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THE EFFECTS OF SOLUBLE ACTIVIN TYPE IIB RECEPTOR ON BONE FORMATION AND BODY COMPOSITION

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ABSTRACT

Tero Puolakkainen

The effects of soluble activin type IIB receptor on bone formation and body composition

University of Turku, Faculty of Medicine, Institute of Biomedicine, Medical Biochemistry and Genetics, Turku Doctoral Programme for Molecular Medicine (TuDMM), Annales Universitatis Turkuensis, Medica-Odontologica, 2018, Turku, Finland

Bone tissue is a dynamic organ which undergoes constant remodeling through a coupled series of events in order to maintain proper skeletal structure and calcium-phosphate homeostasis. In addition to its mechanical properties, extensive research has revealed bone to modulate multiple metabolic processes as well, and it is widely accepted that bone tissue engages in active crosstalk with different tissue types.

Activins are pleiotropic growth factors belonging to the transforming growth factor β-superfamily and have recently been associated with numerous pathologic states affecting the musculoskeletal system. As activins are highly expressed in bone, the clarification of the true roles of activins in the pathogenesis of skeletal disorders could lead to the development of novel therapeutics targeting these growth factors. To address this, we investigated the effect of soluble activin type IIB receptor (ActRIIB-Fc), an activin-signaling inhibitor, on bone formation and body composition in different animal models.

In this series of translational studies we show that treatment with ActRIIB-Fc improves bone mass and strength as well as induces an increase in muscle mass in a mouse model of Duchenne muscular dystrophy. We also demonstrate that ActRIIB-Fc enhances fracture healing in a closed, diaphyseal fracture mouse model. Finally, we showed that treatment with ActRIIB-Fc rescues bone loss and fat gain induced by estrogen deficiency in an ovariectomy-mouse model. Our novel results propose that ActRIIB-Fc affects multiple tissue types in clinically relevant models and could be a potential therapeutic approach in different musculoskeletal and metabolic disorders, in which bone, fat and muscle are affected.

Keywords: TGF-β, Activin, Bone formation, Animal models, Fracture healing, Metabolism

TIIVISTELMÄ

Tero Puolakkainen

Liukoisen tyypin IIB aktiviinireseptorin vaikutus luun muodostumiseen ja kehon koostumukseen

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen biokemia ja genetiikka, Molekyylilääketieteen tohtoriohjelma (TuDMM), Annales Universitatis Turkuensis, Medica-Odontologica, 2018, Turku, Finland

Luu on dynaaminen elin, joka säätelee monia biologisia prosesseja. Luukudos uusiutuu jatkuvasti luuta muodostavien osteoblastien ja luuta hajottavien osteoklastien yhteistoiminnan avulla. Tämän uudistumisen tarkoituksena on ylläpitää luun biomekaanisia ominaisuuksia ja kalsium- ja fosfaattitasapainoa. Viimeaikaiset tutkimustulokset ovat osoittaneet, että luu säätelee myös useita aineenvaihdunnan mekanismeja ja luukudos on suorassa vuorovaikutuksessa useiden eri kudostyyppien kanssa.

Aktiviinit ovat transformoiva kasvutekijä β-kasvutekijäperheen jäseniä, jotka on viimeaikaisissa tutkimuksissa yhdistetty erilaisiin tukikudosten häiriöihin kuten postmenopausaaliseen osteoporoosiin. Aktiviinien kudostason vaikutukset luustossa tunnetaan kuitenkin edelleen puutteellisesti. Aktiviinien merkityksen selvittäminen näissä sairaustiloissa voisi johtaa uusien, näiden kasvutekijöiden toimintaa rajoittavien lääkeaineiden kehittämiseen. Intraperitoneaalisesti annettu liukoinen tyypin IIB aktiviinireseptori (ActRIIB-Fc) estää reseptorin ligandien toimintaa hiirellä. Tässä työssä tutkimme liukoisen ActRIIB-Fc:n vaikutusta luun muodostukseen ja kehon koostumukseen käyttäen erilaisia hiirimalleja.

Ensimmäisessä osatyössä osoitimme, että ActRIIB-Fc lisäsi luun määrää ja tiheyttä sekä lisäsi merkittävästi luurankolihaksen kasvua lihasrappeumahiirimallissa. Toisessa osatyössä tuloksemme osoittivat, että ActRIIB-Fc nopeuttaa hiiren sääriluunmurtuman paranemista lisäämällä luun muodostusta. Viimeisessä osatyössä osoitimme, että ActRIIB-Fc lisää luun muodostusta mutta vähentää rasvan kertymistä elimistöön. Tuloksemme osoittavat, että liukoinen ActRIIB-Fc tarjoaa kiinnostavan hoitovaihtoehdon erilaisiin tuki- ja liikuntaelimistön sekä aineenvaihduntasairauksiin, jotka vaikuttavat luustoon, lihakseen sekä rasvaan.

