fracture in the mouse tibia. J Orthop Res. **11:** 305-312.

70. Hadjiargyrou, M., F. Lombardo, S. Zhao, W. Ahrens, J. Joo, H. Ahn, M. Jurman, D. White, and C. Rubin. (2002). Transcriptional profiling of bone regeneration: insight into the molecular complexity of wound repair. J Biol Chem. **277:** 30177-30182.

71. Zhong, N., R. P. Gersch, and M. Hadjiargyrou. (2006). Wnt signaling activation during bone regeneration and the role of Dishevelled in chondrocyte proliferation and differentiation. Bone. **39:** 5-16.

72. Le, A. X., T. Miclau, D. Hu, and J. Helms. (2001). Molecular aspects of healing in stabilized and non-stabilized fractures. J Orthop Res. **19:** 78-84.

73. Essers, M. A., L. M. de Vries-Smits, N. Barker, P. E. Polderman, B. M. Burgering, and H. C. Korswagen. (2005). Functional interaction between β-catenin and FoxO in oxidative stress signaling. Science. **308:** 1181-1184.

74. Iyer, S., E. Ambrogini, S. M. Bartell, L. Han, P. K. Roberson, R. de Cabo, R. L. Jilka, R. S. Weinstein, C. A. O'Brien, S. C. Manolagas, and M. Almeida. (2013). FoxOs attenuate bone formation by suppressing Wnt signaling. J Clin Invest. **123:** 3409-3419.

75. Gmel, G., A. Bissery, R. Gammeter, J. C. Givel, J. M. Calmes, B. Yersin, and J. B. Daeppen, . (2006). Alcohol-attributable injuries in admissions to a swiss emergency room-an analysis of the link between volume of drinking, drinking patterns, and preattendance drinking. Alcohol Clin Exp Res. **30:** 501-509.

76. Fleming, E. A., G. Gmel, P. Bady, B. Yersin, J. C. Givel, D. Brown, and J. B. Daeppen. (2007). At-risk drinking and drug use among patients seeking care in an emergency department. J Stud Alcohol Drugs. **68:** 28-35.

77. Kuendig, H., M. Hasselberg, L. Laflamme, J. B. Daeppen, and G. Gmel. (2008). Acute alcohol consumption and injury: risk associations and attributable fractions for different injury mechanisms. J Stud Alcohol Drugs. **69:** 218-226.

78. Dong Y. F., D. Y. Soung, E. M. Schwarz, R. J. O'Keefe, and H. Drissi. (2006). Wnt induction of chondrocyte hypertrophy through the Runx2 transcription factor. J Cell Physiol. **208:** 77-86.

79. Manolagas, S. C. and A. M. Parfitt. (2010). What old means to bone. Trends Endo Metab. **21:** 369-374.

80. Poole, K. E. and J. Reeve. (2005). Parathyroid hormone-a bone anabolic and catabolic agent. Curr Opin Pharmacol. **5:** 612-617.

81. Prahalad, A. K., R. J. Hickey, J. Huang, D. J. Hoelz, L. Dobrolecki, S. Murthy, T. Winata, and J. M. Hock. (2006). Serum proteome profiles identifies parathyroid hormone physiologic response. Proteomics. **6:** 3482-3493.

82. Copp, D. H. and B. (1962). Calcitonin-a hormone from the parathyroid which lowers the calcium-level of the blood. Nature. **193:** 381-382.

83. Friedenstein, A. J., R. K. Chailakhjan, and K. S. Lalykina. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. **3:** 393-403.

84. Owen, M. and A. J. Friedenstein. (1988). Stromal stem cells: marrow-derived osteogenic precursors. Cibia Found Symp. **136:** 42-60.

85. Caplan, A. I. and S. P. Bruder. (2001). Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med. **7:** 259-264.

86. Huang, Y., X. Zhang, K. Du, F. Yang, Y. Shi, J. Huang, T. Tang, D. Chen, and K. Dai. (2012). Inhibition of β-catenin signaling in chondrocytes induces delayed fracture healing in mice. J Orthop Res. **30:** 304-310.

87. Essers, M. A., S. Weijzen, A. M. de Vries-Smits, I. Saarloos, N. D. de Ruiter, J. L. Bos, and B. M. Burgering. (2004). FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J. **23:** 4802-4812.

88. Furukawa-Hibi, Y., Y. Kobayashi, C. Chen, and N. Motoyama. (2005). FOXO transcription factors in cell-cycle regulation and the response to oxidative stress. Antioxid Redox Signal. **7:** 752-760.

89. Greer, E. L. and A. Brunet. (2005). FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene. **24:** 7410-7425.

90. Calnan, D. R. and A. Brunet. (2008). The FoxO code. Oncogene. **27:** 2276-2288.

91. Paik, J., R. Kollipara, G. Chu, H. Ji, Y. Xiao, Z. Ding, L. Miao, Z. Tothova, J. W. Horner, D. R. Carrasco, S. Jiang, D. G. Gilliland, L. Chin, W. H. Wong, D. H. Castrillon, and R. A. DePinho. (2007). FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell. **128:** 309-323.

