ACTIVIN RECEPTOR SIGNALING IN BONE DEVELOPMENT AND REGENERATION

by
Brian C. Goh

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

March, 2017

ABSTRACT OF THE DISSERTATION

Activin Receptor Signaling in Bone Development and Regeneration

by

Brian C. Goh

Doctor of Philosophy in Cellular and Molecular Medicine

Johns Hopkins University, 2017

Bone and skeletal muscle mass are highly correlated in mammals, suggesting the existence of common signaling networks that serve to coordinate the development of these two anatomically adjacent tissues. Myostatin and its related activin ligands of the TGB-beta superfamily are known regulators of skeletal muscle mass and are also expressed in bone. Given the well-established role of myostatin in muscle, these ligands may serve a similar function in bone. To investigate the role of this pathway in bone, pharmacological and genetic approaches were employed to determine the role of activin receptor signaling in bone.

To identify which components of the activin signaling pathway were expressed in bone, we used immunohistochemistry. ACVR2A and ACVR2B were localized to osteoblasts and osteocytes in both trabecular and cortical bone. Primary mouse calvarial osteoblasts also expressed the major activin receptor signaling components, including ACVR2A, ACVR2B, and ACVR1B (ALK4). Furthermore, upon exposure to activin ligands, osteoblasts demonstrated active signaling by increased levels of phosphorylated Smad2/3. Osteoblasts deficient in ACVR2A by Cre-mediated recombination exhibited

accelerated differentiation, evidenced by increased alkaline phosphatase activity, mineral deposition, and increased transcriptional expression of *Osterix*, *Osteocalcin*, and *Dmp1* compared to controls. To determine the importance of activin receptor signaling in bone *in vivo*, we analyzed the skeletal phenotypes of mice selectively deficient in ACVR2A and ACVR2B in osteoblasts and osteocytes (osteocalcin-Cre). Whereas ACVR2B deficient mice (Ob.ACVR2B$^{-/-}$), had no significant changes in bone parameters, mice lacking ACVR2A (Ob.ACVR2A$^{-/-}$) had significantly increased femoral trabecular bone volumes at six and twelve weeks of age. In addition, Ob.ACVR2A$^{-/-}$ mice also exhibited increases in cortical parameters at twelve weeks of age, which resulted in enhanced mechanical properties. Compound mutant mice lacking both ACVR2A and ACVR2B demonstrated increases in trabecular bone volumes similar to Ob.ACVR2A$^{-/-}$ mice. Taken together, these data indicate that activin receptor signaling in osteoblasts is predominantly mediated through ACVR2A and acts as a negative regulator of bone mass.

We then evaluated activin-targeted biologics for their efficacy in muscle and bone healing. Therapeutic potential of soluble activin receptor administration was investigated utilizing a composite musculoskeletal injury model, consisting of a pin-stabilized femur osteotomy and an overlying volumetric muscle defect. With ACVR2B/Fc treatment, the bone fracture callus volume and mineral content were significantly increased as compared to vehicle controls while mechanical properties were unchanged between groups. Furthermore, muscle mass was significantly increased while injury fibrosis and fat infiltration were reduced with ACVR2B/Fc administration – supporting the possibility of using soluble activin receptors as a dual muscle and bone anabolic agent.

In conclusion, the results from these studies demonstrate that the activin signaling pathway occurs primarily through ACVR2A in osteoblasts and acts to negatively regulate bone mass. Furthermore, inhibition of this pathway may act as a dual anabolic agent and is a potential therapeutic to augment composite muscle and bone healing.

Thesis Advisor: Thomas L. Clemens, PhD

Thesis Co-Advisor/Second Reader: Douglas J. DiGirolamo, PhD

Acknowledgements

I would like to thank many individuals for their help and support throughout my time in graduate school. Firstly, I would like to express my sincerest gratitude to my thesis advisor, Dr. Tom Clemens, for his immense patience, dedication, and guidance in academics, research, and life. Secondly, I would like to extend that same sense of gratitude to my co-advisor, Dr. Doug DiGirolamo, for his help and support in all aspects of this project. Together, their mentorship has been unparalleled and they have given me the tools and confidence to move forward in my career.

Apart from my advisors, I would also like to thank the other members of my thesis committee, Drs. Se-Jin Lee, Mary Armanios, and Lee Riley III, for their continued guidance and advice in my progress. Their constructive criticism and thoughtful questions have strengthened my research.

I would also like to thank members of the lab, particularly Drs. Ryan Riddle Sr. and Ryan Tomlinson Jr., for their advice and willingness to listen to my endless rants and complaints about science. I also want to extend my deepest gratitude to all other lab members, including Vandana Singhal, Julie Leslie Frey, Meredith Zoch, Soohyun Park, Drs. Zhu Li, Qian Zhang, and Zhi Li. Without each of them, I would be completely and utterly lost in lab.

I would also like to thank my friends and family for their support as I pursue my academic endeavors. They have been there for me through the highs and lows of graduate school. Lastly, I would like to thank Paige Nichols for all of her love and encouragement for as long as I have known her. Without her, I would not be where I am today and I am truly blessed to have her in my life.

Table of Contents

List of Tables and Figures

Chapter 1

1 Introduction

1.1 Overview of Skeletal Biology and Repair

The skeleton is a highly specialized organ system that facilitates locomotion, mineral homeostasis, and the protection of vital organs. Bone is a composite tissue comprised of both organic and inorganic components that are constantly renewed and replaced by resident bone cells. [1, 2] Type I collagen is the predominant organic component of the bony extracellular matrix and acts as a scaffold for mineral nucleation. This mineral phase endows a rigid structure and mechanical strength to the underlying collagen, in addition to acting as a repository for calcium, magnesium, and phosphate ions necessary for homeostasis. [3]

The skeleton is composed of over 200 individual bones that are classified into four distinct categories: short, long, irregular, and flat. Together with cartilage, they form the axial and appendicular skeleton that we know and recognize. Each of these bones develop in utero and grow postnatally through either intramembranous or endochondral ossification. During embryological development, bone formation begins with the

condensation of mesenchymal precursors at primitive, anatomic bone sites to form the skeleton template. Intramembranous ossification occurs at sites of flat bone formation, such as the skull and sternum. During this process, mesenchymal progenitor cells differentiate directly into osteoblasts to form primary ossification centers. [4, 5] In contrast to flat bones, long and short bones develop via endochondral ossification – where progenitor cells first differentiate into chondrocytes that form an initial cartilage template. As these chondrocytes proliferate and subsequently hypertrophy, they begin to secrete the extracellular matrix components necessary for cartilage development. [5, 6] Osteoprogenitor cells, vascular endothelial cells, and hematopoietic stem cells then invade this preliminary cartilage to initiate mineralization. Further angiogenesis occurs within the cartilage template while newly formed osteoblasts begin to secrete osteoid – unmineralized extracellular matrix containing primarily collagen type I. Differentiated osteoblasts then facilitate mineralization of newly formed osteoid through the deposition of hydroxyapatite onto the bone matrix to form the calcified skeleton. [7-9]

Skeletal tissues are categorized into two distinct architectures, cortical and trabecular bone. These bone compartments are distinct in their structure and function as cortical bone is very dense and is the primary form found within the shaft of long bones, providing ample strength to these tissues. Trabecular bone, however, is much less dense and organized into intersecting trabeculae that give a spongy appearance when viewed grossly. The high surface area of trabecular bone allows for rapid mineral metabolism and calcium homeostasis. Trabecular bone is typically found in the epiphyses of long bones, proximal to the growth plate, and provides compressive strength to the proximal

and distal portions of the long bones. [2, 10] While the cortical and trabecular bone compartments are uniquely distinct in shape, together they function as one unit providing mechanical strength, stability, and a mineral reservoir.

Skeletal tissue is populated by three primary cell types – osteoblasts, osteocytes, and osteoclasts – each with distinct functions. Together, they work in concert to model the bone during development and remodel bone throughout adult life. Osteoblasts, which are derived from mesenchymal precursors, function to produce an extracellular collagen matrix and facilitate matrix mineralization. [11] A distinct proportion of these osteoblasts then become entombed in mineral, where they differentiate into osteocytes, comprising of over 90-95% of all cells in the skeleton. [12, 13] Osteocytes are connected to all other bone cells via their dendritic processes that channel through bone canaliculi to form an extensive cellular network. Although the precise functions of the osteocyte are not clear, most evidence suggests that they are responsible for sensing mechanical loads and more recently, participate in mineral metabolism. [12] The third bone cell type is the osteoclast – large, multinucleated cells that originate from bone marrow macrophages and monocytes. These cells are responsible for the removal of bone through degradation of the organic and inorganic components of the skeleton. [2, 10, 14] Utilizing a ruffled membrane to create an isolated extracellular microenvironment, osteoclasts acidify the underlying calcified tissue to liberate inorganic mineral and subsequently degrade the remaining organic proteins. [15]

Together, these three cells work in concert to remodel the skeleton through the coupling of bone formation and resorption. This continual renewal of calcified tissues

allows microdamaged bone to be replaced to maintain the health and integrity of the skeleton while ensuring homeostatic serum mineral levels.

1.2 Physiology of Fracture Healing

Bone fractures are the most common traumatic injuries. While many of these injuries will go on to repair themselves with reduction and setting, approximately ten percent will not heal properly. Non-unions and critically sized defects further result in devastating consequences in morbidity and mortality. [16, 17] Unlike many other tissue repair processes, fracture healing closely mirrors many of the embryological events, including endochondral and intramembranous ossification, that occur during primary development. [18, 19] Bone fractures lead to profuse bleeding and hematoma formation at the site of injury. From there, inflammatory cytokines, including tumor necrosis factor-α and interleukins -1, -6 -11, and -18, are released into the injury site to recruit inflammatory cells and prime the environment for repair. Mesenchymal stem cells (MSC) derived from a number of sources including the bone marrow [20], muscle [21], periosteum [22], and general circulation [23] are then recruited to the injury site. Within the skeletal injury, there is also a differential in oxygen tension with the lowest oxygen concentration in the center of the fracture and highest oxygen tensions distally, where the blood supply remains intact. Because of this differential, endochondral ossification occurs in the center of the fracture while intramembranous ossification occurs closest to the bone ends, where oxygen tension is highest. Tissue hypoxia within the center of the

fracture site induces MSC differentiation into chondrocytes to create a soft, unmineralized callus. Hypoxia also induces vascular invasion and angiogenesis to restore perfusion. As the cartilage develops, resident chondrocytes hypertrophy and eventually terminally differentiate. The newly formed matrix is then mineralized, forming transient immature woven bone. Osteoblasts and osteoclasts then remodel the callus to transform the disorganized, woven bone into hardy, lamellar bone that recapitulates the original anatomic structure. [2, 17, 24, 25] (Figure 1.1)

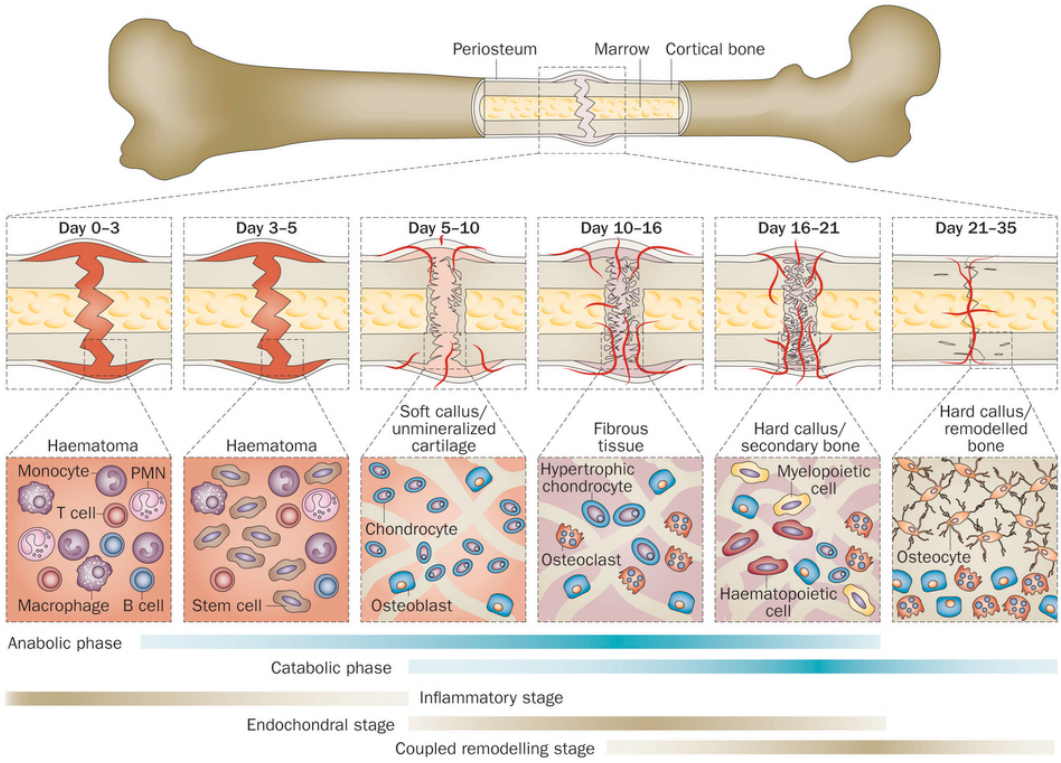

Figure 1.1: Physiology of Fracture Repair

The major events in the progression of bone fracture healing with the primary cell types present at each stage. [10]

Note: Reproduced with permission from "Fracture healing: mechanisms and interventions" by T.A. Einhorn and L.C. Gerstenfeld, 2015, *Nature Reviews Rheumatology* 11, 45-54. Copyright 2014 by Nature Publishing Group.

1.3 Overview of Skeletal Muscle Biology

The counterpart to the rigid skeleton is the adjacent skeletal muscle, the most abundant tissue of the human body. Together, they enable the coordinated movements of daily life. Skeletal muscle is organized into hierarchical structures consisting of myofibers that contract together to act as individual functional units. Each myofiber is formed by the fusion of several myoblasts to create a multinucleated fiber and is composed of several smaller myofibrils. These myofibrils contain multiple repeating sarcomeres – the fundamental unit of muscle contraction. Each sarcomere contains the longitudinal arrangement of the contractile proteins actin and myosin to form the thin and thick filaments, respectively. These filaments slide past one another during the contraction cycle and their coordinated summation generates the locomotion of the human body. [26, 27]

Like the primitive skeleton, skeletal muscle originates from mesenchymal precursors, in particular the paraxial mesoderm. During embryogenesis, a portion of the mesoderm clusters into distinct somites that form the dermatomyotome and later form separate myotomes. These myotomes contain skeletal muscle progenitor cells that migrate to the limb buds and areas of axial muscle development. The progenitor cells then expand and differentiate into myocytes under the direction of myogenic regulatory factors (MRFs) and other transcription factors that regulate myogenesis. [28] The newly

formed myocytes then fuse and mature into multinucleated muscle fibers to form distinct muscle groups. [27, 29]

1.4 Muscle and Bone Interactions

Mature bone is subject to continuous remodeling throughout life. This cycle of bone resorption and formation works to remove damaged skeletal tissue and maintain serum calcium levels, but also allows continual skeletal adaptations to hormonal and mechanical cues. These skeletal changes are highly correlated with the adjacent musculature with peak bone mass accompanying peak muscle mass. Conversely, the opposite is also evident as osteopenia and sarcopenia nearly always coexist. [30-34] Studies investigating spinal cord injuries have demonstrated that severe muscle atrophy nearly always is accompanied by significant bone loss directly beneath the muscle lesion. [35, 36] Furthermore, implanted electrical stimulators that can induce muscle contractions will partially prevent spinal cord injury-induced bone loss. [37] This in-step synchronization suggests that there are common mechanical and paracrine regulatory pathways that exist between muscle and bone, enabling their coordination in form and function.

The prevailing notion for the coordination between muscle and bone is that this relationship is mediated through mechanical forces – either emanating from the surrounding muscles or from physical impact. [38] For any particular movement, bones act as rigid levers that allow muscles to contract and pull against, enabling the complex

movements of daily living. The skeletal response to muscle contractions has been well-documented and was described by early pioneers such as Julius Wolff. His theory, which would be later known as Wolff's Law, states that the skeleton will continually adapt to the various loads under which it is placed. These ideas were taken further and later integrated into Harold Frost's mechanostat theory, where elastic mechanical deformation of bone allows the skeleton to adapt and withstand the maximum forces experienced. [39-42] This process of skeletal adaptation occurs in both loading and unloading scenarios. Adaptation is particularly important in instances of uncontrolled bone loss such as osteoporosis, as interventions that induce mechanical loading, including exercise and vibration therapy, prevent bone loss. In both Wolff's and Frost's theories, mechanical loading is the most important determinant of the geometry and strength of the skeleton and is a primary link between muscle and the bone beneath it.

This link is evident in adult life, where physical activity and loading experienced in sports or exercise are known to impact these two tissues. In several studies that compared the musculoskeletal systems of active individuals to those of sedentary individuals, subjects that competed in sports and weight-bearing exercises had significantly higher bone mineral density as well as muscle function. [43-47] Interesting, while it was assumed that this correlation between mechanical forces from muscles and bone strength was a post-natal phenomenon, recent evidence from Zelzer et al has demonstrated that mechanical adaptation occurs even in utero. Using genetic muscle paralysis models, their group has shown that fetal muscle contractions are necessary for bone shaping and that the bone geometrical outline is lost with muscle paralysis. Without

these muscle-derived forces, the resulting bones are mechanically inferior and are unable to optimize to their load-bearing capacity. [48] Likewise, the physical forces created by muscle contractions are also necessary for proper joint development. Other studies have demonstrated that administration of neuromuscular blocking agents during limb development impairs proper joint formation, further supporting the notion that the interaction between muscle and bone tissue is necessary for proper musculoskeletal development. [49] The findings of these studies as well as loading studies previously discussed suggest that the coordination been muscle and bone mass is largely dependent on the mechanical forces between these two tissues.

While the precise biological signals that transduce these mechanical events are unknown, these signals are likely to utilize common regulatory pathways. Because muscle and bone develop from the same embryological origins, there are several overlapping growth factors and morphogens that are shared in muscle and bone development. For instance, the Wnt [50-52], Growth Hormone/Insulin-like Growth Factor (GH/IGF) [53-55], and Notch [56-58] pathways all play critical roles in muscle and bone development and homeostasis. In the case of the GH pathway, IGF1 acts as a potent inducer of muscle hypertrophy and also plays a central role in the differentiation of osteoblasts into osteocytes to promote bone formation. [59-61] Likewise, other studies suggest a role for other molecules, such as vitamin D, in muscle and bone development. [62-64] Locally produced vitamin D exerts its effects in both an autocrine and paracrine manner to modulate bone remodeling by enhancing osteoblast differentiation and mineralization. [65-67] However, apart from its role in bone, vitamin D has also been

implicated in muscle homeostasis. In mouse models in which the vitamin D receptor was eliminated, the corresponding skeletal muscle was approximately 20% smaller and these mice exhibited impaired motor coordination. [68, 69] While the exact mechanism of vitamin D's role in skeletal muscle has yet to be elucidated, there are clear associations between vitamin D and muscle performance. These example ligands are just a few of the many overlapping signaling pathways that function in muscle and bone tissues.

Because of the significant overlap in regulator pathways, one or more of these pathways are likely responsible for the synchronization of bone and muscle. Even in the case of mechanical stimulation, the physical forces emanating from the surrounding musculature are translated into a biological signal and one of these pathways likely mediates that response. In fact, new studies describe the first direct evidence of crosstalk between muscle and bone as demonstrated during development. Olwin et al have shown that Indian Hedgehog, a bone-derived signaling molecule, is necessary for secondary myogenesis in the developing chick hindlimb. Indian Hedgehog from the developing bone acts directly on myoblasts to regulate muscle precursor apoptosis. [70] This study is the first to establish a direct communication between muscle and bone tissues in development and possibly in adult life.

1.5 Activin Signaling as a Potential Mechanism Coupling Muscle and Bone

The transforming growth factor-beta (TGF-β) superfamily is an important signaling pathway that is common to muscle and bone regulation. TGF-β ligands are comprised of over forty members and are divided into three main sub-families that include TGF-βs, bone morphogenic proteins (BMPs), and activins. [71] The TGF-β pathway is responsible for many critical cellular processes such as proliferation, differentiation, morphogenesis, and tissue regeneration. However, the realization of TGF-β ligands' paradoxical nature – being both a stimulator and inhibitor, depending on condition – revealed more sophisticated levels of complexity in signaling that are time, concentration, and target cell specific. [72, 73] These layers of intricacy have made it difficult to succinctly describe the TGF-β pathway but have enriched our understanding of the multifunctional capabilities of TGF-β ligands.