Avainsanat: TGF-β, Aktiviini, Luun muodostuminen, Eläinmalli, Murtuman paraneminen, Aineenvaihdunta

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ABBREVIATIONS

ActRI/II	Activin type I/II receptor
ALP	Alkaline phosphatase
ALK	Activin receptor-like kinase
BAT	Brown adipose tissue
BMP	Bone morphogenetic protein
BMD	Bone mineral density
BMU	Basic multicellular unit
Bsp	Bone sialoprotein
BV/TV	Bone volume per tissue volume (%)
Col1(A1)	Collagen type I
CSD	Critical-sized defect
DMD	Duchenne muscular dystrophy
DMP-1	Dentin matrix protein 1
ECM	Extracellular matrix
FDA	Food and drug administration
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
GDF	Growth differentiation factor
GTT	Glucose tolerance test
HSC	Hematopoietic stem cell
IGF	Insulin-like growth factor
IL	Interleukin
ITT	Insulin tolerance test
M-CSF	Macrophage-colony stimulating factor
Mdx	DMD^{Mdx} mouse
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
OB	Osteoblast
OC	Osteoclast
OCN	Osteocalcin
OPG	Osteoprotegerin
ORX	Orchiectomized
Osx/Sp7	Osterix/Transcription factor 7
OVX	Ovariectomy/Ovariectomized
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PPAR	Peroxisome proliferator-activated receptor
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROI	Region of interest
Runx2	Runt-related transcription factor 2
SOST	Sclerostin
TGF-β	Transforming growth factor β
TNF	Tumor necrosis factor
Traf	Tumor necrosis factor receptor associated factor
Tra(c)p/Acp5	Tartrate resistant acid phosphatase
Tb.	Trabecular
UCP-1	Uncoupling protein 1
WAT	White adipose tissue
Wnt	Wingless type MMTV integration site family
µCT	Micro-computed tomography

LIST OF ORIGINAL PUBLICATIONS

List of original publications

This thesis is based on the following original publications. The original publications have been reproduced with the permission of the copyright holders.

I **Puolakkainen T**, Hongqian M, Kainulainen H, Pasternack A, Rantalainen T, Ritvos O, Heikinheimo K, Hulmi JJ, Kiviranta R. Treatment with soluble activin type IIB receptor improves bone mass and strength in a mouse model of Duchenne muscular dystrophy. *BMU Musculoskeletal Disorders 2017 18:20 DOI: 10.1186/s12891-016-1366-3*

II **Puolakkainen T**, Rummukainen P, Lehto J, Ritvos O, Hiltunen A, Säämänen A-M, Kiviranta R. Soluble activin type IIB receptor improves fracture healing in a closed tibial fracture mouse model. *PLoS One 2017; 12(7): e0180593 DOI: 10.1371/journal.pone.0180593*

III **Puolakkainen T**, Rummukainen P, Ritvos O, Savontaus E, Kiviranta R. Treatment with soluble activin type IIB receptors ameliorates bone loss and fat gain in OVX mice. *Manuscript, 2018*

1 INTRODUCTION

While completing his surgical fellowship training during 1774-1779, the Italian physician Michele Troja investigated the phenomena of bone regeneration and published his most known work *De novorum ossium regeneratione experimenta*. In this manuscript he narrated his observations of the different events associated with fracture healing using different animal models. He suggested that the periosteal layer of bone tissue and the cellular activity within was primarily responsible for the osteogenesis and fracture healing that took place. These findings were vindicated and acknowledged by his successors but the true mechanisms behind these phases remained obscure for centuries.

In 1965 Marshall R. Urist published his distinguished findings on how implanting demineralized bone matrix intramuscularly exerts the formation of new bone. A few years later the bone morphogenetic protein family (BMP), a group of similarly structured cytokines with osteoinductive properties which were the culprits for Urist's findings, was identified. This revolutionized current concepts on osteogenesis and led not only to a more thorough understanding of the phases of bone formation but also to the development of clinical applications for treating orthopedic injuries. Urist himself predicted that BMPs would be globally used in operating theaters to treat patients suffering from bone pathologies. In 2002, a year after his death, recombinant human BMP-2 was approved by the Food and Drug Administration (FDA) to enhance bone formation in spine fusion procedures.