92. Lam, E. W., R. E. Francis, and M. Petkovic. (2006). FOXO transcription factors: key regulators of cell fate. Biochem Soc Trans. **34:** 722-726.

93. Suh, K. T., S. W. Kim, H. L. Roh, M. S. Youn, and J. S. Jung. (2005). Decreased osteogenic differentiation of mesenchymal stem cells in alcohol-induced osteonecrosis. Clin Orthop Relat Res. **431:** 220-225.

94. Huang, H. and D. J. Tindall. (2007). Dynamic FoxO transcription factors. J Cell Sci. **120:** 2479-2487.

95. Eisenmann, D. M. (2005). Wnt signaling. Wormbook, ed. The *C. elegans* Research Community. Wormbook, doi/10.1895/wormbook.1.7.1, wormbook.org.

96. Tzioupis C. and Giannoudis P. V. (2007). Prevalence of long-bone non-unions. Injury **38**: S3-S9.

97. Lane J. M., Boskey A. L., Li W. K. P., Eaton B., and Posner A. S. (1979). A temporal study of collagen, proteoglycan, lipid and mineral constituents in a model of endochondral osseous repair. Metabol Bone Bio **1**: 319-324.

98. Perrien D. S., Brown E.C., Fletcher T. W., Irby D. J., Aronson J., Gao G. G., Skinner R. A., Hogue W. R., Feige U., Suva L. J., Ronis M. J., Badger T. M., and Lumpkin C. K. (2002). Interleukin-1 and tumor necrosis factor antagonists attenuate ethanol-induced inhibition of bone formation in a rat model of distraction osteogenesis. J Pharmacol Exp Ther **303**: 904-908.

99. Perrien D. S., Liu Z., Wahl E. C., Bunn R. C., Skinner R. A., Aronson J., Fowlkes J., Badger T. M., and Lumpkin C. K. (2003). Chronic ethanol exposure is associated with a local increase in TNF-alpha and decreased proliferation in the rat distraction gap. Cytokine **23**: 179-189.

100. Perrien D. S., Wahl E. C., Hogue W. R., Feige U., Aronson J., Ronis M. J., Badger T. M., and Lumpkin C. K. (2004). IL-1 and TNF antagonists prevent inhibition of fracture healing by ethanol in rats. Toxicol Sci **82**: 656-660.

101. Nyquist F., Halvorsen V., Madsen J. E., Nordsletten L., and Obrant K. J. (1999). Ethanol and its effects on fracture healing and bone mass in male rats. Acta Orthop Scand **70**: 212-216.

102. Brown E. C., Perrien D. S., Fletcher T. W., Irby D. J., Aronson J., Gao G. G., Hogue W. J., Skinner R. A., Suva L. J., Ronis M. J., Hakkah R., Badger T. M., and Lumpkin C. K. (2002). Skeletal toxicity associated with chronic ethanol exposure in a rat model using total enteral nutrition. J Pharmacol Exp Ther **301**: 1132-1138.

103. Mori R., Tanaka K., de Kerckhove M., Okamoto M., Kashiyama K., Tanaka K., Kim S., Kanata T., Komatsu T., Park S., Ikematsu K., Hirano A., Martin P., and Shimokawa I. (2014). Reduced FOXO1 expression accelerates skin wound healing and attenuates scarring. Am J Pathol **184**: 2465-2479.

104. Lehtinen M. K., Yuan Z., Boag P. R., Yang Y., Villen J., Becker E. B., DiBacco S., de la Iglesia N., Gygi S., Blackwell T. K., and Bonni A. (2006). A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. Cell **125**: 987-1001.

105. Zuscik M. J., Hilton M. J., Zhang X., Chen D., and O'Keefe R. J. (2008). Regulation of chondrogenesis and chondrocyte differentiation by stress. J Clin Invest **118**: 429-438.

106. Schieber M. and Chandel N. S. (2014). ROS function in redox signaling and oxidative stress. Curr Biol **24**: 453-462.

107. Kayal R. A., Siqueira M., Alblowi J., McLean J., Krothapalli N., Faibish D., Einhorn T. A., Gerstenfeld L. C., and Graves D. T. (2010). TNF-alpha mediates diabetes-enhanced chondrocyte apoptosis during fracture healing and stimulates chondrocyte apoptosis through FOXO1. J Bone Miner Res **25**: 1604-1615.

108. Ko K. I., Coimbra L. S., Tian C., Alblowi J., Kayal R. A., Einhorn T. A., Gerstenfeld L. C., Pignolo R. J., and Graves D. T. (2015). Diabetes reduces mesenchymal stem cells in fracture healing through a TNFα-mediated mechanism. Diabetologia **58**: 633-642.

109. Dong Y., Long T., Wang C., Mirando A. J., Chen J., O'Keefe R. J., and Hilton M. J. (2014). NOTCH-mediated maintenance and expansion of human bone marrow stromal/stem cells: a technology designed for orthopedic regenerative medicine. Stem Cells Transl Med **3**: 1456-1466.