In canonical TGF-β signaling, ligands first bind to dimerized, cognate type II receptors. This binding then recruits complementary type I receptors to form a heterotetrameric signaling complex. Proximity of these receptors then allows the Ser/Thr kinase domains of the type II receptors to transphosphorylate the nearby type I receptors. This phosphorylation event activates the type I receptors to then recruit secondary messengers (receptor-Smads), which are in turn phosphorylated by the activated type I receptors. These secondary messengers signal through receptor-Smads 2/3, in the case of TGF-βs and activins, or receptor-Smads 1/5/8 in the case of BMPs. [72-74] Following the

TGF-β signaling cascade, phosphorylated Smad 2/3 then interacts with Runx2, the master transcriptional activator of osteoblast differentiation, to control mesenchymal precursor differentiation. [75] TGF-β superfamily ligands have been widely recognized to be critical in mammalian development and are necessary for proper embryogenesis. For instance, during embryogenesis, an asymmetric BMP gradient is created to enable dorsal patterning and form the dorsal-ventral axis of the embryo. [76] In addition to embryo patterning, TGF-β ligands have also been widely implicated in skeletal development and homeostasis as disruptions in TGF-β signaling have been linked to multiple musculoskeletal diseases as well as cancer metastases. [77, 78]

TGF-β signaling is primarily responsible for the maintenance and expansion of the progenitor pool necessary for osteoblast formation. [78] Skeletal tissues, including bone and cartilage, contain large amounts of deposited inactive TGF-β that, when released and activated, can act on target cells. TGF-β ligand is stored within the extracellular matrix in a latent form. When TGF-β ligands are first synthesized, they interact with a latency associated peptide (LAP), which then binds to several other proteins to form the large latent complex (LLC). This inactive LLC is secreted into the extracellular matrix in skeletal tissues and awaits activation and release. [79] Latent TGF-β can be activated in a number of different ways but is broadly categorized as either integrin-independent or integrin-dependent mechanisms. Integrin-independent mechanisms of activation include cleavage by metalloproteases, acidification, or exposure to reactive oxygen species. [80-82] Integrin-dependent mechanisms primarily function through the αVβ6 integrin. [83, 84] This location specific TGF-β activation

enables dynamic microenvironment changes to regulate the balance of osteoblast and osteoclast differentiation at specific sites of bone remodeling. Through this mechanism, TGF-β is able to closely balance bone formation with bone resorption, enabling site-specific adaptations to physical and hormonal cues.

While the TGF-β ligands are known to regulate bone homeostasis and maintenance, other members of the TGF-β superfamily, such as BMPs, are also known for their role in skeletal development. The vertebrate skeleton, which is composed of cartilage and bone, is derived from many embryonic lineages. Progenitor cells from the neural crest, paraxial mesoderm, and lateral plate mesoderm expand and consolidate into mesenchymal condensations that will form the template of the future skeletal sites. [85] This process is governed by many different growth factors and morphogens but the BMPs play a primary role in skeletal development by regulating osteoblast and chondrocyte differentiation, cartilage and bone formation, and axial patterning. In vertebrates, mesodermal cells develop into the paraxial mesoderm, which is then segmented in a rostral-caudal progression to form distinct somites. This patterning, anterior–posterior, dorsal–ventral, and proximal–distal, is predominately mediated by BMP gradients that enable site-specific cell differentiation. [86, 87]

1.6 Activins and Their Receptors

Activins are integral signaling components of the gonadal endocrine system and act primarily as stimulants of ovarian follicle maturation in females and spermatogenesis in males. Activins were first identified in purification fractions of ovarian follicular fluid and shown to strongly induced follicle-stimulating hormone (FSH) release. [88] Since being first identified, many other non-gonadal functions of activins have also been identified, including erythropoiesis, immune function, wound repair, liver proliferation, and angiogenesis. [89]

In the context of skeletal muscle, one of the most well-known activin ligands is myostatin as it has been discovered to be one of the most potent regulators of skeletal muscle. Myostatin was first described by Dr. Se-Jin Lee and colleagues in 1994 as a secreted protein that is made exclusively by skeletal muscle and acts as a negative regulator of muscle mass. Furthermore, with myostatin disruption, mice develop an extreme double-muscled phenotype with dramatic increases in muscle weights and myofiber size due to muscle hypertrophy and hyperplasia. [90] Upon further investigation, it was determined that myostatin signaling occurs through the activin type II receptors, with activin receptor type IIB (ACVR2B) having a much greater affinity for myostatin than activin receptor type IIA (ACVR2A). [91, 92] This finding has led many investigators, including those in the pharmaceutical industry, to develop soluble decoy activin type II receptors to sequester myostatin and promote muscle growth in patients with sarcopenia or other various myopathies. [93-96] (Figure 1.2)

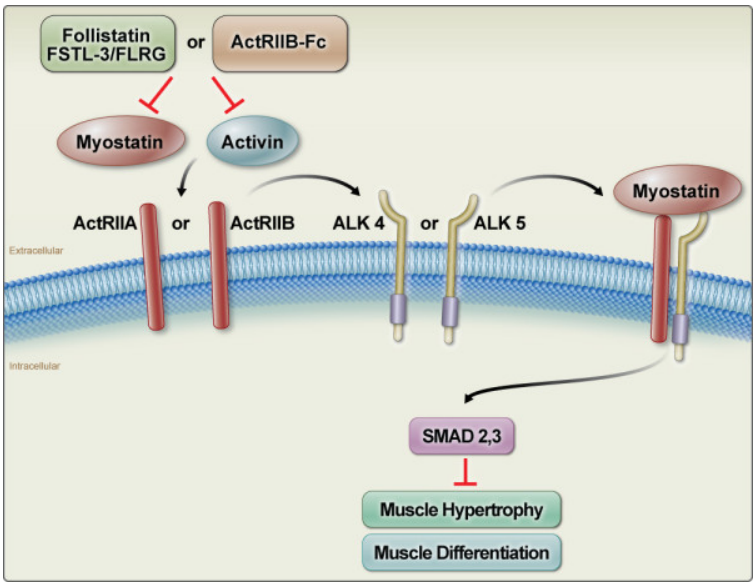

Figure 1.2: Myostatin and Activin Signaling in Skeletal Muscle

Myostatin and activin ligands bind to the activin type II receptors to transduce signaling through receptor-Smads to inhibit muscle development. [84]

Note: Reproduced with permission from "Treating cancer cachexia to treat cancer" by S.J. Lee and D.J. Glass, 2011, *Skeletal Muscle* 1, 2. Copyright 2011 by Biomed Central Ltd.

Because the myostatin and activin ligands exert such profound effects in skeletal muscle, we and others have begun to explore the hypothesis that activins might also regulate growth and development of adjacent bone tissue. Activins are first seen in the developing skeleton in prechondrogenic limb condensations and then during chondrogenesis. [97-99] The primary activin ligand, Activin A, is a dimer of two activin

β_A subunits and is present in proliferating chondrocytes, indicating that activins are important signaling molecules during chondrogenesis and skeletal development. [100] Studies using transgenic mice have also suggested that activins play a critical role in skeletal formation. Activin β_A global null mice are born with cleft palates and without incisors. [101, 102] Likewise, activin receptor type IIA (ACVR2A) deficient mice exhibited mandibular hypoplasia as well as other facial and skeletal abnormalities resembling the characteristics of Pierre-Robinson syndrome in humans. [101] Similar to BMPs, activin signaling also plays a significant role in axial patterning during embryogenesis. Activin receptor type IIA and IIB are involved in mediating the spatiotemporal expression of multiple Hox genes in the anterior-posterior axis. [103] While it is clear that activin receptor signaling is important in skeletal development and homeostasis, the exact effects of activin signaling, whether stimulatory or inhibitory, has had some controversy.

Reports by several groups have suggested that activins act in a stimulatory role during osteoblastogenesis. [104-106] Likewise, other *in vivo* data has also indicated that activin administration was stimulatory in nature, as local injection of activin A improved fibula fracture and calvarial defect healing in rats. [107, 108] However, more recent evidence using osteoblast cultures and bone marrow stromal cells has suggested that activins are in fact inhibitory in nature. [109, 110] Eijken et al recently demonstrated that administration of activin ligands to human osteoblast cultures significantly inhibited their ability to mineralize the surrounding collagen matrix. The investigators further proposed that the inhibitory effect is primarily an autocrine mechanism mediated by locally

produced osteoblast-derived activin ligands. To confirm this assertion, the investigators administered recombinant follistatin, a natural activin antagonist, to osteoblast cultures and demonstrated enhanced matrix mineralization, further supporting an inhibitory role for activins. [109] In addition to these results, systemic inhibition of activin ligands using pharmacological approaches has proven to have a significant anabolic effect in bone. Lotinun et al demonstrated that soluble activin receptor administration resulted in a near doubling of the bone volume and the bone formation rate in the distal femur of Cynomolgus monkeys. Additionally, systemic activin ligand suppression also reduced osteoclast numbers and osteoclast surfaces. Similar anabolic bone changes were also evident in the femoral neck and thoracic vertebrae of treated primates. [111-113] However, it remains unclear if the increases in bone volume are the result of increased mechanical forces secondary to muscle hypertrophy or are a primary effect on bone homeostasis. Because of these discordant observations, more investigation is necessary to better understand the exact effects that activins exert on skeletal tissues.

1.7 Targeting Activin Signaling in Muscle and Bone Injuries

Previous studies have demonstrated that systemic activin inhibition induces anabolic changes in both bone and muscle. These results strongly support the rationale for their use in settings of complex injuries involving muscle and bone. Multi-tissue

injuries are some of the most challenging cases that orthopaedic surgeons face and often result in poor outcomes. One of the primary contributors to these subpar results is the lack of treatment options. Furthermore, treatment modalities are often viewed as two individual components – one for muscle injury and another for the underlying bone defect. However, these two tissues do not exist as two separate entities but rather are intimately related in form, function, and regeneration. It is well known that fractures with surrounding soft tissue damage have much higher complication rates. [114] Additionally, clinical evidence demonstrates that overlying muscle flap transplantation significantly improves fracture healing and union rates. [115-118] This synergy between muscle and bone during development and repair further strengthens a holistic treatment approach to composite musculoskeletal injuries.

While much is known about the fracture repair process, understanding the surrounding injury environment including the precise muscle-bone interactions during composite injury healing is critical to developing new treatment strategies. In a composite injury, the surrounding musculature is a source of vascular supply to provide nutrients to the underlying bone. However, emerging research has shown that the adjacent muscle may also provide osteoprogenitors to aid in fracture healing, particularly when the periosteum is insufficient for bone healing. [119]

The importance of revascularization in muscle and bone regeneration is well-documented. Following typical long bone fracture, angiogenesis precedes osteogenesis and is required for successful endochondral ossification. [120-123] The surrounding musculature has been proposed as a source of angiogenic signals and cells that provide

the underlying fracture with oxygen, nutrients, and osteoprogenitors. [124, 125] Migrating progenitors cells such mesenchymal stem cells and pericytes are delivered to the fracture site via this newly formed vascular network. The importance of the overlying muscle and neovascularization to fracture healing is also well established clinically. Non-union is four times more likely in fractures with impaired vascular function. [126] Additionally, muscle flap coverage during composite musculoskeletal injuries has been shown to improve callus vascularization and rates of bony union as compared to skin closure, further emphasizing the role of adjacent muscle to bone healing. [127-130] In several different fracture models, fracture calluses tend to be the largest and densest at the site of bone and muscle interfaces, which suggests that the adjacent muscle is strong regulator of callus formation. [131]

Composite musculoskeletal injuries are particularly difficult to manage, as damage to one tissue severely impairs the healing of the other. In this regard, therapeutics that target both bone and muscle tissues simultaneously would represent an ideal treatment modality. As discussed above, systemic soluble activin receptor administration has been shown to increase muscle and bone mass simultaneously, which would be an ideal treatment approach in composite injuries. As such, we conducted a comprehensive series of studies to explore the use of activin-targeted biologics for the treatment of composite musculoskeletal injuries.

In summary, the aims of this dissertation are to better understand the role of activin signaling in skeletal tissues and investigate its therapeutic potential in composite muscle and bone injuries. Using pharmacological and genetic approaches, the studies

discussed will provide insight into the role of activin signaling in bone development and regeneration.

Chapter 2

2 Activin Receptor Type II A (ACVR2A) Functions Directly in Osteoblasts as a Negative Regulator of Bone Mass

2.1 Abstract

Bone and skeletal muscle mass are highly correlated in mammals, suggesting the existence of common anabolic signaling networks that coordinate the development of these two anatomically adjacent tissues. The activin signaling pathway is an attractive candidate to fulfill such a role. In the present study, we generated mice with conditional deletion of ACVR2A, ACVR2B, or both, specifically in osteoblasts, to determine the contribution of activin receptor signaling in regulating bone mass. Immunohistochemistry localized ACVR2A and ACVR2B to osteoblasts and osteocytes in bone. Primary osteoblasts expressed activin signaling components, including ACVR2A, ACVR2B, and ACVR1B (ALK4) and demonstrated increased levels of phosphorylated Smad2/3 upon exposure to activin ligands. In vitro, osteoblasts lacking ACVR2B did not show significant changes. However, osteoblasts deficient in ACVR2A exhibited enhanced differentiation by alkaline phosphatase activity, mineral deposition, and transcriptional expression of Osterix, Osteocalcin, and Dmp1. To investigate activin signaling in osteoblasts in vivo, we analyzed the skeletal phenotypes of mice lacking these receptors in osteoblasts and osteocytes (Osteocalcin-Cre). Similar to the lack of

effect in vitro, ACVR2B deficient mice demonstrated no significant change in any bone parameter. By contrast, mice lacking ACVR2A had significantly increased femoral trabecular bone volume at six weeks of age. Further, compound mutant mice lacking both ACVR2A and ACVR2B demonstrated sustained increases in trabecular bone volume, similar to ACVR2A single mutants, at six and twelve weeks of age. Taken together, these data indicate that activin signaling, predominantly through ACVR2A, functions directly in osteoblasts as a negative regulator of bone mass.

2.2 Significance

Bone and skeletal muscle mass are highly correlated throughout mammalian growth and involution, suggesting the existence of common anabolic pathways that synchronize the development and maintenance of these two tissues. Evidence presented in this study suggests the activin signaling pathway is a likely candidate to fulfill such a role. The profound effects of this pathway, predominantly through myostatin and ACVR2B, are well established in regulating skeletal muscle mass. Utilizing genetic mouse models, we demonstrate analogous negative regulatory function of the activin signaling pathway in osteoblasts, predominantly through activin ligands and ACVR2A, to regulate bone mass.

2.3 Introduction

The musculoskeletal system evolved in mammals to perform diverse functions that include locomotion, breathing, protecting internal organs, and coordinating global energy expenditure. After the third decade of life, muscles and bones begin to lose their mass, leading to unfavorable alterations in their function. Aging is universally accompanied by the loss of bone (osteopenia) and skeletal muscle (sarcopenia), which together constitute important global medical problems. Sarcopenia results in reduced walking speed, poor balance, and instability that together predispose to falls and fractures. Thus, the coexistence of osteoporosis and sarcopenia severely compounds the problem of frailty in the elderly population [132].

During organogenesis, muscle and bone develop in close association from common mesodermal precursors and accumulate their final adult mass according to specific genetic instructions and environmental cues. Bone forms in a discrete stepwise process: mesenchymal precursors, derived from the mesoderm, first migrate to the future sites of bone where they condense. Following condensation, these precursors differentiate into chondrocytes or osteoblasts to form cartilage or bone, respectively, depending upon positional cues [133]. Once formed, the skeleton is continually remodeled throughout life. These processes of modeling and remodeling are achieved by the actions of osteoblasts and osteoclasts, and are coordinated through the actions of many autocrine/paracrine factors including Wnts, Hedgehogs, Notch, bone morphogenetic protein (BMP) family members, transforming growth factor-β (TGF-β)

family members, insulin-like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), and interleukin-6 (IL-6), among others [134]. This developing skeleton becomes encased in muscle tissue, which matures along with the adjacent modeling skeleton [135-137]. Similar to the regulation of bone development and maintenance of mass, skeletal muscle development and maintenance is regulated by morphogens and growth factors, many of which overlap with those involved in skeletogenesis, such as FGFs, IGF-1 and TGF-β [138].

Among the most important factors controlling muscle development is the activin/myostatin family of molecules. Activin, and closely related inhibin, are members of the TGF-β superfamily of molecules [139-141] first discovered over 70 years ago [88, 139, 141-144]. Like other TGF-β family members, activin and inhibin are produced as large precursors containing a signaling domain, a propeptide and a mature C-terminal segment that possesses biological activity. Following cleavage of the propeptide, the structure of functional activin and inhibin is a disulfide linked C-terminal dimer. In the case of activin, this dimer consists of two β subunits and for inhibin, one α and one β subunit which are encoded by distinct genes [139]. Myostatin (previously GDF-8) was discovered subsequently in a screen for novel TGF-β family members [90]. Although structurally similar to other TGF-β family members, its expression is restricted almost entirely to skeletal muscle, where it functions as a negative regulator of muscle growth [90]. All three of these molecules function through serine/threonine kinase activin receptors that resemble other TGF-β family receptors. Type II receptors are responsible for ligand binding, which can be tempered by soluble endogenous inhibitors (e.g.

follistatin), and type I receptors mediate signal transduction [145-149]. Tissue specificity and activity is regulated at multiple levels in the system, including the spatiotemporal expression patterns of various components and distinct combinations of receptor/ligand binding.

Myostatin negatively regulates skeletal muscle development by activating ACVR2B and initiating Smad2/3 signaling. Smad2/3 can then activate the MAPK pathway to inhibit proliferation through the p21/Rb cascade [150, 151], or directly affect MyoD by sequestering it in the nucleus, thus halting differentiation [152]. The importance of the myostatin/activin superfamily in skeletal muscle development is dramatically illustrated by the grotesque "double muscled" phenotype seen in myostatin null animals [90] or in mice carrying global mutations in Acvr2a and Acvr2b [91]. These receptors appear to serve redundant functions in skeletal muscle, as heterozygous loss of Acvr2b in combination with homozygous loss of Acvr2a results in further increases in muscle mass. Additional studies using pharmacologic approaches to block access of myostatin to its receptor also support the importance of this pathway in regulating muscle development and size [91, 153-155]. In each of these genetic and pharmacologic models, the anabolic effects on muscle are achieved by inhibition of the inhibitory effect of myostatin on myoblast proliferation [150, 151] and terminal differentiation [152].

Mice and humans that develop large muscles also form large, dense bones, and the maintenance of bone and muscle mass is tightly coupled in both healthy [156-158] and disease states [159]. Conversely, reduced bone mass is commonly associated with a number of myopathies including Pompe disease [160], multiple sclerosis [161], spinal

muscular atrophy [162]. These observations suggest the possibility that common signaling networks control both skeletal muscle and bone development and perhaps enable these adjacent tissues to develop in synchrony. Previous studies have shown indirect evidence that the effects of activin receptor signaling are shared within the musculoskeletal system and may also affect bone mass. In support of this notion, myostatin deficient mice not only exhibit dramatic increases in muscle mass but also significant increases in bone volume [163-166]. Additionally, activin/myostatin decoy receptor administration has been shown to cause anabolic changes in both the muscle and bone compartments [112, 167, 168]. Recent studies also suggest that the increases in bone volume after decoy receptor treatment are a direct effect and independent from muscle changes [169]. Moreover, the anabolic effects of activin signaling blockade on the skeleton do not appear to be mediated by myostatin. Treatment with a myostatin-specific neutralizing antibody did not yield significant bone changes despite drastic increases in muscle mass [170]. Additionally, decoy receptor administration in myostatin null mice demonstrated significant increases in bone volume without dramatic increases in muscle mass [170].

Taken together, studies to date unequivocally show that inhibition of activin or myostatin signaling in skeletal muscle, either by genetic or pharmacological means, increases both muscle and bone mass. However, whether the bone anabolic effects seen in these models is due to direct actions on osteoblasts or, alternatively, results indirectly through changes brought about by increased muscle mass remains unclear. It is this question that we aimed to investigate in the current study.

2.4 Results

2.4.1 Soluble Activin Type II Receptors Increase Bone Mass in vivo and Osteoblast Differentiation in vitro

To begin to investigate the role of activin receptor signaling within the musculoskeletal system, we first determined the effects of systemic inhibition of activin ligands through intraperitoneal injections of ACVR2A/Fc and ACVR2B/Fc. Consistent with previous reports, after just four weeks of soluble activin receptor treatment, there were significant increases in all wet muscle weights in both receptor treatment groups (Figure 2.1A). ACVR2A/Fc treated mice showed approximately double the bone volume fraction and approximately 25% increase in cortical thickness (Figure 2.1B). ACVR2B/Fc treated mice nearly tripled trabecular bone volume, with no significant changes in cortical thickness (Figure 2.1C). To begin to tease out whether these skeletal anabolic effects were direct or resulted through increased muscle force or due to release of excess humoral factors following the increase in muscle mass, we also examined calvarial bone volume, a skeletal site that is both non-load bearing and relatively isolated from surrounding musculature. Calvarial bone volume was significantly increased following both ACVR2A/Fc and ACVR2B/Fc treatment (Figure 2.1D). To further demonstrate that soluble activin receptors affect osteoblasts directly, we then performed *in vitro* studies in primary osteoblasts using ACVR2B/Fc to exploit its broad binding

affinity for activin ligands. Here, ACVR2B/Fc administration reduced basal levels of phosphorylated SMAD2 (Figure 2.1E). Furthermore, ACVR2B/Fc (50 ng/mL) treated osteoblasts demonstrated modest yet consistent increases in alkaline phosphatase and alizarin red staining (Figure 2.1F). Taken together, these experiments strongly suggest that the anabolic effects of activin receptor signaling blockade observed in the skeleton are due to direct effects on bone cells, particularly osteoblasts.