Since Urist's seminal findings, our understanding of the regulation of bone metabolism has evolved greatly. Multiple newly-identified growth factors and their signaling pathways in bone cells have already been targeted for novel therapeutic approaches in treating bone disorders. However, we have yet only scratched the surface of the complex regulation of bone formation. It is essential to keep in mind that bone, as well as being partly autonomous, is also highly dependent on surrounding and systemic signals. Specific interactions, mechanisms of crosstalk and cross-regulation between the different tissue types, cells, cytokines and other factors have started to emerge. Therefore, future studies must be aimed to define the intriguing communication between these interplaying factors that occurs during the bone remodeling cycle, to understand the role of bone as a local and systemic modulator of normal physiology and disease.

2 REVIEW OF LITERATURE

2.1 Bone biology

2.1.1 Composition and structure

Current knowledge on the biology of bone tissue (*os* in Latin) has evolved greatly in the past few decades due to extensive basic, translational and clinical research. Once known as a hard tissue type with tasks only in mechanical support of the body and in enabling locomotion, seminal research has revealed bone tissue to be truly a dynamic organ as it actively participates in the regulation of different metabolic processes, the retaining of minerals, the secretion of growth factors, and modulation of pH homeostasis. Furthermore, bone tissue has been shown to interact with other tissue types proving it to be a vital component in normal and pathophysiology and not being limited to its anatomic boundaries. In order for bone to function properly, it is constantly being remodeled under strict regulation to maintain homeostatic equilibrium (Arnett 2003, Guntur and Rosen 2012).

The composition of the extracellular matrix (ECM), the bulk of bone tissue, can be classified into three entities; the mineralized inorganic, the organic matrix and the remaining part composed of water. The mineralized component represents about 65-70% of total bone volume and mostly consists of mineral known as hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], which gives bone tissue high compressive strength (Buck and Dumanian 2012) The organic matrix (20-30% of total bone volume) is mainly composed of type I collagen, which forms the support grid for the matrix, and so-called ground substance, which includes extracellular fluid and proteoglycans such as decorin, biglycan, betaglycan, syndecans and glypicans, and proteins, such as osteocalcin (OCN), bone sialoprotein (BSP), osteonectin (OCN), osteoprotegerin (OPG) and osteopontin. These molecules play roles in the regulation of biomineralization (Cohen 2006).The ECM also acts as a storage for different growth factors and cytokines, from which they diffuse or dissolve to maintain and support homeostasis.

Bone can be divided into cortical (or compact) bone and cancellous (or trabecular) bone. Cortical bone, which accounts for about 80% of the human skeleton, is referred to as the densely packed form of bone and is surrounded by the external periosteum and the internal endosteum. The microarchitecture of cortical bone is very well organized and it is mostly composed of osteons. Oste-

ons are the principal functional unit made up of concentric layers of compact bone lamellae and Haversian canals. Haversian canals are interconnected through a network of canaliculi or microchannels that work as communication systems between osteons. The shape of cortical bone alters during growth and aging as the diameter of cortical bone grows while the marrow space also expands. This is due to the fact that in the periosteum bone formation exceeds bone resorption and vice versa in the endosteum (Clarke 2008). Additionally cortical bone turnover rate is slower than that of cancellous bone due to having a smaller surface area relative to its total matrix volume (Bala, Zebaze et al. 2015). Cancellous bone is known as the highly vascularized and porous type of bone. Due to its more porous structure, trabecular bone has weaker biomechanical properties than cortical bone. The primary anatomical units of trabecular bone are called the trabeculae, rod or plate-like bone structures, which are interconnected in a honeycomb-styled pattern in order to build a dense network of trabeculae (Clarke 2008). This trabecular network forms a supporting framework for the surrounding bone marrow.

2.1.2 Cell types

Osteoblasts

Osteoblasts are highly differentiated stem cells originating from common mesenchymal progenitors, known as mesenchymal stem cells (MSCs) that have the capability to form and mineralize new bone tissue. Specific transcription factors are essential in regulating the proliferation and differentiation of these cells. Runt-related transcription factor 2 (Runx2) has been defined as the “master switch” for mesenchymal precursor cells to commit to the osteo-chondrocyte lineage and proliferate into immature osteoblasts (Komori 2010). Subsequently, osterix (Osx) is one of the main transcription factors promoting the differentiation of immature pre-osteoblasts into mature osteoblasts (Nakashima, Zhou et al. 2002). A large number of different cytokines and growth factors, which induce the expression and/or activity of these transcription factors, are also involved in the phases of osteoblast development and function. These factors include BMPs of the transforming growth factor β-superfamily (TGF-β), wingless type mouse mammary turos virus integration site family member (Wnt)-family members, parathyroid hormone (PTH) and members of the Hedgehog-family. There are no usable surface markers to identify osteoblasts at their specific differentiation stages. Thus osteoblastic differentiation is followed by measuring the expression

of osteoblasts-specific genes such as alkaline phosphatase *(Alp1),* collagen type I *(Col1A1)*, *Bsp* and *Ocn* (Rodan and Noda 1991). These proteins are also important for bone formation. Mature osteoblasts are destined to three different fates: 1) they either undergo cellular apoptosis, 2) are embedded inside newly formed bone tissue and differentiate into osteocytes, or 3) become flat cells that appose on inactive bone surfaces and are then known as bone lining cells.