110. Wang C., Inzana J. A., Mirando A. J., Ren Y., Liu Z., Shen J., O'Keefe R.J., Awad H. A., and Hilton M. J. (2016). NOTCH signaling in skeletal progenitors is critical for fracture repair. J Clin Invest **126**: 1471-1481.

111. Roper P. M., Abbasnia P., Vuchkovska A., Natoli R. M., and Callaci J. J. (2016). Alcohol-related deficient fracture healing is associated with activation of FoxO transcription factors in mice. J Orthop Res [Ahead of print]

112. Brown M. L., Yukata K., Farnsworth C. W., Chen D., Awad H., Hilton M. J., O'Keefe R. J., Xing L., Mooney R. A., and Zuscik M. J. (2014). Delayed fracture healing and increased callus adiposity in a C57BL/6J murine model of obesity-associated type 2 diabetes mellitus. PLoS One **9**: e99656.

113. Jiao H., Xiao E., and Graves D. T. (2015) Diabetes and its effect on bone and fracture healing. Curr Osteoporos Rep **13**: 327-335.

114. Mangialardi G., Spinetti G., Reni C., and Madeddu P. (2014). Reactive oxygen species adversely impacts bone marrow microenvironment in diabetes. Antioxid Redox Signal **10**: 1620-1630.

115. Tothova Z., Kollipara R., Huntly B. J., Lee B. H., Castrillon D. H., Cullen D. E., McDowell E. P., Lazo-Kallanian S., Williams I. R., Sears C., Armstrong S. A., Passegue E., DePinho R. A., and Gilliland D. G. (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell **128**: 325-339.

116. Kitada M. and Koya, D. (2013). SIRT1 in type 2 diabetes: mechanisms and therapeutic potential. Diabetes Metab J **37**: 315-325.

117. Iyer S., Han L., Bartell S. M., Kim H. N., Gubrij I., de Cabo R., O'Brien C. A., Manolagas S. C., and Almeida M. (2014). Sirtuin1 (Sirt1) promotes cortical bone formation by preventing β-catenin sequestration by FoxO transcription factors in osteoblast progenitors. J Biol Chem **289**: 24069-24078.

VITA

Philip Roper was born in Lafayette, Louisiana on August 27, 1986 to Tony Roper, Jr and Janice (Lucerto) Forney. After his parents' divorce, Dr. Roper and his mother moved to Chicago where he was raised by his mother and step-father, John Forney. Dr. Roper then went on to graduate from the University of Illinois in 2008 earning a Bachelor of Science in Molecular Biology and Chemistry.

In August of 2008, Dr. Roper joined the, then, Cell Biology, Neurobiology and Anatomy Department at Loyola University Chicago, which would become the Integrative Cell Biology Graduate Program. Then in 2009 he joined the laboratory of Dr. John J. Callaci. It was under Dr. Callaci's tutelage that Dr. Roper began to study the effects of alcohol on the skeleton and bone fracture healing, and how alcohol mediated these effects through modulations of intracellular signaling pathways within bone-specific stem cells. During this time, Dr. Roper received a predoctoral position on training grant for the Alcohol Research Program, was awarded a Predoctoral Individual Research Service Award by the National Institute of Alcohol Abuse and Alcoholism, authored several basic science research articles, and presented at numerous national and international meetings.

DISSERTATION APPROVAL SHEET

The dissertation submitted by Philip M. Roper has been read and approved by the following committee:

John J. Callaci, Ph.D., Director
Associate Professor, Department of Orthopaedic Surgery and Rehabilitation
Loyola University Chicago

Mashkoor Choudhry, Ph.D.
Professor, Department of Microbiology and Immunology
Loyola University Chicago

Edward M. Campbell, Ph.D.
Associate Professor, Department of Microbiology and Immunology
Loyola University Chicago

Mitchell F. Denning
Professor, Department of Molecular Pharmacology and Therapeutics
Loyola University Chicago

Pamela L. Witte, Ph.D.
Professor, Department of Microbiology and Immunology
Loyola University Chicago

The final copies have been examined by the director of the dissertation and the signature that appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

_____ _____

Date Director's Signature

DISSERTATION APPROVAL SHEET

The dissertation submitted by Philip M. Roper has been read and approved by the following committee:

John J. Callaci, Ph.D., Director
Associate Professor, Department of Orthopaedic Surgery and Rehabilitation
Loyola University Chicago

Mashkoor Choudhry, Ph.D.
Professor, Department of Microbiology and Immunology
Loyola University Chicago

Edward M. Campbell, Ph.D.
Associate Professor, Department of Microbiology and Immunology
Loyola University Chicago

Mitchell F. Denning
Professor, Department of Molecular Pharmacology and Therapeutics
Loyola University Chicago

Pamela L. Witte, Ph.D.
Professor, Department of Microbiology and Immunology
Loyola University Chicago

The final copies have been examined by the director of the dissertation and the signature that appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

6-16-16
Date

Director's Signature