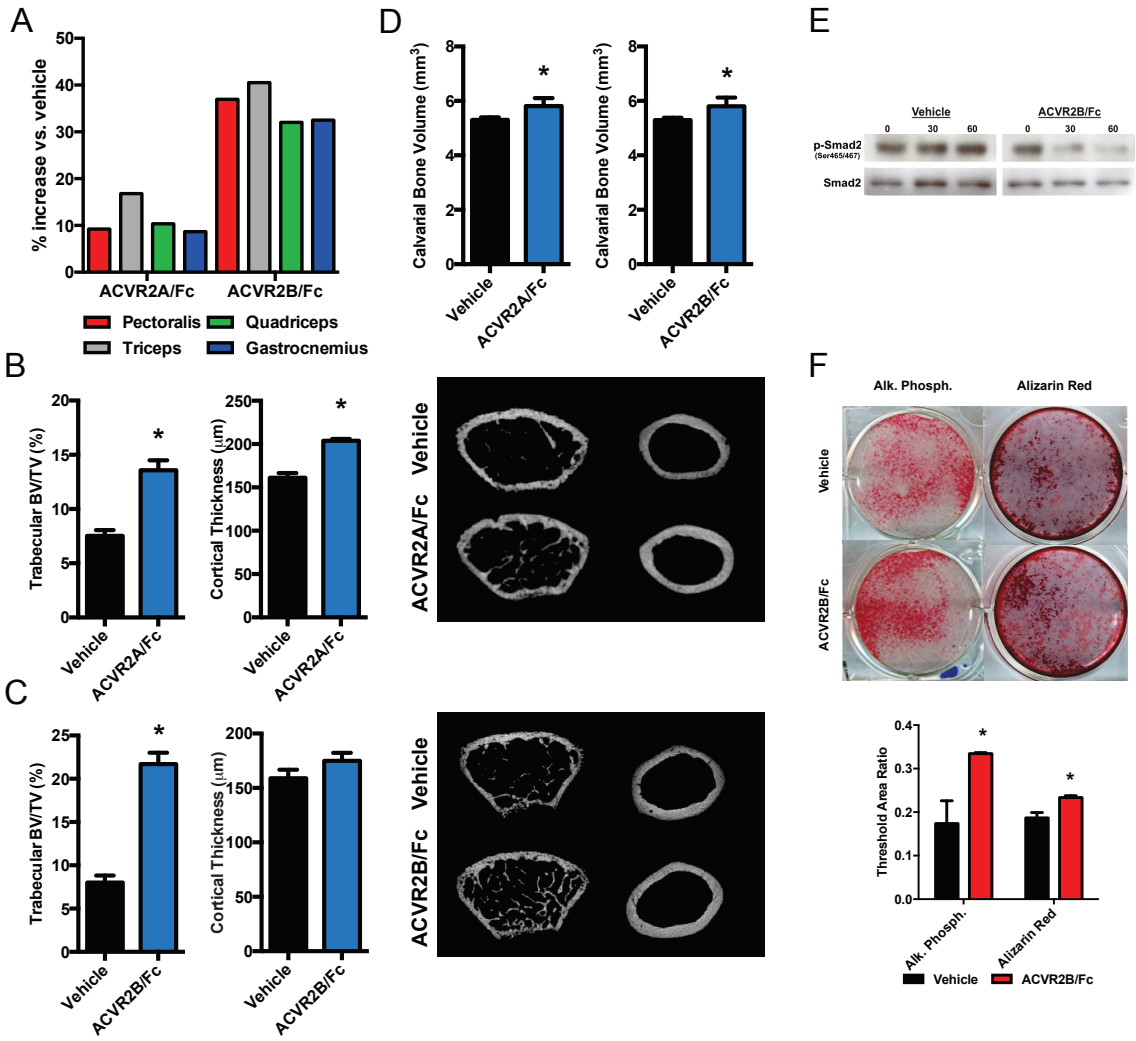

Figure 2.1: Soluble Activin Receptor Administration Increases Bone Volume *in vivo* and Enhances Osteoblast Differentiation *in vitro*

ACVR2A/Fc and ACVR2B/Fc treatment demonstrates significant increases in skeletal muscle weights as compared to vehicle treated controls (A). ACVR2A/Fc administration exhibits a near doubling in trabecular bone volume/tissue volume and a significant increase in cortical thickness (B). Similarly, ACVR2B/Fc treatment demonstrates a near tripling in trabecular bone volume/tissue volume but no significant increase in cortical thickness (C). Both ACVR2A/Fc and ACVR2B/Fc treatment demonstrate significant increases in calvarial bone volume (D). ACVR2B/Fc treatment shows a dose-dependent reduction of Smad2 phosphorylation *in vitro* (E). Further *in vitro* analysis demonstrates that ACVR2B/Fc treatment enhances alkaline phosphatase activity and mineral deposition by alizarin red staining in osteoblast differentiation cultures (F).

2.4.2 Activin Receptor Signaling Components Are Expressed and Functional in Osteoblasts

We next surveyed the transcriptional expression of the components of activin receptor signaling in differentiated osteoblast cultures and compared these results to skeletal muscle, a tissue in which the function of this pathway is well established. Activin type II receptors were expressed in differentiated osteoblasts, with ACVR2A expression at levels equal to skeletal muscle and ACVR2B at significantly lower, near negligible, levels in osteoblasts as compared to muscle. The cognate type I receptor to the activin type II receptors (ACVR1B/ALK4) and the monomeric components of activin ligands (InhBA and InhBB) were also highly expressed in differentiated osteoblasts (Figure 2.2A). Further supporting the notion that activin signaling is important for osteoblast function, the expression of activin receptors also increased throughout the course of osteoblast differentiation, particularly for ACVR2A (Figure 2.2B). It should also be noted that, despite the two-fold increase in ACVR2B during osteoblast differentiation, its expression remained orders of magnitude lower than ACVR2A. Expression of ACVR2A (Figure 2.2C) and ACVR2B (Figure 2.3A) was also evident *in vivo* and localized to osteoblasts and osteocytes within the cortical and trabecular bone by immunohistochemistry. Finally, functionality of activin receptor signaling in osteoblasts was demonstrated *in vitro* by phosphorylation of Smad2 following activin ligand (Activin A, AB, or B) treatment (Figure 2.2D). These experiments indicate that the activin type II receptors, predominantly ACVR2A, are expressed and functional within the osteoblast *in vivo* and *in vitro.*

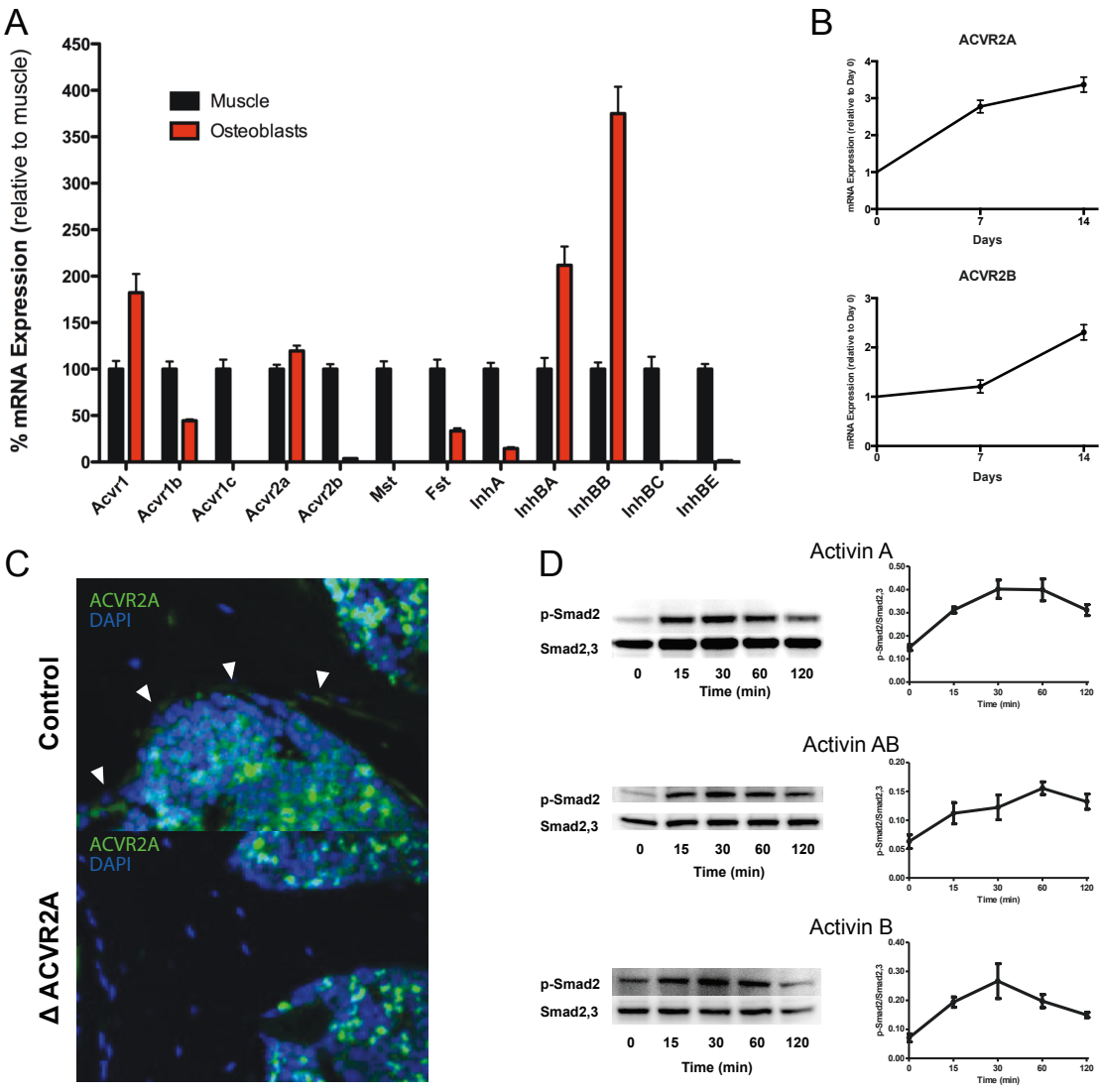

Figure 2.2: Activin Receptor Signaling Components Are Expressed and Functional in Osteoblasts

Transcriptional expression assays demonstrate that the activin signaling components are expressed in primary osteoblasts as compared to skeletal muscle (A). ACVR2A and ACVR2B increase transcriptional expression with osteoblast differentiation (B). Immunohistochemistry localizes ACVR2A (white arrowheads) to osteoblasts and osteocytes in bone sections (C). Phosphorylation of Smad2 following activin ligand administration demonstrates activin receptor functionality in osteoblasts (D).

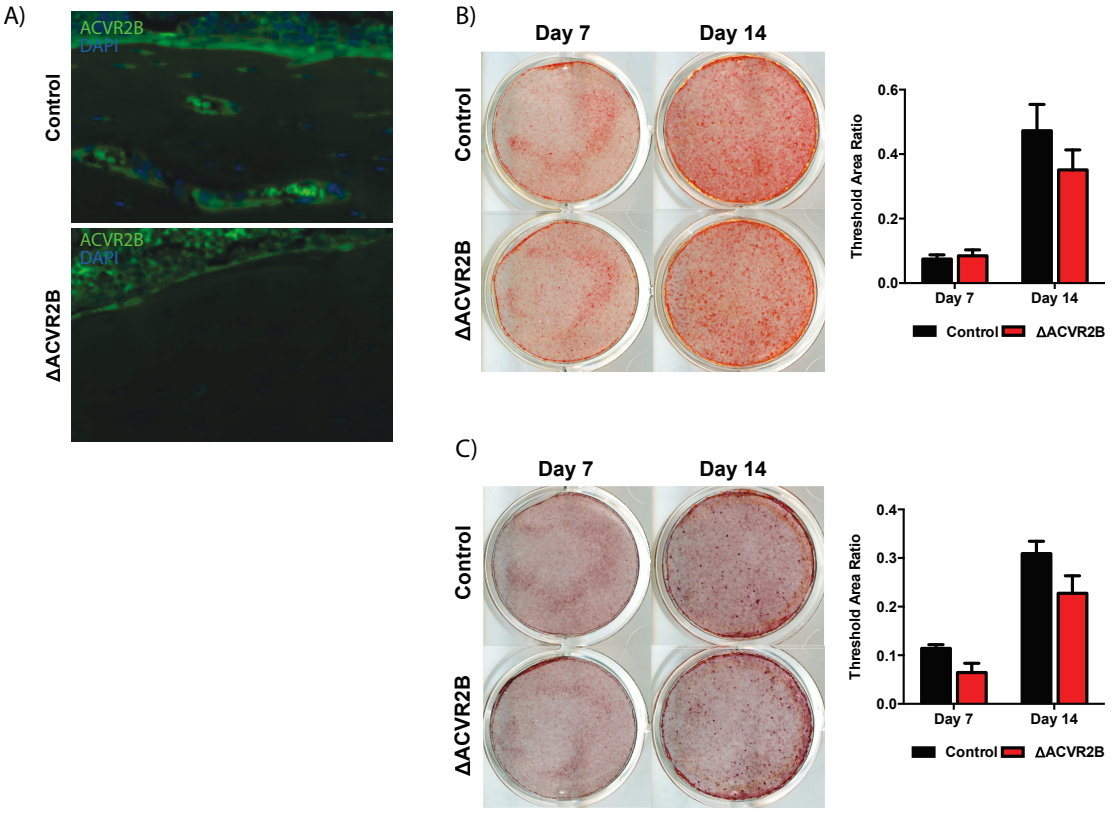

Figure 2.3: Disruption of ACVR2B Does Not Affect Osteoblast Differentiation *in vitro*

ACVR2B was localized to osteoblasts and osteocytes by immunohistochemistry (A). However, disruption of ACVR2B did not affect osteoblast differentiation as shown by alkaline phosphatase activity staining (B) and mineral deposition by alizarin red staining (C).

2.4.3 Disruption of ACVR2A Enhances Osteoblast Differentiation in vitro

To next determine the role of activin receptor signaling in osteoblast function, we differentiated primary calvarial osteoblasts from either ACVR2A or ACVR2B floxed neonates and deleted the receptors using an adenoviral vector expressing the Cre recombinase. Proliferation, as assessed by BrdU incorporation, demonstrated a slight decrease in ΔACVR2A osteoblasts (Figure 2.4A) while ΔACVR2B osteoblasts demonstrated no significant change (Figure 2.4B). ΔACVR2B osteoblasts were unaffected in other functional assays as well, demonstrating no difference in alkaline phosphatase activity (Figure 2.3B) or mineral deposition (Figure 2.3C). However, differentiated osteoblasts lacking ACVR2A did show dramatic increases in alkaline phosphatase staining (Figure 2.4C) as well as enhanced matrix mineralization by alizarin red staining (Figure 2.4D). In accord with these functional assays, transcriptional expression of osteoblast markers such as *Osterix*, *Osteocalcin*, and *DMP1* were significantly upregulated at Day 7 in ΔACVR2A osteoblasts (Figure 2.4E). Together, these data demonstrate that disruption of ACVR2A enhances osteoblast differentiation and maturation *in vitro*.

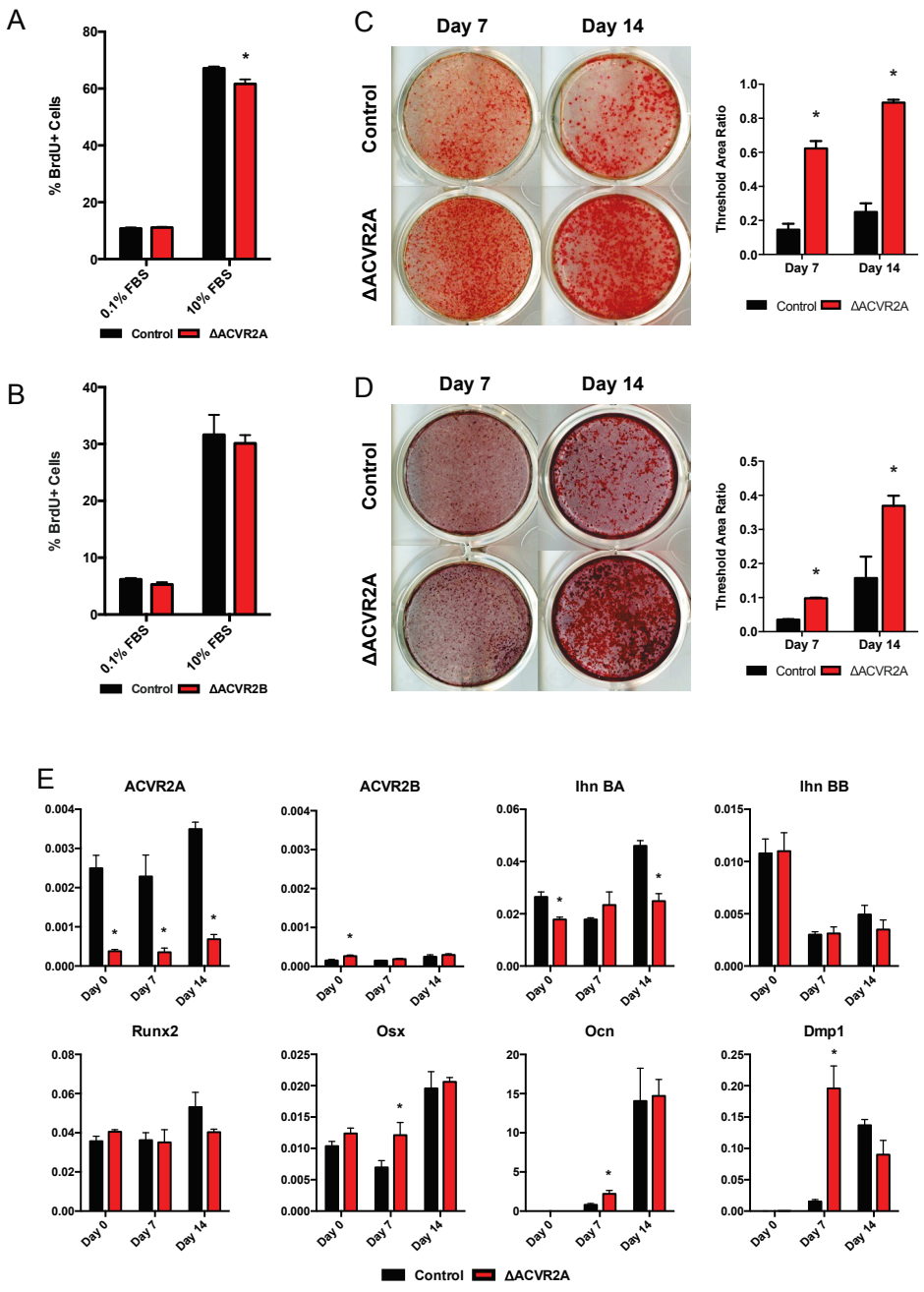

Figure 2.4: Disruption of ACVR2A Enhances Osteoblast Differentiation *in vitro*

ΔACVR2A osteoblasts exhibit a slight decrease in proliferation (A) while disruption of ACVR2B does not alter osteoblast proliferation (B). Osteoblast differentiation is significantly enhanced with ACVR2A disruption as shown by increased alkaline phosphatase activity (C) and mineral deposition (D). Disruption of ACVR2A induces increased transcriptional expression of osteoblast differentiation markers such as *Osterix*, *Osteocalcin*, and *Dmp1* at Day 7 (E).

2.4.4 ΔACVR2A Mice Demonstrate Increased Bone Volume

To determine the consequence of osteoblast-specific disruption of ACVR2A *in vivo*, mice lacking ACVR2A within the osteoblast lineage were generated by crosses of Oc-Cre-Tg+; ACVR2A$^{flox/flox}$ with ACVR2A$^{flox/flox}$ mice. Transgenic ACVR2A$^{flox/flox}$ mice carrying Oc-Cre^{+} (ΔACVR2A) were born within the expected Mendelian ratios and had normal lifespans. Allele-specific PCR was performed to confirm that recombination occurred only within skeletal tissues (Figure 2.5A). MicroCT analysis of the distal femur revealed that ΔACVR2A male mice at six weeks of age exhibited increased trabecular bone volume/tissue volume (BV/TV) (34.2 ± 4.1%) and trabecular number (24.1 ± 1.9%). Furthermore, twelve-week-old male ΔACVR2A mice demonstrated sustained increases in femoral trabecular bone volume fraction (47.9 ± 5.4%), trabecular thickness (13.3 ± 0.7%), trabecular number (27.8 ± 1.7%), and decreases in trabecular spacing (-6.2 ± 0.1%) (Figure 2.6A-D). Cortical parameters within these males were not significantly changed at six weeks of age. However, at twelve weeks of age, the ΔACVR2A male mice demonstrated increases in tissue area (13.0 ± 0.4%), bone area (15.9 ± 0.7%), polar moment of inertia (31.6 ± 0.3%), and cortical thickness (8.6 ± 0.3%) (Figure 2.6E-H). Similar changes, albeit of lesser magnitude, were observed in female mice at six and twelve weeks of age (data not shown). Histomorphometric analysis of the contralateral distal femur from male mice confirmed the increase in trabecular bone volume in ΔACVR2A mice at six and twelve weeks of age (Table 1). Surprisingly, there were no significant changes in osteoblast or osteoclast numbers, nor dynamic parameters,

suggesting the cellular changes that lead to increased bone volume in ΔACVR2A mice likely occurred much earlier in development. Overall, these findings demonstrate that ACVR2A in osteoblasts acts as an important negative regulator of skeletal mass in mice.