Osteoclasts

Osteoclasts are defined as multinucleated cells derived from the myeloid lineage of hematopoietic stem cells (HSCs) and are responsible for the resorbing the mineralized bone tissue. As osteoclasts attach to the bone surface, they form a sealed vacuole, known as the sealing zone, to isolate the area to be resorbed from the surrounding bone. Osteoclasts generate an acidic environment within the sealing zone using vacuolar proton pump to dissolve the bone mineral. To degrade the organic matrix in the formed resorption lacuna osteoclasts secrete proteolytic enzymes with acidic pH optimum, such as matrix cathepsin K (CTSK) and matrix metalloproteinases, that are also required for proper bone resorption (Lotinun, Kiviranta et al. 2013). The phases of osteoclast development, such as the proliferation from myeloid progenitor cells to osteoclast progenitor cells, the fusion of these cells and their differentiation into osteoclasts are regulated by several signaling pathways. For example the roles of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) are well established in regulating osteoclast differentiation via multiple different signaling pathways, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and phosphoinositide 3-kinase – protein kinase B (PI3K-AKT) (Kim and Kim 2016). Other pathways participating in bone resorption have also been identified such as the tumor necrosis factor receptor (TNF)-associated factor (TRAF)-dependent cascade. Specific markers genes for osteoclast functions include tartrate-resistant acid phosphatase (*Tra(c)p/Acp5)*, *Ctsk* and *RANKL* (Takeshita, Kaji et al. 2000).

Osteocytes

Osteocytes are fully matured osteoblasts that undergo a terminal differentiation process and become embedded within the osteoid and eventually bone from where they respond not only to mechanical stimuli but are also are thought to play a major role in the regulation and control of bone formation and resorption

(Bellido 2014). Osteocytes, making up of around 98% of all bone cells, are widely distributed within the bone matrix and are connected to each other via dendritic processes and gap junctions forming a complex osteocytic network that may also signal to other bone cells. Osteocytes were for long thought to be mainly mechanotransductory cells sensing the mechanical strains applied to bone (Mullender and Huiskes 1995). However, recent work as demonstrated that osteocytes have multiple functions in regulating bone and mineral metabolism (Schaffler, Cheung et al. 2014). Osteocytes produce key factors that are essential in the regulation of bone remodeling. For example, osteocytes express dentin matrix acidic phosphoprotein 1 (DMP-1), sclerostin (SOST) and RANKL that control both bone formation and resorption phases. Furthermore osteocytes control phosphate homeostasis via secreting phosphatonin fibroblast growth factor (FGF)-23 (Bonewald and Wacker 2013). Abnormalities in the form and function of osteocytes are associated with pathophysiological conditions affecting bone tissue including osteoporosis (Neve, Corrado et al. 2012). Therefore, osteocytes express factors that regulate both osteoblasts and osteoclasts via numerous signaling and communication pathways and are involved in orchestrating the regulation of bone remodeling and calcium-phosphate balance.

Bone marrow

Bone marrow is an essential hematopoietic organ consisting of a variety of different cell types and is located in the confined central cavity of axial and long bones. Bone marrow is primarily responsible for hematopoiesis and therefore is mostly composed of HSCs and their differentiated forms. In addition, the marrow is rich in adipocytes, bone trabeculae, blood vessels as well as MSCs. Bone marrow provides a unique microenvironment that supports the hematopoiesis, also referred to as the bone marrow stem cell niche, and is essential in the regulation of normal bone homeostasis (Kajimura and Saito 2014). Moreover, the functional roles of bone marrow fat have been an attractive area of research. Previously it was thought that marrow adipocytes are metabolically inert but current theories suggest bone marrow adipocytes to actively regulate metabolic processes. In fact bone marrow adiposity has recently been associated with metabolic perturbations such as diabetes and osteoporosis (Zhang, McFarlane et al. 2012, Walji, Turecamo et al. 2016). However, the relationship between marrow adipose tissue and other fat depots is elusive and further research is warranted to unravel the regulatory crosstalk between the bone marrow niche and other tissue types.