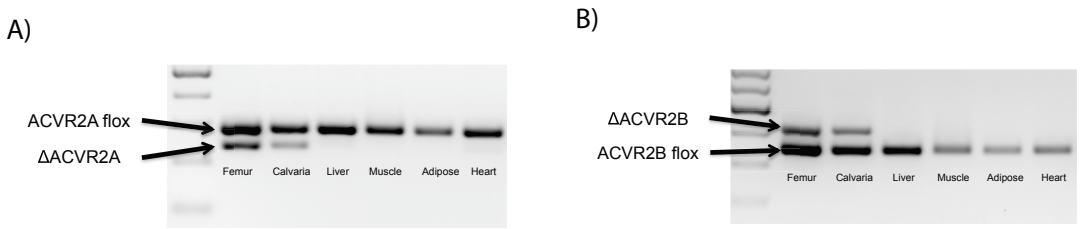

Figure 2.5: Allele-Specific PCR Confirms Cre-Lox Recombination Occurs Exclusively in Skeletal Tissues

Osteocalcin-directed Cre-Lox recombination of ACVR2A (A) and ACVR2B (B) occurs exclusively in skeletal tissues. Allele-specific PCR results of femur and calvaria samples shown.

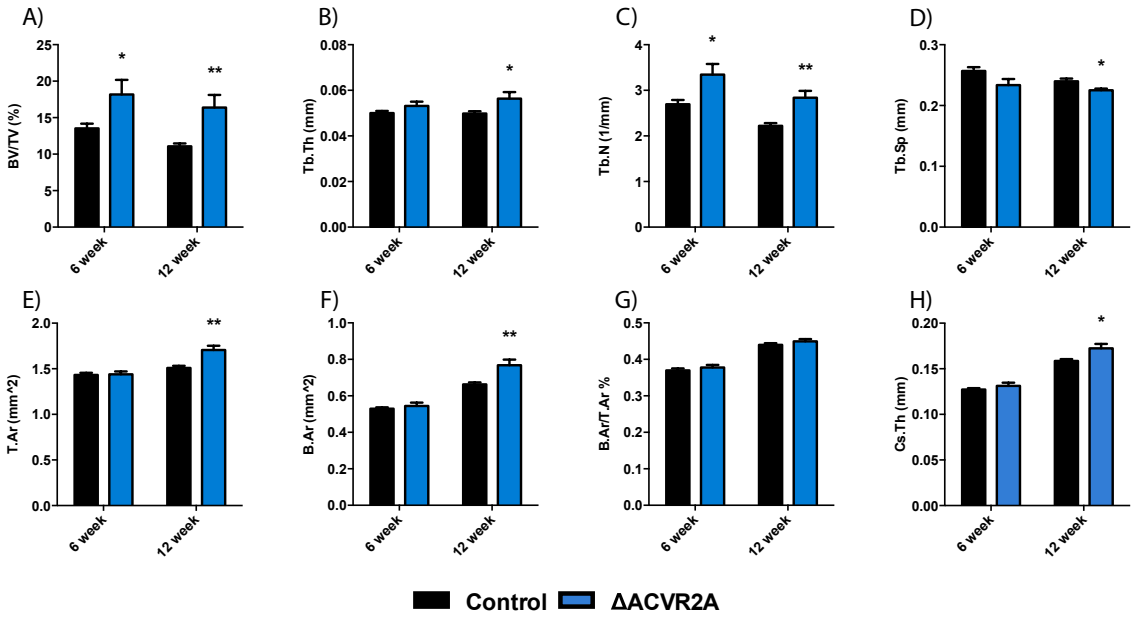

Figure 2.6: Femurs of ΔACVR2A Male Mice Exhibit Increased Bone Volume

Femurs from ΔACVR2A mice exhibit increases in trabecular bone parameters (A-D), including bone volume/tissue volume (A) and trabecular number (D) at six weeks of age. Femurs of ΔACVR2A mice at twelve weeks of age demonstrate increases in trabecular bone volume/tissue volume (A), trabecular thickness (B), trabecular number (C), and a decrease in trabecular spacing (D). Femoral cortical parameters (E-H) were unchanged at six weeks of age in ΔACVR2A mice. However, cortical tissue area (E), bone area (F), and cross-sectional thickness (H) were increased while cortical bone area/tissue area (G) was unchanged at six and twelve weeks of age.

	6 weeks		12 weeks	
	Control	*ΔACVR2A*	*Control*	*ΔACVR2A*
BV/TV	5.09 ± 0.28	6.28 ± 0.57*	7.87 ± 0.57	9.99 ± 0.77*
Tb.Th	18.56 ± 0.48	20.88 ± 1.14	22.83 ± 0.90	25.70 ± 1.14*
Tb.Sp	356.18 ± 22.68	323.32 ± 19.59	275.55 ± 16.18	239.75 ± 14.45*
OV/BV	0.99 ± 0.23	0.86 ± 0.07	0.33 ± 0.05	0.51 ± 0.07*
OS/BS	4.17 ± 0.74	3.48 ± 0.27	2.54 ± 0.42	3.18 ± 0.38
O.Th	2.28 ± 0.25	2.53 ± 0.19	1.60 ± 0.15	2.06 ± 0.09*
N.Ob/BS	254.44 ± 39.35	237.30 ± 79.45	144.80 ± 39.62	170.88 ± 65.61
Ob.S/BS	3.68 ± 0.51	2.98 ± 0.90	2.11 ± 0.60	2.44 ± 1.07
ES/BS	2.96 ± 0.61	3.18 ± 0.52	2.15 ± 0.42	2.83 ± 0.62
E.De	5.52 ± 0.46	4.23 ± 0.27*	3.95 ± 0.64	4.15 ± 0.36
N.Oc/BS	95.43 ± 16.49	112.08 ± 19.95	75.73 ± 14.79	93.24 ± 20.41
Oc.S/BS	2.83 ± 0.64	3.00 ± 0.53	2.13 ± 0.42	2.74 ± 0.61
MAR	2.04 ± 0.13	1.71 ± 0.06*	1.13 ± 0.07	1.11 ± 0.07
dLS/BS	9.77 ± 0.83	10.07 ± 1.17	10.65 ± 0.95	12.60 ± 1.68
sLS/BS	7.18 ± 0.61	8.70 ± 1.27	5.97 ± 0.34	5.55 ± 0.66
MS/BS	13.36 ± 0.96	14.42 ± 1.44	13.64 ± 1.07	15.37 ± 1.83
BFR/BS	3.85 ± 0.37	3.41 ± 0.30	3.21 ± 0.35	3.43 ± 0.46
Omt	1.17 ± 0.15	1.47 ± 0.08*	1.47 ± 0.16	1.90 ± 0.13*
Mlt	0.37 ± 0.08	0.37 ± 0.04	0.26 ± 0.04	0.49 ± 0.13*

Table 1: Bone Histomorphometry

Histomorphometry was performed in trabecular bone of the distal femur in ΔACVR2A male mice. BV/TV: Bone Volume/Tissue Volume (%); Tb.Th: Trabecular Thickness (mm); Tb.Sp: Trabecular Spacing (mm); OV/BV: Osteoid Volume/Bone Volume (%); OS/BS: Osteoid Surface/Bone Surface (%); O.Th: Osteoid Thickness (mm); N.Ob/BS: Osteoblast Number/Bone Surface (no./100mm); Ob.S/BS: Osteoblast Surface/Bone Surface (%); ES/BS: Erosion Surface/Bone Surface (%); E.De: Erosion Depth (mm); N.Oc/BS: Osteoclast Number/Bone Surface (no./100mm); Oc.S/BS: Osteoclast Surface/Bone Surface (%); MAR: Mineral Apposition Rate (day); dLS/BS: Double Labeled Surface/Bone Surface (%); sLS/BS: Single Labeled Surface/Bone Surface (%); MS/BS: Mineralizing Surface/Bone Surface (%); BFR/BS: Bone Formation Rate/Bone Surface ($\mu m^3/\mu m^2$/day); Omt: Osteoid Maturation Time (day); Mlt: Mineralization Lag Time (day). Values shown are mean ± S.E.M. *P<0.05 vs. age-matched control

2.4.5 ΔACVR2B Mice Exhibit No Skeletal Changes

Despite exhibiting no effect on osteoblasts *in vitro*, we further explored whether ACVR2B may behave differently *in vivo*, as it was identified in osteoblasts by immunohistochemistry. We disrupted ACVR2B in the osteoblast lineage using the same osteocalcin-driven Cre by crossing Oc-Cre-Tg+; $ACVR2B^{flox/flox}$ with $ACVR2B^{flox/flox}$ mice. Transgenic $ACVR2B^{flox/flox}$ mice carrying Oc-Cre^{+} (ΔACVR2B) were born within the expected Mendelian ratios and with normal lifespans. Allele-specific PCR was performed to confirm that recombination occurred only within skeletal tissues (Figure 2.5B). Micro CT analysis of the distal femur of ΔACVR2B mice showed virtually no changes at six and twelve weeks of age (Figure 2.7 – males, female data not shown), suggesting that ACVR2B does not function as the primary receptor for activin signaling in the osteoblast lineage.

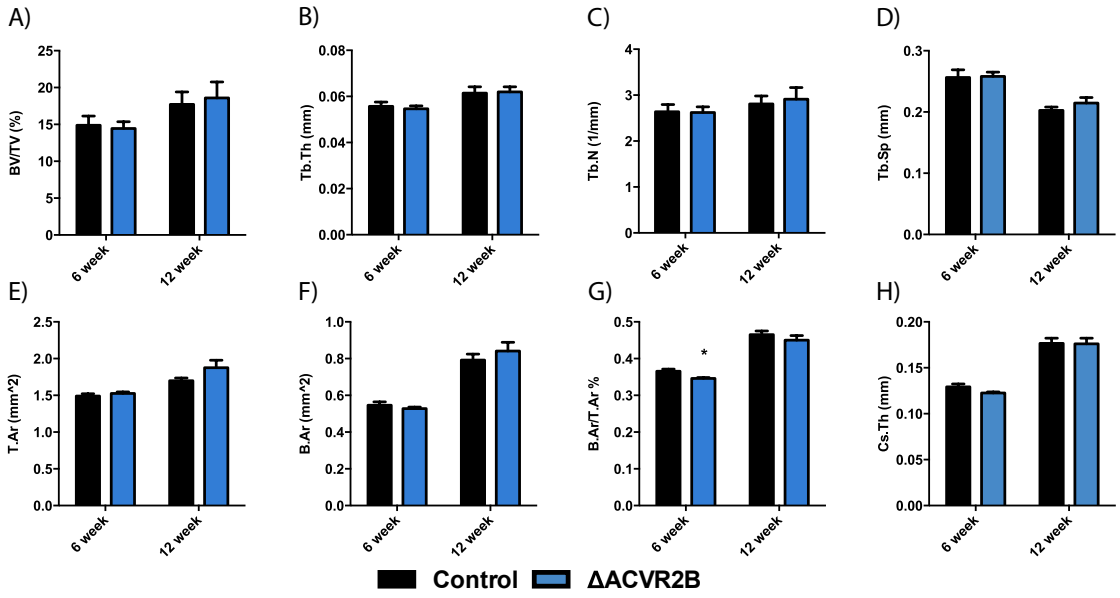

Figure 2.7: ACVR2B Disruption Does Not Affect Bone Volume *in vivo*

Femurs of ΔACVR2B male mice show no significant changes in trabecular (A-D) or cortical bone parameters (E-H) at six or twelve weeks of age. Trabecular bone parameters include bone volume/tissue volume (A), trabecular thickness (B), trabecular number (C), and trabecular spacing (D). Apart from a slight reduction in bone area/tissue area at 6 weeks of age (G), there are no significant changes observed in cortical parameters, including tissue area (E), bone area (F), bone area/tissue area (G), and cross-sectional thickness (H) in six or twelve-week-old ΔACVR2B male mice.

2.4.6 Compound ΔACVR2A/2B Mice Exhibit a Phenotype Similar to ΔACVR2A Mice

To unequivocally determine if ACVR2B is able to function in osteoblasts *in vivo* – specifically, to partially compensate for the loss of ACVR2A – we generated compound mutants of ACVR2A and ACVR2B (ΔACVR2A/2B) in the osteoblast lineage by crossing Oc-Cre-Tg+; ACVR2A/2B$^{flox/flox}$ with ACVR2A/2B$^{flox/flox}$ mice. ΔACVR2A/2B male mice showed significant increases in trabecular bone volume fraction (22.6 ± 1.1%), trabecular number (25.0 ± 3.9%), and decreased trabecular spacing (-15.1 ± 3.6%) at six weeks of age. At twelve weeks of age, ΔACVR2A/2B male mice demonstrated sustained increases in trabecular bone volume fraction (24.8 ± 2.2%) and trabecular number (18.4 ± 6.0%) (Figure 2.8A-D). However, unlike ΔACVR2A mice, the ΔACVR2A/2B mice demonstrated no significant changes in cortical parameters at twelve weeks of age (Figure 2.8E-H). As was the case for ΔACVR2A mice, female ΔACVR2A/2B mice displayed skeletal changes similar to males with slightly lesser magnitude (not shown). These data demonstrate that compound ΔACVR2A/2B mutant mice have increases in trabecular bone parameters similar to ΔACVR2A mutants, further supporting the notion that ACVR2A is the predominant activin signaling receptor in osteoblasts.

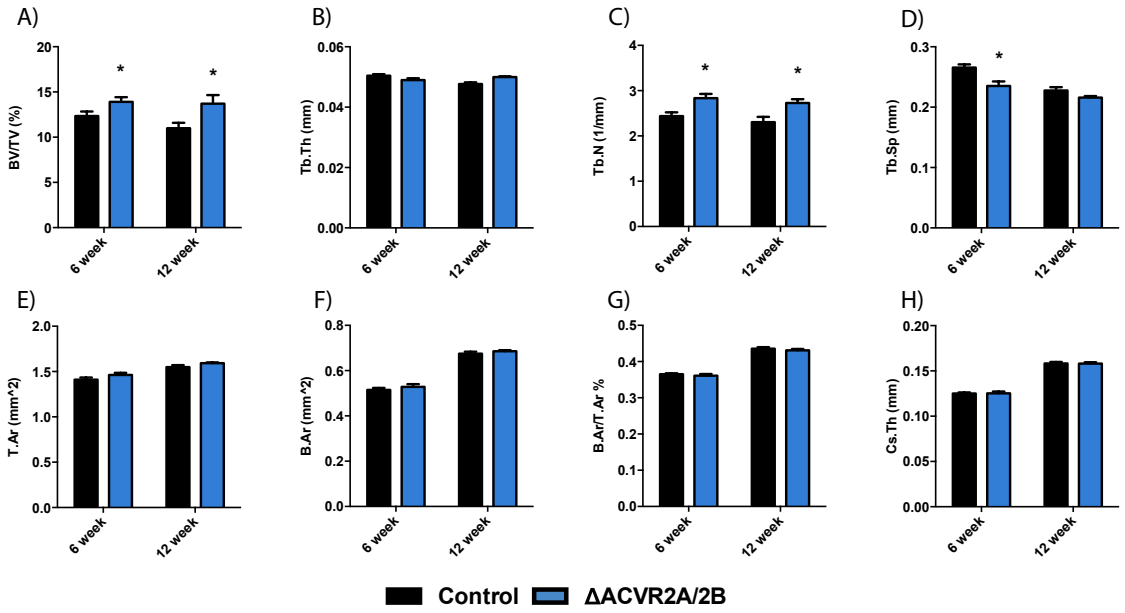

Figure 2.8: Femurs of ΔACVR2A/2B Mice Exhibit Similar Changes in Bone Volume as ΔACVR2A Mice

Femurs of ΔACVR2A/2B male mice exhibit similar increases in trabecular bone parameters (A-D) as ΔACVR2A mice. ΔACVR2A/2B mice demonstrate increases in trabecular bone volume/tissue volume (A), trabecular number (C), and a decrease in trabecular spacing (D) at six weeks of ago. These increases were sustained in trabecular bone volume/tissue volume (A) and trabecular number (C) at twelve weeks of age. However, no significant changes were seen in trabecular thickness (B) at six or twelve weeks of age. Cortical bone parameters, including tissue area (E), bone area (F), bone area/tissue area (G), and cross-sectional thickness (H), were unchanged in six and twelve-week-old ΔACVR2A/2B male mice.

2.4.7 Skeletal Changes in ΔACVR2A Mice Are Accompanied by Increased Mechanical Attributes

An important consideration for future therapeutic targeting of this pathway in bone is whether the additional bone mass observed in the mutant mice is mechanically competent. Thus, we also evaluated changes in mechanical properties of femurs from ΔACVR2A male mice by three-point bending. ΔACVR2A mutants demonstrated significant increases in ultimate moment (24.8 ± 2.0%) and bending rigidity (30 ± 2.7%) with non-significant trends in ultimate stress (10.1 ± 0.5%). In addition, pre-yield parameters including strain (21.3 ± 1.6%), energy (45.8 ± 6.3%), and toughness (36.1 ± 4.0%) were also significantly increased compared to controls (Figure 2.9A-G). The ultimate bending energy and Young's modulus were, however, unchanged (Figure 2.9D and 2.9H, respectively). Overall, the mechanical testing indicates that the additional bone produced in mice with osteoblast-specific disruption of ACVR2A is of high mechanical competency and increases bone strength.

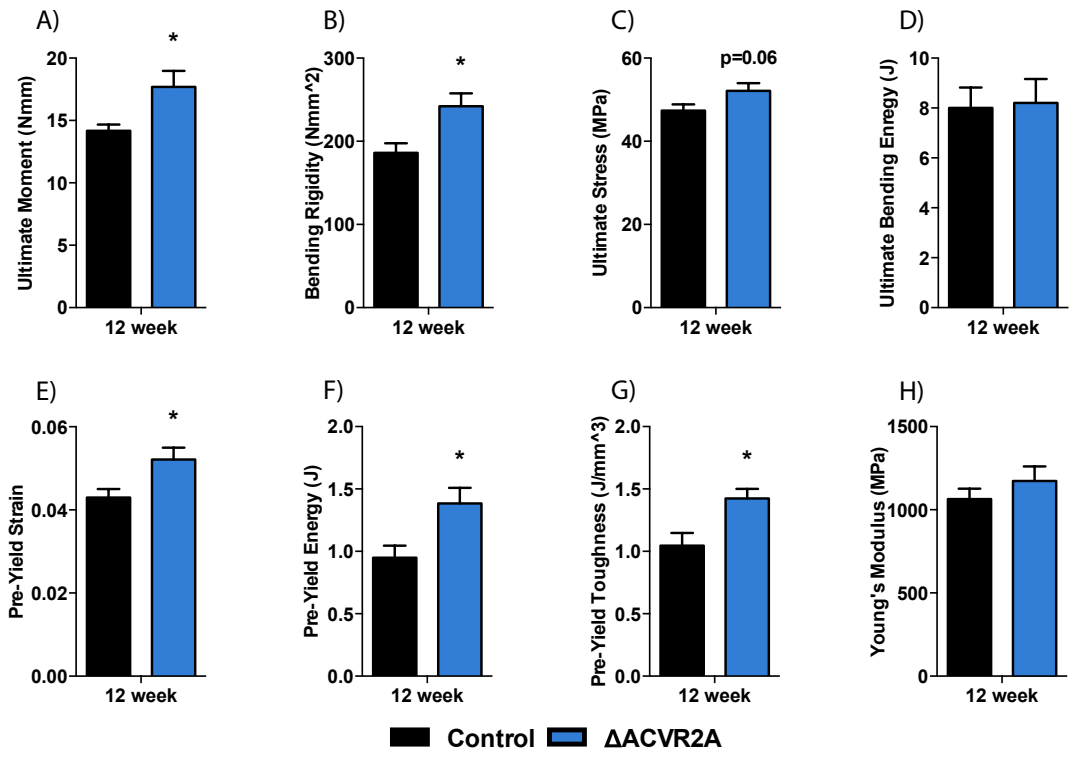

Figure 2.9: Skeletal Changes in ΔACVR2A Mice Are Accompanied by Increased Mechanical Properties

Three-point bending analysis of femurs from ΔACVR2A mice demonstrates significant increases in mechanical properties, such as the ultimate moment (A), bending rigidity (B), pre-yield strain (E), pre-yield energy (F), and pre-yield toughness (G), and non-significant trends in ultimate stress (C). There were, however, no significant changes in the ultimate bending energy (D) or Young's modulus (H) with ACVR2A disruption.