2.1.3 Bone modeling and remodeling

Bone grows in longitudinal and appositional dimensions via modeling where bone adapts to mechanical strain and results in bone formation independent of bone resorption taking place at the same site. Bone remodeling on the other hand is dependent on the coupling of bone resorption to bone formation, that is, site-specific osteoclast and osteoblast activity is strictly regulated for a net-neutral effect on bone mass (Hattner, Epker et al. 1965). Bone remodeling takes place in specialized units known as basic multicellular units (BMU) as first named by Harold Frost over 50 years ago but today are also known as bone remodeling compartments (BRC) and bone remodeling units (BRU) (Figure 1). The BMU itself is defined by an anatomical canopy structure located along the surface of the bone where intricate interactions between osteoclasts, osteoblasts, osteocytes, bone lining cells and other factors occur (Hauge, Qvesel et al. 2001, Eriksen 2010). The measures that take place in these compartments can broadly be divided into four phases; the resting phase, the resorption phase, the reversal phase and the bone formation phase after which the cycle repeats itself. In the resting phase BMUs are inactive and bone resorption or apposition does not take place. Then activation signals, such as monocyte chemoattractant protein-1 (MCP-1), RANKL and M-CSF-1, are secreted and promote proliferation and differentiation of osteoclast precursors to mature osteoclasts osteoclastogenesis (Hodge, Kirkland et al. 2007, Miyamoto, Ninomiya et al. 2009). Fully matured osteoclasts form a sealed resorption lacuna underneath them, which is sealed from the extracellular space by the tight sealing zone. Integrin αvβ3 is essential for this tight attachment but multiple other mechanisms are also involved (Batsir, Geiger et al. 2017). Due to its dual structure consisting of mineral and organic phase, bone resorption is a complex process requiring multiple molecular players. For example, vacuolar-ATPase (V-ATPase) is primarily responsible for lowering the pH inside the vacuole by proton transporting to dissolve the mineral matrix (Vaananen, Karhukorpi et al. 1990, Sun-Wada and Wada 2013), chloride specific ion channel (ClC7) participates in transferring counter anions to and from the resorption zone (Kornak, Kasper et al. 2001) and cathepsin K is essential in degrading type I collagen and other bone matrix proteins (Bossard, Tomaszek et al. 1996). These events lead to the disintegration of bone matrix leaving eroded pits known as Howship's resorption lacunae on the surface of bone where osteoclasts reside.

At the end of the resorption phase, osteoclast activity is arrested and the reversal phase commences. Despite the identification of numerous osteogenic molecules secreted by osteoclasts, the precise mechanism on how the coupling from resorption to bone formation occurs is unclear. Current theories describe the role of reversal cells, mononucleated cells that colonize eroded bone surfaces, being key factors in mediating communication between osteoclasts and osteoblasts and their activity (Andersen, Abdelgawad et al. 2013). This is supported by the evidence of reversal cells possessing the capability to mature into osteoblasts and displaying a higher density of cell populations in eroded pits compared to quiescent bone surfaces (Delaisse 2014). Studies have also shown that osteoclasts express anabolic cytokines that could potentially act as recruiters for osteoblastic precursors and therefore reinforce their multifunctional role in the bone coupling system (Henriksen, Karsdal et al. 2014). For example, the release of TGF-β1 during bone resorption initiates migration and homing of mesenchymal stem cells, which acts as a key signal in order to initiate bone formation (Tang, Wu et al. 2009). The reversal phase is an essential signaling event in the bone remodeling cycle linking the resorption and formation phases and functions of osteoclasts and osteoblasts.

During the final stage of the bone remodeling cycle, the bone formation phase, osteoblasts precursor cells proliferate and differentiate to mature osteoblasts, which then form new bone matrix to replace the resorbed bone. Osteoblasts are able to produce organic matrix, referred to as osteoid or unmineralized bone tissue, and then continue to gradually mineralize osteoid by dispensing small vesicles containing calcium and phosphate ions. The progressive filling of the eroded pit and the formation of a new osteon brings the bone remodeling cycle to a momentary cease before initiatory signaling of osteoclast precursors takes place and the cycle repeats itself (Clarke 2008).

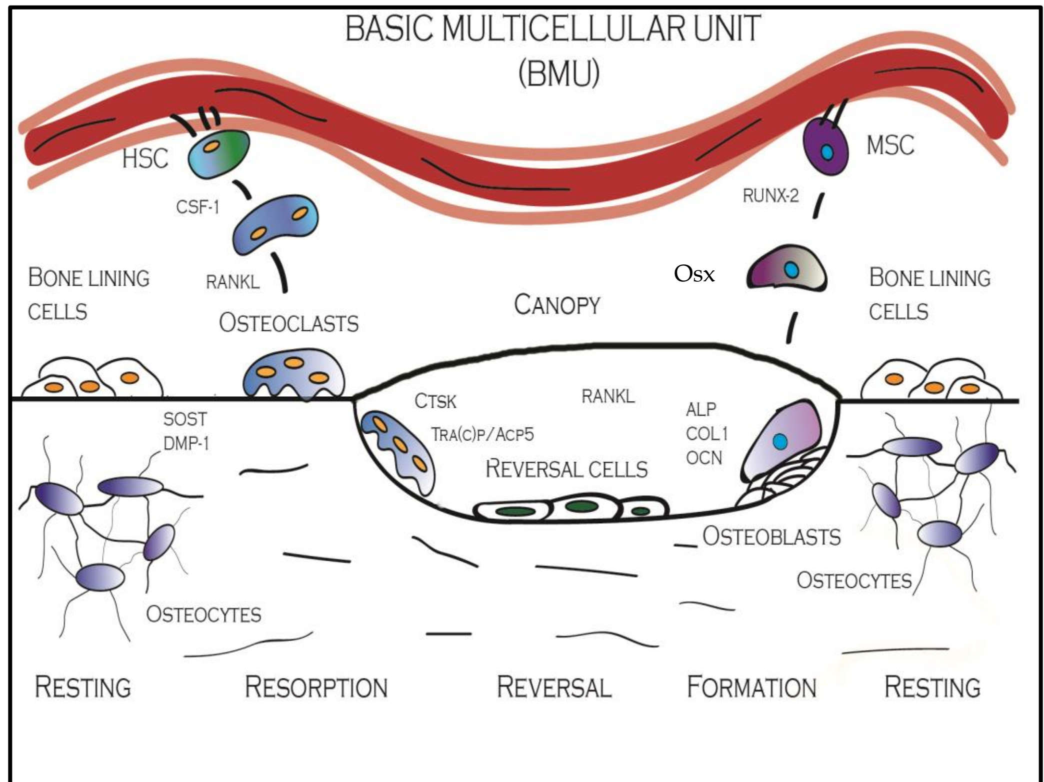

Figure 1: A simplified diagram representing the phases and functions of a basic multicellular unit. Bone lining cells and osteocytes secrete signaling factors to attract osteoclast progenitors to the area that then differentiate to multinucleated osteoclasts. Mature osteoclasts then commence the resorption phase by forming a sealed resorption lacuna on the surface of the bone. Bone mineral dissolves in the acidic environment of the lacuna followed by degradation of organic matrix by proteolytic enzymes. When osteoclasts move forward reversal cells are then recruited to the exposed bone surface. These cells attract osteoblastic precursors to the area to commence bone formation to replace the resorbed bone. After the mineralization phase, bone lining cells and osteocytes are primarily responsible for re-initiating the cycle. (HSC Hematopoetic Stem Cell, M-CSF Macrophage Colony Stimulating Factor, RANKL Receptor Activator of Nuclear Factor Kappa-B Ligand, SOST Sclerostin, DMP-1 Dentin Matrix Protein-1, CTSK Cathepsin K, Tra(c)p/Acp5 Tartrate Resistant Acid Phosphatase, ALP Alkaline Phosphatase, COL1 Collagen 1, OCN Osteocalcin, Osx Osterix, RUNX2 Runt Related Transcription Factor 2, MSC Mesenchymal Stem Cell)

2.1.4 Bone Pathology

2.1.4.1 Osteoporosis

As described by the National Institutes of Health, osteoporosis is defined as a "skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture" (Nih Consensus Development Panel on Osteoporosis Prevention and Therapy 2001). This broad definition encompasses a number of different pathological states, where the microarchitectural quality of bone tissue is impaired making the subjects more prone to fractures. In order to differentiate osteoporosis from other similar diseases such as osteopenia, another important definition or term has also been established: according to the World Health Organization osteoporosis is defined as "a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture." A patient is considered to have osteoporosis if the bone mineral density T-score is -2.5 or lower when comparing to healthy, young women, where the T-score represents the amount of standard deviations differing from healthy bone. However, this does not directly take into account bone structure and quality or other clinical risk factors that affect the overall fracture risk. Global bone and mineralized tissue communities still argue over the definition of the term osteoporosis as many claim that the definition should also address other essential parameters such as the endocrine regulation of bone metabolism, the clinical investigation of patients and other pathologic attributes of the disease (Lorentzon and Cummings 2015).

Osteoporosis is characterized by a negative balance between bone formation and resorption ultimately resulting in net loss of bone mass and impaired bone structure. This balance of balance resorption and formation is greatly influenced by a variety of medical, genetic and behavioral factors. There is some controversy on the etiological factors predisposing patients to osteoporosis but peak bone mass and the changes associated with aging seem to be important. Menopausal estrogen deficiency, continuous use of glucocorticoids, smoking, inadequate vitamin D intake and history of previous fractures also increase the risk of osteoporosis (Lane 2006). Osteoporosis occurs mostly in postmenopausal women but it has been recently recognized as a health concern in older men as well due to underdiagnosis and limitations in screening (Willson, Nelson et al. 2015).