2.4.8 Activin Receptor Signaling in Osteoblasts Contributes Significantly to the Anabolic Effects Observed with Soluble Receptor Administration

Finally, we returned to the question of what proportion of the anabolic effect observed from soluble activin receptor treatment results from direct effects on the osteoblast versus indirect effects from skeletal muscle, or perhaps even other secondary systems. To do so, we treated ΔACVR2A/2B mutant mice with ACVR2B/Fc for four weeks, as done in our initial experiments, and compared the percentage change in bone volume with that observed in wild type mice treated with the soluble receptor. In wild type animals treated with ACVR2B/Fc, the trabecular bone volume fraction increased by 114 ± 16.0%. By contrast, ΔACVR2A/2B mice treated with ACVR2B/Fc gained 62.3 ± 14.5% in trabecular BV/TV (Figure 2.10A). Similarly, increases in trabecular number were differentially affected between control (82.0 ± 8.5%) and ΔACVR2A/2B (50.1 ± 11.1%) mice (Figure 2.10C). In contrast to the changes observed in trabecular bone, there were no differential increases in muscle mass (gastrocnemius, tibialis anterior, and quadriceps) between control and ΔACVR2A/2B mice with ACVR2B/Fc administration (Figure 2.10E-G). Taken together, these data would suggest that a substantial portion of the effect of soluble activin receptor administration on bone volume (>50%) results from the direct actions of this pathway in osteoblasts.

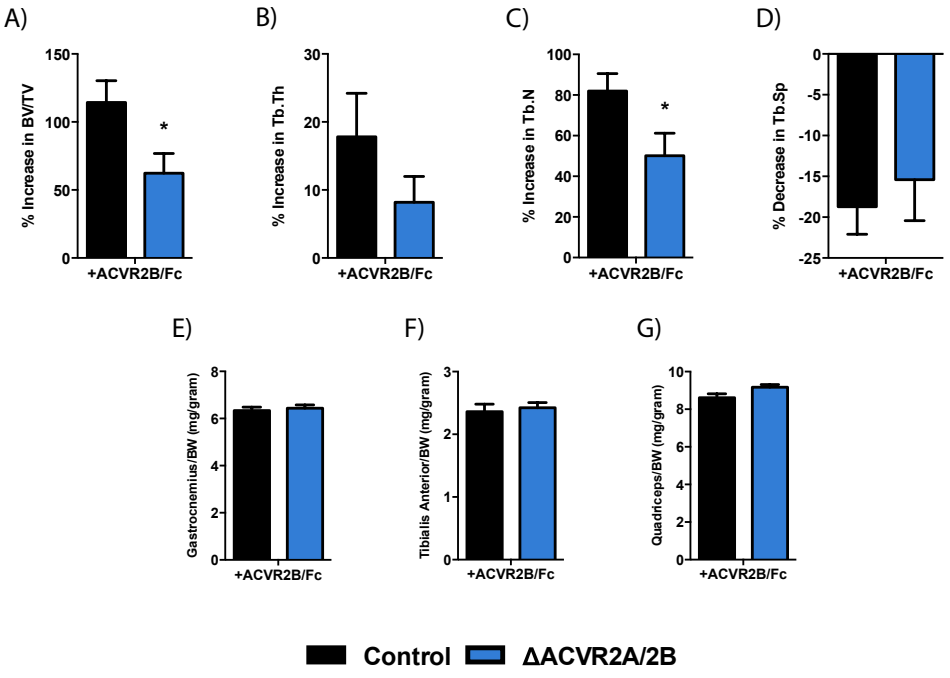

Figure 2.10: Activin Receptor Signaling is a Significant Contributor to Anabolic Changes Observed with Soluble Receptor Administration

Soluble activin receptor treatment with ACVR2B/Fc demonstrates attenuated increases in bone volume/tissue volume (A), trabecular thickness (B), and trabecular number (C) in ΔACVR2A/2B mice. There were no differential changes in trabecular bone spacing (D) or differential increases in skeletal muscle weights, including the gastrocnemius (E), tibialis anterior (F), or quadriceps (G) muscles.

2.5 Discussion

We have described, for the first time, a role for activin receptor signaling in osteoblasts that is analogous to the known function of this pathway as a negative regulator of muscle development and mass. Curiously, early studies suggested that activin A exerted a stimulatory effect on osteoblastogenesis [104, 106], but more recent reports using human osteoblast preparations suggest that activin inhibits osteoblastogenesis and mineralization [109]. Initial *in vivo* studies in bone were also conflicting. For example, local injection of activin A into rat fibula fractures [107] or over the calvaria of rat neonates [108] increased bone accumulation. In more recent studies, systemic infusion with a soluble ACVR2A resulted in increased bone mass in both mice [167] and monkeys [111, 112]. Moreover, administration of soluble ACVR2A increased markers of bone formation in humans [113]. Histomorphometric analysis of bones from mice and monkeys treated with this ACVR2A fusion peptide demonstrated that the anabolic effects were attributable to increased osteoblast activity [112, 167]. Our initial studies with soluble activin receptor administration support these previous studies of pharmacologic blockade of activin signaling, even over the considerably shorter course of treatment we used. We utilized this 4-week treatment in hopes of minimizing the effect of long-term adaptation of bone mass in response to the increased mechanical forces that follow the additional muscle mass gained from systemic activin receptor blockade. Combined with the observed increases in bone mass in the calvarium, a

skeletal site that is neither load bearing nor encased in skeletal muscle like the femur, we were encouraged to further investigate the direct activity of this pathway in osteoblasts.

Further interrogation of this pathway in osteoblasts – both through pharmacologic and genetic blockade of activin receptor activity – demonstrated that activin receptor signaling is, indeed, a negative regulator of osteoblast function. Interestingly, both *in vitro* and *in vivo*, this effect appears to be nearly entirely dependent on ACVR2A. Previous studies have suggested that myostatin and/or ACRV2B may also play a role in regulating bone mass. For example, myostatin null mice have increased bone mass [163], and infusion of a soluble ACVR2B fusion molecule increased bone mass in a mouse model of androgen deprivation [171]. In our hands, as well, administration of soluble ACVR2B resulted in profound increases in trabecular bone volume. However, we consider it unlikely that ACVR2B and myostatin are physiologically relevant in osteoblasts to regulate bone mass for the following reasons. In the case of the myostatin null mice, increased bone mass was only observed in the regions of long bones immediately adjacent to entheses [163], strongly suggesting that the effect is secondary to increased mechanical force generated as a result of the double muscled phenotype in myostatin null mice. Additionally, we observed no detectable expression of myostatin in primary mouse osteoblasts, and ACVR2B expression was orders of magnitude lower than ACVR2A expression (Figure 2.2A). Finally, while ACVR2B and various soluble forms of this receptor do predominantly bind and sequester myostatin, ACVR2B is the more promiscuous of the two activin receptors and also binds activin ligands with nearly as high affinity as it binds myostatin [172].

Our studies with genetic disruption of ACVR2A and ACVR2B in osteoblasts bear out the notion that ACVR2A functions as a negative regulator of osteoblast function. Both *in vitro* and *in vivo*, disruption of ACVR2B had no discernable effect on osteoblast function. Moreover, compound mutants that lacked both activin receptors in osteoblasts displayed changes in trabecular bone that were strikingly similar to ΔACVR2A mice. Of interest, however, was the fact that the increased cortical bone phenotype observed in ΔACVR2A mice at 12 weeks of age (Figure 2.6E-H) was not observed in the double ΔACVR2A/2B mice (Figure 2.8E-H). One possible explanation for this change – which, presumably, is exactly the opposite of what one would expect if ACVR2B were able to partially compensate for ACVR2A loss – is that complete loss of type II activin receptors from osteoblasts alters other TGF-β family and/or BMP signaling in osteoblasts, as these receptor families are well known to be promiscuous in ligand binding and signaling activity. In support of this notion, Lowery and colleagues have recently demonstrated that selective disruption of BMPR2 in osteoprogenitor cells actually impaired activin signaling, with no effect on BMP signaling, resulting in a high bone mass phenotype [173]. Their result also indirectly supports our overall finding that activin signaling in osteoblasts acts as a negative regulator of their function.

Despite the clear skeletal phenotype observed in ΔACVR2A mice, we were unable to detect any significant changes in bone cell numbers at either time point observed, in either sex. We believe that these changes must have occurred much earlier in development for a number of reasons. Both genetic and pharmacologic blockade of activin signaling *in vitro* resulted in rapid and robust differentiation of osteoblasts. We

presume it is likely that the same effect would have taken place *in vivo* with an early burst of osteoblast differentiation and bone formation that was then damped by yet unknown compensatory mechanisms. In support of this idea, the only statistically significant histomorphometric parameters observed were a slightly repressed mineral apposition rate at six weeks of age, increased osteoid maturation time at both six and twelve weeks of age, and increased mineralization lag time at twelve weeks of age (Table 1); all opposite of what one would typically expect in mice with higher bone mass, but consistent with a repression of osteoblast activity. Future studies are already underway to examine the role of activin receptor signaling at varying points in the osteoblast lineage, the current study having focused on the mature osteoblast/osteocyte using a human osteocalcin Cre driver, and will explore these early developmental changes in greater detail. Regardless, our current study provides clear evidence of the importance of ACVR2A in osteoblasts as a negative regulator of bone development and mass.

In an attempt to begin to understand the precise contribution of activin receptor signaling in osteoblasts to the observed anabolic effects of soluble activin receptor administration in the skeleton, we also treated the double ΔACVR2A/2B mice with soluble ACVR2B. The results of this experiment suggest that over half of the observed increase in bone mass following administration of soluble ACVR2B/Fc is due to the direct activity of this pathway in osteoblasts (Figure 2.10A). Interestingly, the absolute final trabecular bone volume fraction following soluble receptor treatment was nearly the same between treated wild type mice and double mutants, suggesting a sort of ceiling by which modulating this pathway can influence bone volume in mice (not shown).

Interestingly, we observed no difference in skeletal muscle wet weights between ACVR2B/Fc treated wild type and ΔACVR2A/2B mice, hinting that changes in activin receptor signaling in osteoblasts do not seem to reciprocally affect skeletal muscle. The additional increase in bone volume in ΔACVR2A/2B mice following ACVR2B/Fc treatment is likely to occur from multiple secondary mechanisms, given the established functions of activin signaling in other systems known to affect bone, e.g. the pituitary and immune system [174, 175]. Future studies are already underway to determine the precise contribution of skeletal muscle to this effect by observing changes in the skeleton following muscle-specific disruption of activin receptor signaling.

Our study is the first to conclusively demonstrate the activity of activin receptor signaling in osteoblasts as a negative regulator of bone development, analogous to the well-established function of this pathway in skeletal muscle. This effect appears to be mediated predominantly through ACVR2A and activin ligands, suggesting an evolutionary split in the control of musculoskeletal development; with ACVR2A and activin ligands controlling the skeleton, while ACVR2B and myostatin exert control over skeletal muscle. Such an arrangement would allow for precise, concerted control of the development and maintenance of the musculoskeletal system, and also provides a particularly attractive therapeutic area to modulate crosstalk between both tissues.

2.6 Methods

2.6.1 Soluble Activin Type II Receptor Animal Studies

All animal protocols were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. Systemic suppression of activin ligands was accomplished by intraperitoneal injections (10 mg/kg BW) of a soluble chimeric fusion protein consisting of either the extracellular ACVR2A or ACVR2B domain conjugated to the Fc domain of a murine IgG antibody (ACVR2A/Fc or ACVR2B/Fc, respectively). Injections were administered weekly for four weeks in twelve-week-old female mice (n = 5). After soluble activin receptor treatment, skeletal tissues were harvested and wet muscle weights were measured.

2.6.2 Transgenic Animal Studies

Osteoblast-specific disruption of ACVR2A, ACVR2B, or both was accomplished by crossing $ACVR2A^{flox/flox}$ or $ACVR2B^{flox/flox}$ mice [176], with mice expressing the Cre recombinase under the direction of the human osteocalcin promoter (Oc-Cre), in which Cre recombinase expression is restricted to mature osteoblasts [177]. Recombination

within the skeletal tissues was confirmed using allele specific PCR and details are available upon request. Control ($ACVR2A^{flox/flox}$ or $ACVR2B^{flox/flox}$) and mutant ($ACVR2A^{flox/flox}$ or $ACVR2B^{flox/flox}$; Oc-Cre^{+}) male and female mice were sacrificed at six and twelve weeks of age (n = 10). Skeletal tissues then were harvested and fixed in formalin overnight and stored until analysis in 70% ethanol. Prior to harvest, six-week-old mice were double labeled by two sequential intraperitoneal calcein injections (10 mg/kg BW) at seven and two days prior to sacrifice for dynamic histomorphometric analysis. Twelve-week-old mice were double labeled with calcein injections at ten and three days before sacrifice. Mice were maintained on a pure C57Bl/6 background.

2.6.3 Microcomputed Tomography (μCT)

Images of skeletal tissues were obtained using a Skyscan 1172 desktop imaging system (Skyscan-Bruker). Scans were performed with an isotropic voxel size of 10 μm at 65 kV and 200 μA through a 0.5mm aluminum filter. Femoral trabecular parameters were measured in a 200 μm region of interest located approximately 50 μm proximal to the distal growth plate. Femoral cortical parameters were measured within a 500 μm region of interest centered at the midshaft. All bone analysis was performed in accordance with the American Society of Bone and Mineral Research (ASBMR) recommended parameters [178].

2.6.4 Bone Histomorphometry

For histomorphometric analysis, femurs were fixed in 10% neutral buffered formalin, dehydrated, and embedded in polymethylmethacrylate (PMMA). Three-micron sections were cut through the primary spongiosium and stained using the Mason-Goldner Trichrome stain. Additional serial sections were cut for fluorescent microscopy. Osteoblast and osteoclast measures were assessed at standardized sites beneath the growth plate using Osteoplan II (Kontron). Static and dynamic parameters were calculated in accordance with the ASBMR guidelines [179].

2.6.5 Osteoblast Isolation and Culture

Osteoblasts were isolated from neonatal calvaria of ACVR2A$^{flox/flox}$ and ACVR2B$^{flox/flox}$ mice by serial digestion in a 1.8 mg/mL collagenase type I (Worthington Biochemical) solution. Calvaria were incubated in 10 mL of collagenase solution at 37 °C for 15 minutes with constant agitation per digestion cycle. The digestion solutions were collected and fresh collagenase was added to the remaining digestion cycles an additional four times. Digestions 3-5, containing osteoblasts, were pooled and cultured in α-MEM medium containing 10% FBS and 1% penicillin/streptomyocin. Osteoblasts were cultured at 37 °C at 5% carbon dioxide in a humidified incubator. Osteoblasts were grown to 70% confluency and then transduced in PBS with either a control adenovirus

encoding green fluorescent protein (GFP) or an adenovirus encoding Cre recombinase (Vector Biolabs) at a titer of 100 MOI. After 1 hour of incubation, osteoblasts were supplemented with α-minimum essential medium (α-MEM) containing 10% fetal bovine serum and 1% penicillin/streptomyosin and then allowed to recover for 48 hours. Osteoblasts were then harvested to assess ACVR2A or ACVR2B deletion efficiency by quantitative PCR and re-plated for proliferation studies and differentiation studies.

2.6.6 Osteoblast Proliferation and Differentiation

Osteoblast proliferation was assessed using BrdU incorporation and flow cytometry. Osteoblasts were seeded at a low density of (5×10^4 cells per well) and cultured in α-MEM containing 0.5% FBS for 24 hours to arrest cell cycle. BrdU (10 μM) (BD Biosciences) was then added to the medium for an additional 24 hours prior to harvest to allow incorporation. Osteoblasts were then fixed, stained with anti-BrdU antibody and 7-AAD, and analyzed by FACS Calibur flow cytometry (BD Biosciences). 10,000 events were collected for each sample and the results were processed using Flowing Software v. 2.5. For differentiation experiments, control and ΔACVR2A or ΔACVR2B osteoblasts were cultured to confluency and then cultured in medium supplemented with 10 mM β-glycerol phosphate and 50 μg/mL ascorbic acid. For alkaline phosphatase and mineral deposition assays, osteoblasts deficient in activin receptor were seeded at confluency and were subsequently differentiated in complete

medium supplemented with 10 mM β-glycerol phosphate and 50 μg/mL ascorbic acid. Cultures were then fixed at Day 7 and 14 using 100% ethanol and assessed for alkaline phosphatase activity and mineral deposition with Fast Red TR/Naphthol AS-MX phosphate (Sigma-Aldrich, Corp., St. Louis, MO) or 40 mM Alizarin Red (Sigma) staining, respectively. Image analysis was performed using FIJI. [180]

2.6.7 Quantitative Real-Time PCR

Total RNA was collected from cells and tissue samples using TRIzol (Invitrogen) following the manufacturer's protocol. One microgram of RNA was then reversely transcribed using an iScript cDNA synthesis kit (Bio-Rad). Two microliters of cDNA were amplified under standard PCR conditions using iQ SYBR Green Supermix (Bio-rad). All cDNA samples were run in triplicate, averaged, and normalized to endogenous β-actin expression levels. Primer sequences were designed using Primer-BLAST (NCBI) and are available upon request.

2.6.8 Cell Lysis and Immunoblotting

For signaling experiments, osteoblasts were cultured to near confluence in α-MEM containing 10% FBS. Cells were then starved in α-MEM containing 0.5% FBS overnight to reduce endogenous cellular activity prior to stimulation. Activin A, AB, and B (R&D Systems) were added to the medium for a final concentration of 20 ng/mL. ACVR2B/Fc was supplemented to a final concentration of 50 ng/mL. Whole cell lysates were then collected at appropriate timepoints in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100 and 10% glycerol). Protein concentrations were measured by BCA Protein Assay (Pierce) and 15 μg of total protein were loaded into each lane using a mini-SDS-PAGE system (Bio-Rad). Following electrophoresis, proteins were transferred to a PDVF membrane using Mini Trans-blot Cell System (Bio-Rad). Membranes were then blocked using 5% BSA at room temperature for 1 hour and then incubated with antibody of interest (Cell Signaling) at 4 °C overnight. Protein signal was detected using secondary antibodies conjugated with horseradish-peroxidase. Chemiluminescence of bound antibody was produced by Supersignal West Femto Substrate (Pierce) and captured by Gel Doc XR+ System (Bio-Rad). Blot analysis was performed using Image Lab (Bio-Rad).

2.6.9 Mechanical Testing

Femurs were harvested from 12-week-old control and ΔACVR2A male mice. Soft tissue was then cleared from the femur and placed on a custom three-point bending apparatus with a span of 5.3 mm. After a preload of 0.3 N, the femur was subjected to force in displacement control at 0.1 mm/s until fracture was achieved (Bose Electroforce 3100). Bone hydration was maintained throughout the testing period using PBS. Force-displacement data and microCT imaging was analyzed using a custom MATLAB (MathWorks) program similar to others [181].

2.6.10 Statistical Analysis

All values are shown as mean ± standard error of the mean. Statistical significance between comparable groups was assessed using Student's *t*-test with an assigned significance level (α) of 0.05.

Chapter 3

3 Evaluation of Activin-Targeted Biologics in Composite Muscle and Bone Injury

3.1 Introduction

Composite injuries involving bone and muscle deficits, such as those seen in combat veterans, are some of the most challenging cases that orthopedic surgeons encounter. These complex musculoskeletal injuries are difficult to treat clinically and often result in compromised function. One of the primary reasons that these injuries have had such poor outcomes is that the treatment approach is oftentimes viewed as two individual tissues rather than one functional unit. However, a more comprehensive approach is beginning to take hold with clinicians as they have long observed that muscle flap transplantation improves fracture healing and bone union rates. [182]

The use of soluble activin receptors is an attractive candidate to address such an issue as systemic administration targets both muscle and bone simultaneously. The dual anabolic effect of soluble activin receptor treatment is unique among therapeutic options and would represent a novel way of addressing composite musculoskeletal injuries.

3.2 Methods

3.2.1 Study Design

All animal protocols were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. To assess the use of soluble activin receptor administration in the setting of composite musculoskeletal injury, a murine model was developed consisting of a pinned femoral fracture with an overlying volumetric muscle defect. Soluble activin receptor type IIB (ACVR2B/Fc) was used for these treatment studies to take advantage of its broad ligand binding profile as well as its affinity for myostatin, since muscle healing was a primary outcome measure. Vehicle and soluble activin receptor mice were treated for eight weeks after surgery and tissues were harvested after the administration protocol. Weekly body weights were recorded and intermittent radiographs were taken during the treatment duration.

3.2.2 Composite Musculoskeletal Injury Model

This model was adapted from previous rodent composite injury models pioneered by Drs. Robert Guldberg and Nick Willett. [183] Twelve-week old female mice were first placed under isoflurane anesthesia (3% induction and 1.5% for maintenance). The

surgical site was assessed by palpation of the left femur and overlying hair was removed by surgical clippers and subsequent Nair Hair Removal application. The site was then cleansed and sterilized using Chloraprep wipes (CareFusion). A skin incision was then made along the lateral length of the femur to expose the underlying musculature. Blunt dissection was used to separate the rectus femoris muscle from the biceps femoris muscle along the fascial plane to expose the lateral aspect of the femur. A 5mm biopsy punch was then used to create a volumetric muscle defect at the femur midshaft through the rectus femoris muscle to the surface of the femur. A 2 mm longitudinal incision was then made to translocate the patellar ligament medially to expose the distal femur head. Once exposed, a 25-gauge needle was inserted just anterior to the intercondylar notch to pilot a canal to the femoral marrow cavity. The needle was then removed and a 12 mm (length) X 0.7 mm (diameter) Kirschner wire with a trocar point (Key Surgical) was inserted into the marrow cavity. After wire insertion, a #10 scalpel blade was then used to remove 4 mm of periosteum with a 2 mm overhang on each side of the proposed osteotomy site. After periosteal stripping, 0.22 mm Gigli wire saw was then used to create an osteotomy at the femur midshaft. Once completed, the rectus femoris and biceps femoris muscles were reapproximated and the patellar ligament was restored to its native position using two simple interrupted sutures of 5-0 PDS II absorbable suture (Ethicon). Reflex 7 mm wound clips (Fine Science) were then used for wound closure. A 1:1 mixture of New-Skin Liquid Bandage and powdered metronidazole was then applied to the wound clips to assist in wound healing and prevent wound dehiscence. Fluid loss was replaced using 5% dextrose in 0.45% saline. For pain management, buprenorphine was administered at

0.1 mg/kg intraperitoneally every eight hours for three days post-operation. Wound clips were removed two weeks after the surgery.

3.2.3 Microcomputed Tomography (μCT)

Images of skeletal tissues were obtained using a Skyscan 1172 desktop imaging system (Skyscan-Bruker). Scans were performed with an isotropic voxel size of 10 μm at 65 kV and 200 μA through a 0.5mm aluminum filter. Femoral trabecular parameters were measured in a 200 μm region of interest located approximately 50 μm proximal to the distal growth plate. Femoral cortical parameters were measured within a 500 μm region of interest centered at the midshaft. All bone analysis was performed in accordance with the American Society of Bone and Mineral Research (ASBMR) recommended parameters [178].

3.2.4 Radiological Analysis

Radiographs were acquired on the MX-20 Specimen Radiography System (Faxitron). Radio tube voltage was set at 26 kV with a 15 sec exposure time. Image analysis was performed using FIJI [180].

3.2.5 Histology

Harvested tissues were fixed overnight at 4° C in 10% neutral-buffered formalin. Samples were then washed and placed into a decalcification solution of 14% EDTA for two weeks. After decalcification, samples were paraffin embedded and cut at five-micron thick sections. Slides were stained in hematoxylin and eosin staining solution according to standard procedures.

3.2.6 Torsional Mechanical Testing

In order to determine the mechanical properties of healed fractures after the treatment protocol, torsional mechanical testing was employed using an Electroforce 3100 System (Bose) with an attached torsional torque apparatus. The each end of the femur was potted into molten woods metal and allowed to set in molds. Upon hardening, the femur assembly was placed into fixtures attached to the torsional torque apparatus. The sample was then advanced at three degrees per second until mechanical failure was achieved.

3.3 Results

3.3.1 ACVR2B/Fc Administration Increases Muscle Mass

Soluble activin receptor administration induces a direct increase in skeletal muscle mass through muscle hypertrophy and hyperplasia. This anabolism is the result of systemic myostatin inhibition. As expected, ACVR2B/Fc treated mice gained muscle mass, which resulted an increase in body weight (18.1 ± 0.7%) (Figure 3.1). In addition, the injured quadriceps gained 40.9 ± 5.0% in wet weight while the uninjured quadriceps gained 19.1 ± 0.1% with ACVR2B/Fc administration.

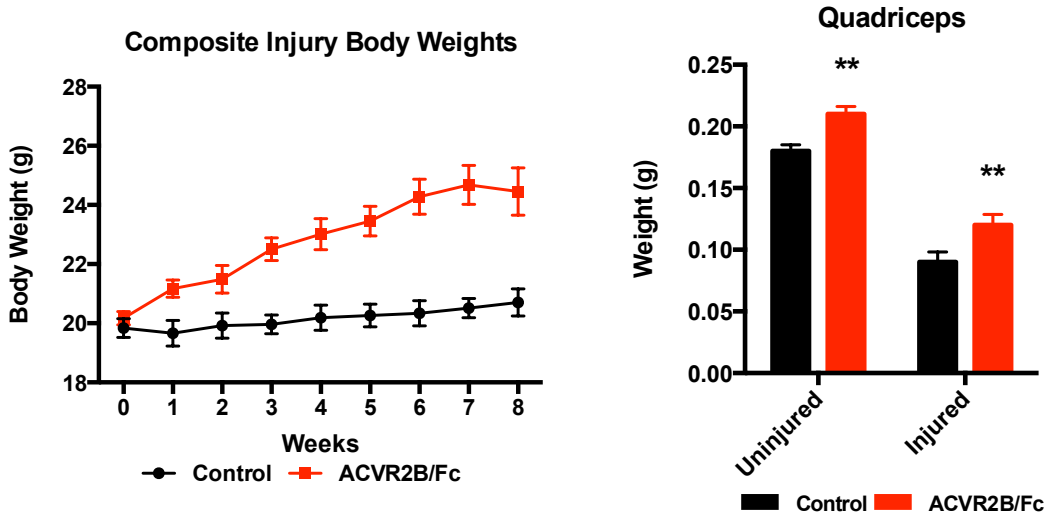

Figure 3.1: Weights with ACVR2B/Fc Treatment in Composite Injury Model

Body weights of vehicle and ACVR2B/Fc treated mice (left). Quadriceps weights of injured and uninjured limbs in vehicle and ACVR2B/Fc treated mice (right).

3.3.2 ACVR2B/Fc Administration Enhances Callus Formation

In addition to gains in muscle mass, ACVR2B/Fc treatment also enhanced callus formation at the fracture site. With ACVR2B/Fc administration, the callus bone volume was drastically enhanced (112 ± 19.7%) as compared to the vehicle treated mice. Additionally, the total mineral content was increased by 109 ± 19.8% with ACVR2B/Fc treatment. The tissue mineral density, however, was unchanged with soluble activin receptor treatment. (Figure 3.2)

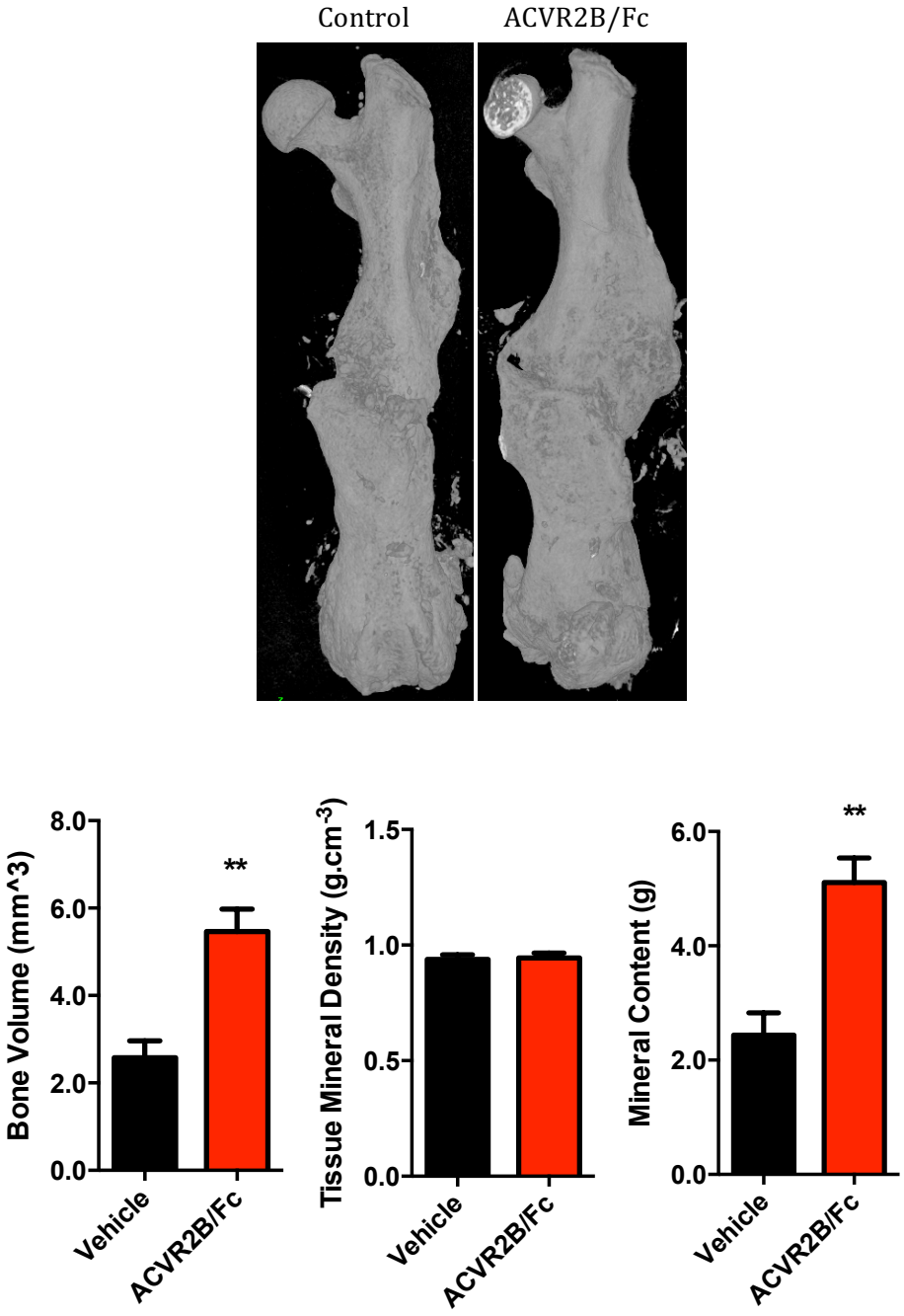

Figure 3.2: Fracture Callus Analysis

Representative images of vehicle and ACVR2B/Fc treated eight weeks post-operation (top). Callus bone volume, tissue mineral density, and total mineral content of injured femurs (bottom).

3.3.3 ACVR2B/Fc Treatment Does Not Affect Fracture Mechanical Properties

Despite the tremendous increase in callus bone volume, upon torsion mechanical testing soluble activin receptor treatment did not enhance the mechanical properties of the fractured femur. The maximum torque, stiffness, and ultimate energy were not significantly changed with ACVR2B/Fc administration (Figure 3.3).

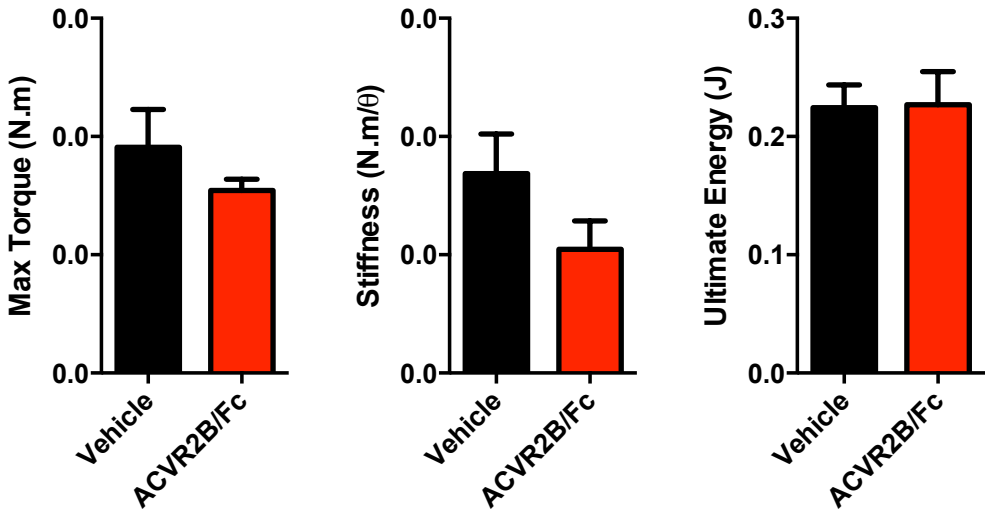

Figure 3.3: Torsional Mechanical Testing Analysis

Maximum torque (left), stiffness (center), and ultimate energy (right) of fracture calluses of vehicle and ACVR2B/Fc treated mice.

3.3.4 ACVR2B/Fc Treatment Enhances Volumetric Muscle Healing

Similar to the anabolic effects seen in the bone, muscle healing was drastically enhanced with systemic activin blockade. Histological sections of the injured quadriceps demonstrate dramatic improvement in muscle mass, reduction of muscle fibrosis, and minimization of fatty infiltrate (Figure 3.4). This muscle hypertrophy and hyperplasia led to an increased number of regenerating myofibers with centrally located nuclei in the ACVR2B/Fc treated mice.

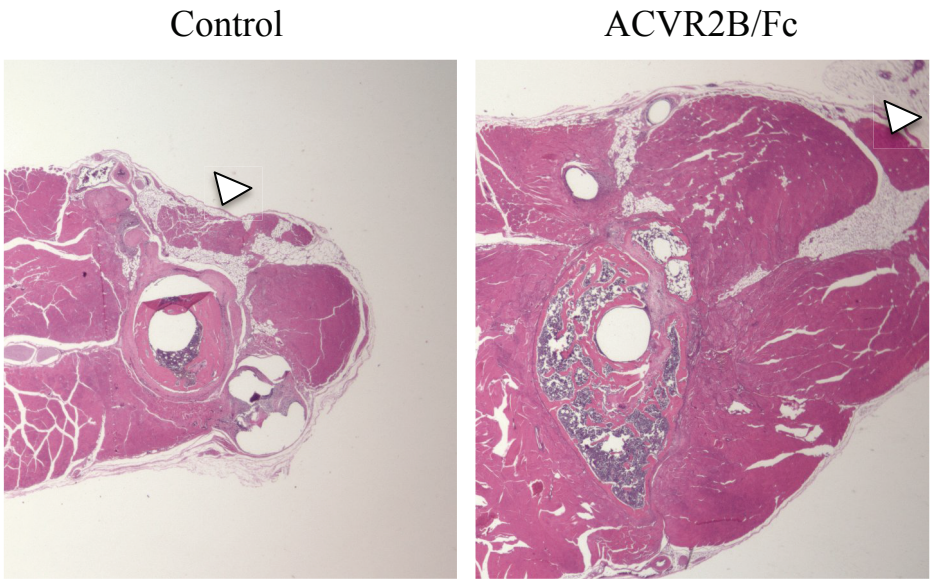

Figure 3.4: Histological Sections of Injured Muscle and Facture Callus

Hematoxylin and eosin stained sections through fracture callus and injured muscle from vehicle (left) and ACVR2B/Fc (right) treated mice.

3.4 Discussion

Activin signaling blockade is clearly a potent anabolic for both muscle and bone tissues, even in the setting of composite musculoskeletal injury. Muscle healing as observed by histology is dramatically improved with ACVR2B/Fc treatment with increased muscle mass, reduced fibrosis, and reduced fat infiltrate. These results are primarily due to ACVR2B/Fc's strong affinity for mysotatin. In addition to muscle anabolism, ACVR2B/Fc also dramatically affected the fracture callus. The bone volume (112 ± 19.7%) and total mineral content (109 ± 19.8%) were significantly increased with soluble activin receptor treatment while the tissue mineral density was unaffected, indicating that there was no change in inherent tissue material properties. However, when subjecting the fractured femurs to torsional testing, it was clear that ACVR2B/Fc treatment did not enhance the mechanical properties of the calluses despite the dramatic increases in bone volume. When looking at histological sections through the fracture callus, there was abundant bone formation but the mineralized tissue was disorganized, woven bone and had not yet fully consolidated. Additionally, histological sections demonstrated larger and more abundant areas of cartilage with ACVR2B/Fc treatment, indicating that the fracture callus had not fully mineralized and matured – likely attributing to the reduced mechanical properties.

Ultimately, these studies demonstrate that soluble activin receptor treatment enhances callus bone volume and mineral content as well as improves volumetric muscle deficit healing in the setting of composite musculoskeletal injury. The dual nature of this

potential treatment lends itself to possible synergy in healing between the bone and muscle compartments. Despite these promising preliminary results, further investigation into the mechanism of ACVR2B/Fc's anabolic effects and effective treatment protocol will be needed to determine if soluble activin receptor administration is a viable therapeutic option in the setting of composite muscle and bone injury.

Chapter 4

4 Conclusions and Future Directions

Through the studies discussed in this dissertation, it is clear that activin receptor signaling is an important regulator of both bone and muscle homeostasis. Importantly, activin signaling is a negative regulator of skeletal tissues and acts in a manner similar to that exhibited in muscle.

The experiments discussed demonstrate that the activin signaling components are present and functional within the osteoblasts. Additionally, disruption of activin receptor signaling, using either pharmacological or genetic approaches, enhances osteoblast function and differentiation both *in vitro* and *in vivo*. Through these analyses, it was determined that activin receptor type IIA (ACVR2A) is the primary signaling mediator in osteoblasts and disruption of this receptor leads to increased bone volume and enhanced mechanical properties. The anabolic effect of ACVR2A disruption, however, cannot fully account for the full changes observed with systemic soluble activin receptor administration and represents approximately half of the total gains in bone volume. The remaining anabolic fraction could be explained by a number of undefined factors, including hormonal changes, mechanical stimuli, and non-osteoblast effects. Further investigation into these possibilities is necessary to fully understand the anabolic effects of systemic soluble activin receptor treatment.

Additional studies that target the activin type II receptors in other musculoskeletal cell types, such as osteoclasts and myoblasts, would also provide valuable information into the role of activin signaling within the musculoskeletal functional unit. These studies would provide the relative contributions of each cell type to the total anabolic effect seen with systemic activin signaling blockade.

Because pharmacological inhibition of activin signaling is known to induce bone and muscle increases, a logical extension would be to use soluble activin receptor administration to improve composite musculoskeletal injury healing. In the setting of composite injury, systemic inhibition of activin signaling via ACVR2B/Fc demonstrated dramatic increases in callus bone volume and mineral content. These changes, however, did not translate into enhanced mechanical strength of the fractured femurs, suggesting that the quality of the bone callus was diminished. Upon further investigation, it became clear that the accelerated bone formation with ACVR2B/Fc treatment was not able to fully consolidate into hardy, lamellar bone in the post-surgical period. Despite these results, soluble activin receptor treatment, however, did have profound effects in muscle healing. ACVR2B/Fc administration significantly increased muscle size, reduced injury fibrosis, and reduced fatty infiltrate into the injury site. Overall, further investigation into the mechanisms and dynamics of ACVR2B/Fc treatment in bone and muscle healing will be necessary to determine if ACVR2B/Fc is a vial therapeutic option for composite musculoskeletal injuries.

Additional soluble activin receptor studies will be also necessary to determine the appropriate treatment dosing regimen to both enhance fracture callus development as

well as improve the mechanical properties of the healed bone. A more precise treatment schedule may improve the callus quality and allow proper bone consolidation, which is necessary to yield a mechanical advantage. Other additional studies in applying the composite injury model to transgenic mouse models, such as the osteoblast-specific ACVR2A/2B knockout, would also yield important information on the role of activin receptor signaling in the context of bone regeneration. These cell-specific transgenic mouse models are powerful tools to dissect the major mechanisms of composite healing, and the proposed soluble activin receptor treatment studies are simply the beginning of our understanding of how activin signaling affects composite muscle and bone regeneration.

In conclusion, the results of the studies discussed in this dissertation demonstrate that activin signaling in osteoblasts, which is primarily mediated through ACVR2A, acts a negative regulator of bone homeostasis, and activin-targeted biologics are potential dual anabolic agents that may have promise in the treatment of composite musculoskeletal injuries.

References

1. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan, *Principles of bone biology*. 2nd ed. 2002, San Diego: Academic Press.
2. Rosen, C.J. and American Society for Bone and Mineral Research., *Primer on the metabolic bone diseases and disorders of mineral metabolism*. 8th ed. 2013, Ames, Iowa: Wiley-Blackwell. xxvi, 1078 p.
3. Wnek, G.E. and G.L. Bowlin, *Encyclopedia of biomaterials and biomedical engineering*. 2nd ed. 2008, New York: Informa Healthcare USA.
4. Mackie, E.J., L. Tatarczuch, and M. Mirams, *The skeleton: a multi-functional complex organ: the growth plate chondrocyte and endochondral ossification.* J Endocrinol, 2011. **211**(2): p. 109-21.
5. Kozhemyakina, E., A.B. Lassar, and E. Zelzer, *A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation.* Development, 2015. **142**(5): p. 817-31.
6. Berendsen, A.D. and B.R. Olsen, *Bone development.* Bone, 2015. **80**: p. 14-8.
7. Mackie, E.J., et al., *Endochondral ossification: how cartilage is converted into bone in the developing skeleton.* Int J Biochem Cell Biol, 2008. **40**(1): p. 46-62.
8. Long, F. and D.M. Ornitz, *Development of the endochondral skeleton.* Cold Spring Harb Perspect Biol, 2013. **5**(1): p. a008334.
9. Kronenberg, H.M., *Developmental regulation of the growth plate.* Nature, 2003. **423**(6937): p. 332-6.