Current pharmaceutical treatment options for osteoporosis can be classified into anabolic and antiresorptive agents based on their mechanisms of action. Anabolic agents target and enhance osteoblast activity aiming to stimulate bone formation. Teriparatide, the 1-34 fragment of human PTH, remains the golden standard for anabolic treatment as it increases bone mineral density (BMD) and decreases fracture risks (Han and Wan 2012). However, due to the coupling of bone formation and resorption it also subsequently increases osteoclast activity and may limit the beneficial effects in some patients (Harslof and Langdahl 2016). This reinforces the fact that there is indeed a lack of anabolic treatment options for osteoporosis. Specific SOST antibodies that enhance Wnt signaling (discussed later in this review) (Padhi, Jang et al. 2011) and PTH receptor activators (Leder, O'Dea et al. 2015) have displayed auspicious results in terms of increasing bone mass and strength, and have emerged as potential novel anabolic agents for treating osteoporosis.

Antiresorptives aim to impair the functions of osteoclasts resulting in suppressed bone turnover. The decrease in the rate of bone resorption also reduces the rate of bone formation ultimately leading to a new equilibrium between the two phases of bone remodeling (Appelman-Dijkstra and Papapoulos 2014). Currently, there is a wide array of antiresorptive approaches, which include bisphosphonates, methods for improving estrogen deficiency, and specific monoclonal antibodies. Bisphosphonates bind to mineralized tissue with an extremely high affinity and suppress osteoclast-mediated bone resorption by inducing osteoclast apoptosis or impairing their capability to form sealing zones (Green 2004). Due to their efficacy and remarkably long half-life in bone tissue, they are often considered the first choice in treating osteoporosis. However, the long-term use of bisphosphonates has been associated with side-effects such as hypocalcemia, atrial fibrillation and osteonecrosis of the jaw (BRONJ) (Drake, Clarke et al. 2008). Furthermore, bisphosphonates impair bone remodeling, which often results in strain-induced microdamage, or microcracks, and can predispose the patient to atypical femoral fractures (Saita, Ishijima et al. 2015).

Estrogen replacement therapy (ERT) is a treatment method which aims to substitute the reduced secretion of estrogen induced by menopause. Estrogen is a key regulator of bone metabolism and suppresses bone resorption (Riggs 2000). A decrease in estrogen production leads to accelerated bone remodeling switching the balance towards increased bone resorption resulting in a negative balance in bone turnover and subsequently decreases BMD (Gambacciani and Ciaponi 2000). Multiple methods for treating estrogen deficiency have been

developed. These include hormone/estrogen replacement therapy (HRT/ERT), selective estrogen receptor modulators (SERMs) and selective tissue estrogen activity regulators (STEARs) with varying molecular mechanisms of action.

Monoclonal antibodies refer to specific compounds which are able to inhibit the actions of certain regulatory factors by directly binding to them. This prevents the bound factor from interacting with its own target receptors and thus prevents the initiation of the specific signaling pathway. In the treatment of osteoporosis, monoclonal antibodies have been produced to target a specific marker that is known to be involved in bone resorption. Denosumab, a RANKL-specific monoclonal antibody has been shown to decrease bone resorption and improve BMD (Kostenuik, Nguyen et al. 2009, Papapoulos, Chapurlat et al. 2012).

In addition to anabolic and antiresorptive agents a third approach has also been investigated for treating osteoporosis: combination therapy with both an anabolic and an antiresorptive agent. This concept is based on the theory that dual therapy could enhance bone mass and strength compared to either type of monotherapy by having either and additive or synergistic effect (Cosman 2014). However recent research has demonstrated controversial results. Some studies have shown that dual therapy increases BMD in all sites compared to a single agent (Cosman, Eriksen et al. 2011, Walker, Cusano et al. 2013), whereas others show that combination therapy offers no additional benefit to single therapy (Black, Greenspan et al. 2003, Finkelstein, Wyland et al. 2010). A recently published meta-analysis of randomized controlled trials regarding the effects of combination therapy to monotherapy compiled some key aspects (Li, Chen et al. 2015). It summarized that the effects of combination therapy are site-specific, that is, key anatomic sites affected by osteoporosis respond differently to treatment. It seems that the spine might benefit more from a combination therapy compared to the hip region or the femoral neck. This is also highly dependent on the used antiresorptive method. However, at the present time, it can be concluded that, while it may provide positive effects for some patient groups, dual therapy does not have a superior effect in the treatment of osteoporosis over the administration of an anabolic or antiresorptive agent alone. This being said, sequential therapies, such as using an antiresorptive drug to preserve bone mass after the anabolic treatment with teriparatide are currently widely used (Liu and Li 2017).