10. Bilezikian, J.P., L.G. Raisz, and T.J. Martin, *Principles of bone biology*. 3rd ed. 2008, San Diego, Calif.: Academic Press/Elsevier.

11. Seibel, M.J., S.P. Robins, and J.P. Bilezikian, *Dynamics of bone and cartilage metabolism*. 2nd ed. 2006, San Diego: Academic Press. xix, 919 p., 6 p. of plates.

12. Bonewald, L.F., *The amazing osteocyte.* J Bone Miner Res, 2011. **26**(2): p. 229-38.

13. Franz-Odendaal, T.A., B.K. Hall, and P.E. Witten, *Buried alive: how osteoblasts become osteocytes.* Dev Dyn, 2006. **235**(1): p. 176-90.

14. Boyle, W.J., W.S. Simonet, and D.L. Lacey, *Osteoclast differentiation and activation.* Nature, 2003. **423**(6937): p. 337-42.

15. Teitelbaum, S.L., *Bone resorption by osteoclasts.* Science, 2000. **289**(5484): p. 1504-8.

16. Tzioupis, C. and P.V. Giannoudis, *Prevalence of long-bone non-unions.* Injury, 2007. **38 Suppl 2**: p. S3-9.

17. Einhorn, T.A. and L.C. Gerstenfeld, *Fracture healing: mechanisms and interventions.* Nat Rev Rheumatol, 2015. **11**(1): p. 45-54.

18. Bolander, M.E., *Regulation of fracture repair by growth factors.* Proc Soc Exp Biol Med, 1992. **200**(2): p. 165-70.

19. Vortkamp, A., et al., *Recapitulation of signals regulating embryonic bone formation during postnatal growth and in fracture repair.* Mech Dev, 1998. **71**(1-2): p. 65-76.

20. Matsumoto, T., et al., *Fracture induced mobilization and incorporation of bone marrow-derived endothelial progenitor cells for bone healing.* J Cell Physiol, 2008. **215**(1): p. 234-42.

21. Henrotin, Y., *Muscle: a source of progenitor cells for bone fracture healing.* BMC Med, 2011. **9**: p. 136.

22. Zhang, X., et al., *Periosteal stem cells are essential for bone revitalization and repair.* J Musculoskelet Neuronal Interact, 2005. **5**(4): p. 360-2.

23. Granero-Molto, F., et al., *Regenerative effects of transplanted mesenchymal stem cells in fracture healing.* Stem Cells, 2009. **27**(8): p. 1887-98.

24. Einhorn, T.A., *The cell and molecular biology of fracture healing.* Clin Orthop Relat Res, 1998(355 Suppl): p. S7-21.

25. Marsell, R. and T.A. Einhorn, *The biology of fracture healing.* Injury, 2011. **42**(6): p. 551-5.

26. Jackman, R.W. and S.C. Kandarian, *The molecular basis of skeletal muscle atrophy.* Am J Physiol Cell Physiol, 2004. **287**(4): p. C834-43.

27. Grefte, S., et al., *Skeletal muscle development and regeneration.* Stem Cells Dev, 2007. **16**(5): p. 857-68.

28. Braun, T. and M. Gautel, *Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis.* Nat Rev Mol Cell Biol, 2011. **12**(6): p. 349-61.

29. Buckingham, M., et al., *The formation of skeletal muscle: from somite to limb.* J Anat, 2003. **202**(1): p. 59-68.

30. Walsh, M.C., G.R. Hunter, and M.B. Livingstone, *Sarcopenia in premenopausal and postmenopausal women with osteopenia, osteoporosis and normal bone mineral density.* Osteoporos Int, 2006. **17**(1): p. 61-7.

31. Tarantino, U., et al., *Sarcopenia and fragility fractures: molecular and clinical evidence of the bone-muscle interaction.* J Bone Joint Surg Am, 2015. **97**(5): p. 429-37.

32. Iolascon, G., et al., *Sarcopenia in women with vertebral fragility fractures.* Aging Clin Exp Res, 2013. **25 Suppl 1**: p. S129-31.

33. Cederholm, T., A.J. Cruz-Jentoft, and S. Maggi, *Sarcopenia and fragility fractures.* Eur J Phys Rehabil Med, 2013. **49**(1): p. 111-7.

34. Di Monaco, M., et al., *Prevalence of sarcopenia and its association with osteoporosis in 313 older women following a hip fracture.* Arch Gerontol Geriatr, 2011. **52**(1): p. 71-4.

35. Qin, W., W.A. Bauman, and C. Cardozo, *Bone and muscle loss after spinal cord injury: organ interactions.* Ann N Y Acad Sci, 2010. **1211**: p. 66-84.

36. Dudley-Javoroski, S. and R.K. Shields, *Muscle and bone plasticity after spinal cord injury: review of adaptations to disuse and to electrical muscle stimulation.* J Rehabil Res Dev, 2008. **45**(2): p. 283-96.

37. Qin, W., et al., *The central nervous system (CNS)-independent anti-bone-resorptive activity of muscle contraction and the underlying molecular and cellular signatures.* J Biol Chem, 2013. **288**(19): p. 13511-21.

38. Turner, C.H., *Three rules for bone adaptation to mechanical stimuli.* Bone, 1998. **23**(5): p. 399-407.

39. Frost, H.M., *Defining osteopenias and osteoporoses: another view (with insights from a new paradigm).* Bone, 1997. **20**(5): p. 385-91.

40. Frost, H.M., *Bone "mass" and the "mechanostat": a proposal.* Anat Rec, 1987. **219**(1): p. 1-9.

41. Frost, H.M., *Bone's mechanostat: a 2003 update.* Anat Rec A Discov Mol Cell Evol Biol, 2003. **275**(2): p. 1081-101.

42. Frost, H.M. and E. Schonau, *The "muscle-bone unit" in children and adolescents: a 2000 overview.* J Pediatr Endocrinol Metab, 2000. **13**(6): p. 571-90.

43. Egan, E., et al., *Bone mineral density among female sports participants.* Bone, 2006. **38**(2): p. 227-33.

44. Krustrup, P., et al., *Long-term musculoskeletal and cardiac health effects of recreational football and running for premenopausal women.* Scand J Med Sci Sports, 2010. **20 Suppl 1**: p. 58-71.

45. Seabra, A., et al., *Muscle strength and soccer practice as major determinants of bone mineral density in adolescents.* Joint Bone Spine, 2012. **79**(4): p. 403-8.

46. Cassell, C., M. Benedict, and B. Specker, *Bone mineral density in elite 7- to 9-yr-old female gymnasts and swimmers.* Med Sci Sports Exerc, 1996. **28**(10): p. 1243-6.

47. Emslander, H.C., et al., *Bone mass and muscle strength in female college athletes (runners and swimmers).* Mayo Clin Proc, 1998. **73**(12): p. 1151-60.

48. Sharir, A., et al., *Muscle force regulates bone shaping for optimal load-bearing capacity during embryogenesis.* Development, 2011. **138**(15): p. 3247-59.

49. Murray, P.D. and D.B. Drachman, *The role of movement in the development of joints and related structures: the head and neck in the chick embryo.* J Embryol Exp Morphol, 1969. **22**(3): p. 349-71.

50. von Maltzahn, J., et al., *Wnt signaling in myogenesis.* Trends Cell Biol, 2012. **22**(11): p. 602-9.

51. Cisternas, P., et al., *Wnt signaling in skeletal muscle dynamics: myogenesis, neuromuscular synapse and fibrosis.* Mol Neurobiol, 2014. **49**(1): p. 574-89.

52. Baron, R. and M. Kneissel, *WNT signaling in bone homeostasis and disease: from human mutations to treatments.* Nat Med, 2013. **19**(2): p. 179-92.

53. Locatelli, V. and V.E. Bianchi, *Effect of GH/IGF-1 on Bone Metabolism and Osteoporsosis.* Int J Endocrinol, 2014. **2014**: p. 235060.

54. Singhal, V., et al., *Osteoblast-restricted Disruption of the Growth Hormone Receptor in Mice Results in Sexually Dimorphic Skeletal Phenotypes.* Bone Res, 2013. **1**(1): p. 85-97.

55. Mavalli, M.D., et al., *Distinct growth hormone receptor signaling modes regulate skeletal muscle development and insulin sensitivity in mice.* J Clin Invest, 2010. **120**(11): p. 4007-20.

56. Tsivitse, S., *Notch and Wnt signaling, physiological stimuli and postnatal myogenesis.* Int J Biol Sci, 2010. **6**(3): p. 268-81.

57. Vasyutina, E., D.C. Lenhard, and C. Birchmeier, *Notch function in myogenesis.* Cell Cycle, 2007. **6**(12): p. 1451-4.

58. Mead, T.J. and K.E. Yutzey, *Notch signaling and the developing skeleton.* Adv Exp Med Biol, 2012. **727**: p. 114-30.

59. Hirukawa, K., et al., *Effect of tensile force on the expression of IGF-I and IGF-I receptor in the organ-cultured rat cranial suture.* Arch Oral Biol, 2005. **50**(3): p. 367-72.

60. Sheng, M.H., K.H. Lau, and D.J. Baylink, *Role of Osteocyte-derived Insulin-Like Growth Factor I in Developmental Growth, Modeling, Remodeling, and Regeneration of the Bone.* J Bone Metab, 2014. **21**(1): p. 41-54.

61. Pedersen, B.K. and M.A. Febbraio, *Muscles, exercise and obesity: skeletal muscle as a secretory organ.* Nat Rev Endocrinol, 2012. **8**(8): p. 457-65.

62. van Driel, M., H.A. Pols, and J.P. van Leeuwen, *Osteoblast differentiation and control by vitamin D and vitamin D metabolites.* Curr Pharm Des, 2004. **10**(21): p. 2535-55.

63. van Driel, M., et al., *Evidence for auto/paracrine actions of vitamin D in bone: 1alpha-hydroxylase expression and activity in human bone cells.* FASEB J, 2006. **20**(13): p. 2417-9.

64. Atkins, G.J., et al., *Metabolism of vitamin D3 in human osteoblasts: evidence for autocrine and paracrine activities of 1 alpha,25-dihydroxyvitamin D3.* Bone, 2007. **40**(6): p. 1517-28.

65. Howard, G.A., et al., *Human bone cells in culture metabolize 25-hydroxyvitamin D3 to 1,25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3.* J Biol Chem, 1981. **256**(15): p. 7738-40.

66. Ichikawa, F., et al., *Mouse primary osteoblasts express vitamin D3 25-hydroxylase mRNA and convert 1 alpha-hydroxyvitamin D3 into 1 alpha,25-dihydroxyvitamin D3.* Bone, 1995. **16**(1): p. 129-35.

67. Panda, D.K., et al., *25-hydroxyvitamin D 1alpha-hydroxylase: structure of the mouse gene, chromosomal assignment, and developmental expression.* J Bone Miner Res, 2001. **16**(1): p. 46-56.

68. Endo, I., et al., *Deletion of vitamin D receptor gene in mice results in abnormal skeletal muscle development with deregulated expression of myoregulatory transcription factors.* Endocrinology, 2003. **144**(12): p. 5138-44.

69. Girgis, C.M., et al., *The vitamin D receptor (VDR) is expressed in skeletal muscle of male mice and modulates 25-hydroxyvitamin D (25OHD) uptake in myofibers.* Endocrinology, 2014. **155**(9): p. 3227-37.

70. Bren-Mattison, Y., M. Hausburg, and B.B. Olwin, *Growth of limb muscle is dependent on skeletal-derived Indian hedgehog.* Dev Biol, 2011. **356**(2): p. 486-95.

71. Guo, X. and X.F. Wang, *Signaling cross-talk between TGF-beta/BMP and other pathways.* Cell Res, 2009. **19**(1): p. 71-88.

72. Shi, Y. and J. Massague, *Mechanisms of TGF-beta signaling from cell membrane to the nucleus.* Cell, 2003. **113**(6): p. 685-700.

73. Massague, J., *TGFbeta signalling in context.* Nat Rev Mol Cell Biol, 2012. **13**(10): p. 616-30.

74. Derynck, R. and K.h. Miyazono, *The TGF-[beta] family*. Cold Spring Harbor monograph series. 2007, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. p.

75. Chen, G., C. Deng, and Y.P. Li, *TGF-beta and BMP signaling in osteoblast differentiation and bone formation.* Int J Biol Sci, 2012. **8**(2): p. 272-88.

76. Beddington, R.S. and E.J. Robertson, *Axis development and early asymmetry in mammals.* Cell, 1999. **96**(2): p. 195-209.

77. Siegel, P.M. and J. Massague, *Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer.* Nat Rev Cancer, 2003. **3**(11): p. 807-21.

78. Derynck, R. and R.J. Akhurst, *Differentiation plasticity regulated by TGF-beta family proteins in development and disease.* Nat Cell Biol, 2007. **9**(9): p. 1000-4.

79. Shi, M., et al., *Latent TGF-beta structure and activation.* Nature, 2011. **474**(7351): p. 343-9.

80. Yu, Q. and I. Stamenkovic, *Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis.* Genes Dev, 2000. **14**(2): p. 163-76.

81. Lyons, R.M., J. Keski-Oja, and H.L. Moses, *Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium.* J Cell Biol, 1988. **106**(5): p. 1659-65.

82. Barcellos-Hoff, M.H. and T.A. Dix, *Redox-mediated activation of latent transforming growth factor-beta 1.* Mol Endocrinol, 1996. **10**(9): p. 1077-83.

83. Munger, J.S., et al., *The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis.* Cell, 1999. **96**(3): p. 319-28.

84. Wipff, P.J. and B. Hinz, *Integrins and the activation of latent transforming growth factor beta1 - an intimate relationship.* Eur J Cell Biol, 2008. **87**(8-9): p. 601-15.

85. Wan, M. and X. Cao, *BMP signaling in skeletal development.* Biochem Biophys Res Commun, 2005. **328**(3): p. 651-7.

86. Liu, A. and L.A. Niswander, *Bone morphogenetic protein signalling and vertebrate nervous system development.* Nat Rev Neurosci, 2005. **6**(12): p. 945-54.

87. Lupo, G., W.A. Harris, and K.E. Lewis, *Mechanisms of ventral patterning in the vertebrate nervous system.* Nat Rev Neurosci, 2006. **7**(2): p. 103-14.

88. Vale, W., et al., *Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid.* Nature, 1986. **321**(6072): p. 776-9.

89. Vale, W., et al., *Activins and inhibins and their signaling.* Ann N Y Acad Sci, 2004. **1038**: p. 142-7.

90. McPherron, A.C., A.M. Lawler, and S.J. Lee, *Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member.* Nature, 1997. **387**(6628): p. 83-90.

91. Lee, S.J., et al., *Regulation of muscle growth by multiple ligands signaling through activin type II receptors.* Proc Natl Acad Sci U S A, 2005. **102**(50): p. 18117-22.

92. Lee, S.J., et al., *Regulation of muscle mass by follistatin and activins.* Mol Endocrinol, 2010. **24**(10): p. 1998-2008.

93. Chen, J.L., et al., *Development of novel activin-targeted therapeutics.* Mol Ther, 2015. **23**(3): p. 434-44.

94. Morine, K.J., et al., *Activin IIB receptor blockade attenuates dystrophic pathology in a mouse model of Duchenne muscular dystrophy.* Muscle Nerve, 2010. **42**(5): p. 722-30.

95. Attie, K.M., et al., *A single ascending-dose study of muscle regulator ACE-031 in healthy volunteers.* Muscle Nerve, 2013. **47**(3): p. 416-23.

96. Lee, S.J. and D.J. Glass, *Treating cancer cachexia to treat cancer.* Skelet Muscle, 2011. **1**(1): p. 2.

97. Roberts, V.J., P.E. Sawchenko, and W. Vale, *Expression of inhibin/activin subunit messenger ribonucleic acids during rat embryogenesis.* Endocrinology, 1991. **128**(6): p. 3122-9.

98. Feijen, A., M.J. Goumans, and A.J. van den Eijnden-van Raaij, *Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins.* Development, 1994. **120**(12): p. 3621-37.

99. Merino, R., et al., *Control of digit formation by activin signalling.* Development, 1999. **126**(10): p. 2161-70.

100. Shuto, T., et al., *Osteoblasts express types I and II activin receptors during early intramembranous and endochondral bone formation.* J Bone Miner Res, 1997. **12**(3): p. 403-11.

101. Matzuk, M.M., T.R. Kumar, and A. Bradley, *Different phenotypes for mice deficient in either activins or activin receptor type II.* Nature, 1995. **374**(6520): p. 356-60.

102. Matzuk, M.M., et al., *Functional analysis of activins during mammalian development.* Nature, 1995. **374**(6520): p. 354-6.

103. Oh, S.P., et al., *Activin type IIA and IIB receptors mediate Gdf11 signaling in axial vertebral patterning.* Genes Dev, 2002. **16**(21): p. 2749-54.

104. Centrella, M., T.L. McCarthy, and E. Canalis, *Activin-A binding and biochemical effects in osteoblast-enriched cultures from fetal-rat parietal bone.* Mol Cell Biol, 1991. **11**(1): p. 250-8.

105. Gaddy-Kurten, D., et al., *Inhibin suppresses and activin stimulates osteoblastogenesis and osteoclastogenesis in murine bone marrow cultures.* Endocrinology, 2002. **143**(1): p. 74-83.

106. Centrella, M., T.L. McCarthy, and E. Canalis, *Glucocorticoid regulation of transforming growth factor beta 1 activity and binding in osteoblast-enriched cultures from fetal rat bone.* Mol Cell Biol, 1991. **11**(9): p. 4490-6.

107. Sakai, R., K. Miwa, and Y. Eto, *Local administration of activin promotes fracture healing in the rat fibula fracture model.* Bone, 1999. **25**(2): p. 191-6.

108. Oue, Y., et al., *Effect of local injection of activin A on bone formation in newborn rats.* Bone, 1994. **15**(3): p. 361-6.

109. Eijken, M., et al., *The activin A-follistatin system: potent regulator of human extracellular matrix mineralization.* FASEB J, 2007. **21**(11): p. 2949-60.

110. Woeckel, V.J., et al., *1alpha,25-dihydroxyvitamin D3 stimulates activin A production to fine-tune osteoblast-induced mineralization.* J Cell Physiol, 2013. **228**(11): p. 2167-74.

111. Fajardo, R.J., et al., *Treatment with a soluble receptor for activin improves bone mass and structure in the axial and appendicular skeleton of female cynomolgus macaques (Macaca fascicularis).* Bone, 2010. **46**(1): p. 64-71.

112. Lotinun, S., et al., *A soluble activin receptor Type IIA fusion protein (ACE-011) increases bone mass via a dual anabolic-antiresorptive effect in Cynomolgus monkeys.* Bone, 2010. **46**(4): p. 1082-8.

113. Ruckle, J., et al., *Single-dose, randomized, double-blind, placebo-controlled study of ACE-011 (ActRIIA-IgG1) in postmenopausal women.* J Bone Miner Res, 2009. **24**(4): p. 744-52.

114. Gustilo, R.B., R.M. Mendoza, and D.N. Williams, *Problems in the management of type III (severe) open fractures: a new classification of type III open fractures.* J Trauma, 1984. **24**(8): p. 742-6.

115. Chan, J.K., et al., *Soft-tissue reconstruction of open fractures of the lower limb: muscle versus fasciocutaneous flaps.* Plast Reconstr Surg, 2012. **130**(2): p. 284e-295e.

116. Harry, L.E., et al., *Comparison of the healing of open tibial fractures covered with either muscle or fasciocutaneous tissue in a murine model.* J Orthop Res, 2008. **26**(9): p. 1238-44.

117. Harry, L.E., et al., *Comparison of the vascularity of fasciocutaneous tissue and muscle for coverage of open tibial fractures.* Plast Reconstr Surg, 2009. **124**(4): p. 1211-9.

118. Yazar, S., et al., *Outcome comparison between free muscle and free fasciocutaneous flaps for reconstruction of distal third and ankle traumatic open tibial fractures.* Plast Reconstr Surg, 2006. **117**(7): p. 2468-75; discussion 2476-7.

119. Davis, K.M., et al., *Muscle-bone interactions during fracture healing.* J Musculoskelet Neuronal Interact, 2015. **15**(1): p. 1-9.

120. Riddle, R.C., et al., *Role of hypoxia-inducible factor-1alpha in angiogenic-osteogenic coupling.* J Mol Med (Berl), 2009. **87**(6): p. 583-90.

121. Wang, Y., et al., *The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development.* J Clin Invest, 2007. **117**(6): p. 1616-26.

122. Kusumbe, A.P., S.K. Ramasamy, and R.H. Adams, *Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone.* Nature, 2014. **507**(7492): p. 323-8.

123. Gerber, H.P., et al., *VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation.* Nat Med, 1999. **5**(6): p. 623-8.

124. Rhinelander, F.W., *Tibial blood supply in relation to fracture healing.* Clin Orthop Relat Res, 1974(105): p. 34-81.

125. Laroche, M., *Intraosseous circulation from physiology to disease.* Joint Bone Spine, 2002. **69**(3): p. 262-9.

126. Lu, C., et al., *Ischemia leads to delayed union during fracture healing: a mouse model.* J Orthop Res, 2007. **25**(1): p. 51-61.

127. Richards, R.R. and E.H. Schemitsch, *Effect of muscle flap coverage on bone blood flow following devascularization of a segment of tibia: an experimental investigation in the dog.* J Orthop Res, 1989. **7**(4): p. 550-8.

128. Richards, R.R., et al., *A comparison of the effects of skin coverage and muscle flap coverage on the early strength of union at the site of osteotomy after devascularization of a segment of canine tibia.* J Bone Joint Surg Am, 1991. **73**(9): p. 1323-30.

129. Richards, R.R., et al., *The influence of muscle flap coverage on the repair of devascularized tibial cortex: an experimental investigation in the dog.* Plast Reconstr Surg, 1987. **79**(6): p. 946-58.

130. Liu, R., A. Schindeler, and D.G. Little, *The potential role of muscle in bone repair.* J Musculoskelet Neuronal Interact, 2010. **10**(1): p. 71-6.

131. Stein, H., et al., *The muscle bed--a crucial factor for fracture healing: a physiological concept.* Orthopedics, 2002. **25**(12): p. 1379-83.

132. DiGirolamo, D.J., D.P. Kiel, and K.A. Esser, *Bone and skeletal muscle: neighbors with close ties.* J Bone Miner Res, 2013. **28**(7): p. 1509-18.

133. Olsen, B.R., A.M. Reginato, and W. Wang, *Bone development.* Annu.Rev.Cell Dev.Biol., 2000. **16**: p. 191-220.

134. Jilka, R.L., *Molecular and cellular mechanisms of the anabolic effect of intermittent PTH.* Bone, 2007. **40**(6): p. 1434-1446.

135. Kablar, B., et al., *MyoD and Myf-5 define the specification of musculature of distinct embryonic origin.* Biochem.Cell Biol., 1998. **76**(6): p. 1079-1091.

136. Hasty, P., et al., *Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene.* Nature, 1993. **364**(6437): p. 501-506.

137. Nabeshima, Y., et al., *Myogenin gene disruption results in perinatal lethality because of severe muscle defect.* Nature, 1993. **364**(6437): p. 532-535.

138. Allen, R.E. and L.L. Rankin, *Regulation of satellite cells during skeletal muscle growth and development.* Proc Soc Exp Biol Med, 1990. **194**(2): p. 81-6.

139. Mayo, K.E., *Inhibin and activin Molecular aspects of regulation and function.* Trends Endocrinol Metab, 1994. **5**(10): p. 407-15.

140. Kingsley, D.M., *The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms.* Genes Dev, 1994. **8**(2): p. 133-46.

141. Vale, W., *The Inhibin/Activin Family of Hormones and Growth Factors*. Peptide Growth Factors and their Receptors, ed. M. Sporn and A. Roberts. 1990, Berlin: Springer-Verlag.

142. McCullagh, D.R., *Dual Endocrine Activity of the Testes.* Science, 1932. **76**(1957): p. 19-20.

143. Vale, W., et al., *Chemical and biological characterization of the inhibin family of protein hormones.* Recent Prog Horm Res, 1988. **44**: p. 1-34.

144. Ling, N., et al., *Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin.* Nature, 1986. **321**(6072): p. 779-82.

145. Krummen, L.A., et al., *Identification and characterization of binding proteins for inhibin and activin in human serum and follicular fluids.* Endocrinology, 1993. **132**(1): p. 431-43.

146. Hill, C.S., *Signalling to the nucleus by members of the transforming growth factor-beta (TGF-beta) superfamily.* Cell Signal, 1996. **8**(8): p. 533-44.

147. Attisano, L., et al., *TGF-beta receptors and actions.* Biochim Biophys Acta, 1994. **1222**(1): p. 71-80.

148. ten Dijke, P., K. Miyazono, and C.H. Heldin, *Signaling via hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors.* Curr Opin Cell Biol, 1996. **8**(2): p. 139-45.

149. McCarthy, S.A. and R. Bicknell, *Inhibition of vascular endothelial cell growth by activin-A.* J Biol Chem, 1993. **268**(31): p. 23066-71.

150. Thomas, M., et al., *Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation.* J Biol Chem, 2000. **275**(51): p. 40235-43.

151. Joulia, D., et al., *Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin.* Exp Cell Res, 2003. **286**(2): p. 263-75.

152. Langley, B., et al., *Myostatin inhibits myoblast differentiation by down-regulating MyoD expression.* J Biol Chem, 2002. **277**(51): p. 49831-40.

153. Bogdanovich, S., et al., *Functional improvement of dystrophic muscle by myostatin blockade.* Nature, 2002. **420**(6914): p. 418-21.

154. Whittemore, L.A., et al., *Inhibition of myostatin in adult mice increases skeletal muscle mass and strength.* Biochem Biophys Res Commun, 2003. **300**(4): p. 965-71.

155. Wolfman, N.M., et al., *Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15842-6.

156. Blain, H., et al., *Appendicular skeletal muscle mass is the strongest independent factor associated with femoral neck bone mineral density in adult and older men.* Exp Gerontol. **45**(9): p. 679-84.

157. Rantalainen, T., et al., *Neuromuscular performance and bone structural characteristics in young healthy men and women.* Eur J Appl Physiol, 2008. **102**(2): p. 215-22.

158. Marin, R.V., et al., *Association between lean mass and handgrip strength with bone mineral density in physically active postmenopausal women.* J Clin Densitom. **13**(1): p. 96-101.

159. Montgomery, E., et al., *Muscle-bone interactions in dystrophin-deficient and myostatin-deficient mice.* Anat Rec A Discov Mol Cell Evol Biol, 2005. **286**(1): p. 814-22.

160. van den Berg, L.E., et al., *Low bone mass in Pompe disease: muscular strength as a predictor of bone mineral density.* Bone. **47**(3): p. 643-9.

161. Formica, C.A., et al., *Reduced bone mass and fat-free mass in women with multiple sclerosis: effects of ambulatory status and glucocorticoid Use.* Calcif Tissue Int, 1997. **61**(2): p. 129-33.

162. Khatri, I.A., et al., *Low bone mineral density in spinal muscular atrophy.* J Clin Neuromuscul Dis, 2008. **10**(1): p. 11-7.

163. Hamrick, M.W., *Increased bone mineral density in the femora of GDF8 knockout mice.* Anat Rec A Discov Mol Cell Evol Biol, 2003. **272**(1): p. 388-91.

164. Hamrick, M.W., A.C. McPherron, and C.O. Lovejoy, *Bone mineral content and density in the humerus of adult myostatin-deficient mice.* Calcif Tissue Int, 2002. **71**(1): p. 63-8.

165. Hamrick, M.W., et al., *Femoral morphology and cross-sectional geometry of adult myostatin-deficient mice.* Bone, 2000. **27**(3): p. 343-9.

166. Hamrick, M.W., C. Pennington, and C.D. Byron, *Bone architecture and disc degeneration in the lumbar spine of mice lacking GDF-8 (myostatin).* J Orthop Res, 2003. **21**(6): p. 1025-32.

167. Pearsall, R.S., et al., *A soluble activin type IIA receptor induces bone formation and improves skeletal integrity.* Proc Natl Acad Sci U S A, 2008. **105**(19): p. 7082-7.

168. Cadena, S.M., et al., *Administration of a soluble activin type IIB receptor promotes skeletal muscle growth independent of fiber type.* J Appl Physiol (1985), 2010. **109**(3): p. 635-42.

169. Digirolamo D, S.V., Clemens T, Lee S.-J, *Systemic administration of soluble 483 activin receptors produces differential anabolic effects in muscle and bone in mice.* J. Bone Miner. Res, 2011. **1167 (Suppl.)**.

170. Bialek, P., et al., *A myostatin and activin decoy receptor enhances bone formation in mice.* Bone, 2014. **60**: p. 162-71.

171. Koncarevic, A., et al., *A soluble activin receptor type IIb prevents the effects of androgen deprivation on body composition and bone health.* Endocrinology, 2010. **151**(9): p. 4289-300.

172. Sako, D., et al., *Characterization of the ligand binding functionality of the extracellular domain of activin receptor type IIb.* J Biol Chem, 2010. **285**(27): p. 21037-48.

173. Lowery, J.W., et al., *Loss of BMPR2 leads to high bone mass due to increased osteoblast activity.* J Cell Sci, 2015. **128**(7): p. 1308-15.

174. Bilezikjian, L.M., et al., *Cell-type specific modulation of pituitary cells by activin, inhibin and follistatin.* Mol Cell Endocrinol, 2012. **359**(1-2): p. 43-52.

175. Licona, P., J. Chimal-Monroy, and G. Soldevila, *Inhibins are the major activin ligands expressed during early thymocyte development.* Dev Dyn, 2006. **235**(4): p. 1124-32.

176. Lee, S.J., et al., *Role of satellite cells versus myofibers in muscle hypertrophy induced by inhibition of the myostatin/activin signaling pathway.* Proc Natl Acad Sci U S A, 2012. **109**(35): p. E2353-60.

177. Zhang, M., et al., *Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization.* J Biol Chem, 2002. **277**(46): p. 44005-12.

178. Bouxsein, M.L., et al., *Guidelines for assessment of bone microstructure in rodents using micro-computed tomography.* J Bone Miner Res, 2010. **25**(7): p. 1468-86.

179. Parfitt, A.M., et al., *Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee.* J Bone Miner Res, 1987. **2**(6): p. 595-610.

180. Schindelin, J., et al., *Fiji: an open-source platform for biological-image analysis.* Nat Methods, 2012. **9**(7): p. 676-82.

181. Schriefer, J.L., et al., *A comparison of mechanical properties derived from multiple skeletal sites in mice.* J Biomech, 2005. **38**(3): p. 467-75.

182. Cierny, G., 3rd, H.S. Byrd, and R.E. Jones, *Primary versus delayed soft tissue coverage for severe open tibial fractures. A comparison of results.* Clin Orthop Relat Res, 1983(178): p. 54-63.

183. Willett, N.J., et al., *Attenuated human bone morphogenetic protein-2-mediated bone regeneration in a rat model of composite bone and muscle injury.* Tissue Eng Part C Methods, 2013. **19**(4): p. 316-25.

CURRICULUM VITAE FOR Ph.D. CANDIDATES

The Johns Hopkins University School of Medicine

BRIAN CHUN KIM GOH Updated: February 7, 2017

EDUCATION HISTORY

PhD expected	2017	Program in Cellular and Molecular Medicine Mentors: Thomas L. Clemens, PhD Douglas J. DiGirolamo, PhD	Johns Hopkins University School of Medicine
MD expected	2017	Doctor of Medicine	Johns Hopkins University School of Medicine
BS	2009	Biochemistry with College Honors, *summa cum laude*	Louisiana State University

PROFESSIONAL EXPERIENCE

2014-present Johns Hopkins Spine Outcomes Research Center
- Advisors: Brian Neuman, MD and Lee Riley, III, MD

2009-2012 Craniofacial and Orthopaedic Tissue Engineering Lab, Johns Hopkins University
- Advisor: Warren Grayson, PhD

2010 Cardiac Surgery Summer Research Program
- Advisor: Duke Cameron, MD

2004-2009 Stem Cell and Circadian Biology Research Lab, Pennington Biomedical Research Center
- Advisor: Jeffrey Gimble, MD, PhD

GRANTS & FUNDING

2014-2015 National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)
Building Interdisciplinary Research Team (BIRT) Revision Award (R01)
- Principle Investigator: Douglas DiGirolamo, PhD
- Collaborating PI: Robert Guldberg, PhD; Georgia Institute of Technology
- "Activin Receptor-Based Therapies for Composite Musculoskeletal Injuries" (R01AR062074 03S1)

2009-present Johns Hopkins University SOM Medical Scientist Training Program (MSTP)
- MD/PhD Candidate with Full Funding (T32GM007309)

HONORS & AWARDS

Johns Hopkins University School of Medicine

2015 Young Investigator Award, American Society for Bone and Mineral Research
2015 Alice L. Jee Award, Orthopaedic Research Society, Sun Valley Workshop
2012-2014 Fellows Forum on Metabolic Bone Diseases, American Society for Bone and Mineral Research and Endocrine Fellows Foundation
2011 Paul and Daisy Soros Fellowship for New Americans

Louisiana State University

2009 *USA Today* All-USA College Academic First Team
2009 Louisiana State University: University Medal
2009 Honors College: College Honors
2008 Barry M. Goldwater Scholar

2008 College of Basic Sciences Outstanding Junior
2008 Top 20 Cited Articles in Journal of Tissue Engineering and Regenerative Medicine
- Goh BC et al. Cryopreservation characteristics of adipose-derived stem cells: maintenance of differentiation potential and viability. 2007 Aug;1(4):322-324.

2007 Louisiana State University Outstanding Sophomore
2007 College of Basic Sciences Outstanding Sophomore
2007 Honors College Sophomore Honors Distinction
2006 Howard Hughes Medical Institute Undergraduate Summer Research Scholar
2006 Louisiana State University Outstanding Freshman
2006 Honors College Outstanding Freshman
2006 University College of Freshman Year Outstanding Freshman

PUBLICATIONS

1. **Goh BC**, Singhal V, Herrera A, Tomlinson R, Clemens TL, Lee SJ, DiGirolamo (2017) Activin Receptor Type IIA (ACVR2A) Functions Directly in Osteoblasts as a Negative Regulator of Bone Mass. *Under Review*.
2. Tomlinson RE, Li Z, Zhang Q, **Goh BC**, Li Z, Thorek DL, Rajhandari L, Brushart TM, Zhou FQ, Minichiello L, Venkatesan A, Clemens TL (2016) NGF-TrkA signaling by sensory nerves coordinates the vascularization and ossification of developing endochondral bone. Cell Reports, 16(10):2723-35.
3. Dewan A, Tomlinson R, Mitchell S, **Goh BC**, Yung R, Kumar, S, Tan E, Faugere M, Dietz H, Clemens T, Sponseller P (2015) Dysregulated TGF-β Signaling Alters Bone Microstructure in a Mouse Model of Loeys-Dietz Syndrome. J Orthop Res, 33(10):1447-54.
4. Yilgor HP, Cook CA, Hutton DL, **Goh BC**, Gimble JM, DiGirolamo DJ, Grayson WL (2013) Biophysical cues enhance myogenesis of human adipose derived stem/stromal cells. Biochem Biophys Res Commun, 438(1):180-5.
5. Singhal V, **Goh BC**, Bouxsein ML, Faugere MC, DiGirolamo DJ (2013) Osteoblast-restricted disruption of the growth hormone receptor in mice results in sexually dimorphic skeletal phenotypes. Bone Res, 1(1):85–97.
6. Salter E, **Goh B**, Hung B, Hutton D, Ghone N, Grayson WL (2012) Bone tissue engineering bioreactors: a role in the clinic? Tissue Eng Part B Rev,18(1):62-75.
7. Smith BJ, Sutton GM, Wu X, Yu G, **Goh BC**, Hebert T, Pelled G, Gazit Z, Gazit D, Butler AA, Gimble JM (2010) Ovariectomy and genes encoding core circadian regulatory proteins in murine bone. Osteoporos Int, 22(5):1633-9.
8. Gimble JM, Ptitsyn AA, **Goh BC**, Hebert T, Yu G, Wu X, Zvonic S, Shi X, Floyd ZE (2009) Delta sleep-inducing peptide and glucocorticoid-induced leucine zipper: potential links between circadian mechanisms and obesity? Obes Rev, 10 Suppl 2:46-51.
9. Hebert TL, Wu X, Yu G, **Goh BC**, Halvorsen YC, Wang Z, Moro C, Gimble JM (2009) Culture effects of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) on cryopreserved human adipose-derived stromal/stem cell proliferation and adipogenesis. J Tissue Eng Regen Med, 3(7):553-561.
10. Wu X, Xie H, Yu G, Hebert T**, Goh BC**, Smith SR, Gimble JM (2009) Expression profile of mRNAs encoding core circadian regulatory proteins in human subcutaneous adipose tissue: correlation with age and body mass index. Int J Obes (Lond), 33(9):971-977.
11. Tandon N, **Goh B**, Marsano A, Chao PG, Montouri-Sorrentino C, Gimble J, Vunjak-Novakovic G (2009) Alignment and elongation of human adipose-derived stem cells in response to direct-current electrical stimulation. Conf Proc IEEE Eng Med Biol Soc, 1:6517-6521.
12. Wu X, Yu G, Parks H, Hebert T, **Goh BC**, Dietrich MA, Pelled G, Izadpanah R, Gazit D, Bunnell BA, Gimble JM (2008) Circadian mechanisms in murine and human bone marrow mesenchymal stem cells following dexamethasone exposure. Bone, 42(5):861-870.

13. **Goh BC**, Wu X, Evans AE, Johnson ML, Hill MR, Gimble JM (2007) Food entrainment of circadian gene expression altered in PPARalpha-/- brown fat and heart. Biochem. Biophys. Res. Commun, 360(4):828-833.
14. **Goh BC**, Thirumala S, Kilroy G, Devireddy RV, Gimble JM (2007) Cryopreservation characteristics of adipose-derived stem cells: maintenance of differentiation potential and viability. J Tissue Eng Regen Med, 1(4):322-324.
15. Wu X, Zvonic S, Floyd ZE, Kilroy G, **Goh BC**, Hernandez TL, Eckel RH, Mynatt RL, Gimble JM (2007) Induction of circadian gene expression in human subcutaneous adipose-derived stem cells. Obesity (Silver Spring), 15(11):2560-2570.
16. McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L, Kloster A, Di Halvorsen Y, Ting JP, Storms RW, **Goh B**, Kilroy G, Wu X, Gimble JM (2006) The immunogenicity of human adipose-derived cells: temporal changes in vitro. Stem Cells, 24(5):1246-1253.
17. Zvonic S, Ptitsyn AA, Conrad SA, Scott LK, Floyd ZE, Kilroy G, Wu X, **Goh BC**, Mynatt RL, Gimble JM (2006) Characterization of peripheral circadian clocks in adipose tissues. Diabetes, 55(4):962-970.
18. Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Di Halvorsen Y, Storms RW, **Goh B**, Kilroy G, Wu X, Gimble JM (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells, 24(2):376-385.

SCIENTIFIC PRESENTATIONS

1. **Goh BC**, Singhal V, Herrera A, Tomlinson R, Clemens TL, Lee SJ, DiGirolamo (2016) Activin Receptor Signaling in Bone Development and Regeneration. Research Rounds for Department of Orthopaedic Surgery, Johns Hopkins Hospital, Baltimore, MD, June 2016.
2. **Goh BC**, Singhal V, Herrera A, Tomlinson R, Clemens TL, Lee SJ, DiGirolamo (2015) Activin Receptor Type IIA (ACVR2A) Functions Directly in Osteoblasts as a Negative Regulator of Bone Mass. American Society for Bone and Mineral Research Annual Meeting, Seattle, WA, October 2015.
3. **Goh BC**, Singhal V, Herrera A, Tomlinson R, Clemens TL, Lee SJ, DiGirolamo (2016) ACVR2A Functions as a Negative Regulator of Bone Mass. Orthopaedic Research Society (ORS), International Sun Valley Workshop, Sun Valley, ID, August 2015.
4. **Goh BC**, Singhal V, Herrera A, Tomlinson R, Clemens TL, Lee SJ, DiGirolamo (2016) ACVR2A Functions as a Negative Regulator of Bone Mass. Gordon Research Conference: Bones and Teeth, Galveston, TX, January 2014.
5. Yilgor HP, Cook CA, Hutton DL, **Goh BC**, Gimble JM, DiGirolamo DJ, Grayson WL (2013) Uniaxial Cyclic Stretch Enhance Adipose-Derived Stem Cell Myogenesis. Tissue Engineering and Regenerative Medicine International Society (TERMIS), Atlanta, GA, November 2013.
6. **Goh BC**, Wu X, Evans AE, Johnson ML, Hill MR, Gimble JM (2007) PPARα's Role in Lipid Metabolism through Circadian Rhythm Analysis. HHMI Summer Undergraduate Research Forum, Baton Rouge, LA, August 2007.

LEADERSHIP & VOLUNTEER EXPERIENCE

2016-present	Young Investigator Subcommittee Member, American Society for Bone and Mineral Research
2014	MD/PhD Admissions Student Committee Member
2011-present	Orthopaedic Surgery Interest Group • Senior Leadership Team (2015-present)
2009-present	Charm City Clinic – Hopkins' First Community Health Access Partnership/Clinic • Founding member and Inaugural Clinical Director (2009-2011)

- Health Resource Case Manager and Preventive Health Screener (2011-present)

2010-2015 Medical Scientist Training Program MD/PhD Advisory Board

- Professional and Ethics Committee (2010-2013)
- Social Committee (2013-2015)

2012 Hospital Sungai Buloh Medical Student Observership

- Medical student observership in Selangor, Malaysia

2010 Highlands Bolivian Medical Mission in Montero, Bolivia

- Providing medical services, screenings, and supplies to Highlands Bolivia

2009 National Outdoor Leadership School

- NOLS Rocky Mountain: Backpacking/Rock Climbing (2004)

1999-2009 Boy Scouts of America

- Eagle Scout (2004)
- Boy Scouts Junior Assistant Scoutmaster (2004-2008)
- BSA Certified Rock Climbing Instructor (2004-2009)