2.1.4.2 Bone fracture healing

Bone fracture is a pathological condition where the integrity and continuity of bone is compromised due to an extrinsic, destructive force. In addition, bones can also fracture spontaneously or after low trauma, referred to as pathologic fractures, due to changes in bone structure caused by intrinsic factors or systemic diseases. Fractures can be classified in multiple ways but the assessment of whether or not the fractured bone ends perforate the surrounding soft tissue, mucosa and skin is a key element. These are referred to as open fractures, or when the surrounding tissues remain intact, closed ones. Furthermore fractures can be classified by the anatomy of the fracture line, the bone where the fracture takes place, and the degree of dislocation of bone fragments.

Fracture healing is a complex process with multiple steps, which take place simultaneously and in succession, aiming to restore the original form and function of the broken bone (Einhorn 1998). These phases are strictly regulated by a number of different growth factors, cytokines and angiogenic factors. The corresponding healing phases greatly depend on the type of fracture in question. For example, closed fractures heal differently than open ones or critical sized defects (CSDs) due to risks of contamination and factors derived from soft tissues participating in the healing phases (Park, O'Connor et al. 1999). The method of healing, cartilage-based endochondral healing or intramembranous healing, is highly dependent on the stabilization of the fractured fragments. It is well documented that in order for bone to heal and regenerate, it must undergo a series of strictly regulated healing phases, which include the inflammatory phase, the angiogenic phase, the callus formation phase for endochondral healing, and the bone formation and remodeling phases. These events overlap during fracture healing and the distinction of one phase from another is convoluted.

Immediately after a bone fracture takes place, the inflammatory phase commences aiming to remove possible pathogens and necrotic tissue and to create a favorable environment to initiate fracture repair. Macrophages, neutrophils, platelets and other members of the innate and adaptive immune system are recruited to the area and secrete characteristic markers of inflammation, such as interleukins (ILs) and tumor necrosis factor (TNF) family members (Gerstenfeld, Cho et al. 2003, Yang, Ricciardi et al. 2007). The inflammatory phase is followed by the angiogenic phase. Platelet derived growth factors (PDGFs) and FGFs participate in the formation of new blood vessels to the area. Angiogenesis and blood vessels, in addition to the periosteum, cortex and bone marrow (Dimitriou, Tsiridis et al. 2005), are an essential source for MSCs which

migrate to the area and start to differentiate into collagen producing fibroblasts and cartilage forming chondrocytes. These form the basis for the callus, a dynamic, fibrocartilaginous capsule that surrounds the fracture area, stabilizing the fracture fragments and bridging them together. The callus serves as a domain where cartilage is formed and resorbed and subsequently replaced by mineralized new bone tissue. In the following remodeling phase the newly-formed, structurally unorganized woven bone is replaced by well-organized lamellar bone, finally restoring the continuity of the broken bone. In fractures, where the fracture ends are properly realigned, the callus and cartilage formation is not distinct and bone regenerates with minimal callus formation (see Figure 2).

The choice of treatment in fracture healing is heavily dependent on the type, degree and severity of the fracture. One essential principle is to ensure realignment of the fracture ends to their anatomically correct locations, and then to secure immobility by either internal or external fixation resulting in bone union. This principle can be applied to many closed, simple fractures regardless of the fracture site. However open, displaced and complex fractures, which have a higher incidence of mortality and are more prone to infections, often require surgical intervention and open reduction of the fracture components. This is done with the use of different rods, plates, wires and screws made of biologically compatible materials, such as titanium alloy

When properly treated, bone fractures tend to heal without further complications. However, some cases, such as complex fracture patterns, provide therapeutic challenges and require additional means to augment bone mass accrual for proper bone union to take place. These include recombinant forms of different growth factors, although they also have their challenges. The different phases of fracture healing are strictly regulated and the correct time to dose a therapeutic agent is not only dependent on the target of the agent but also on the healing phase taking place. Ever since growth factors were implemented into clinical settings the treatment options for non-unions and open fractures have increased. Although promising results of preclinical animal studies have suggested growth factor applications to have great potential to improve healing of the orthopedic and maxillofacial defects (Gothard, Smith et al. 2014), the transition from treating animal experiments to treat the patients is not rectilinear, since analogous results are only found with certain growth factors under specific conditions. The concept that therapeutic use of growth factor would revolutionize the clinical limitations of contemporary treatment methods seems to have subsided and, in principle, autogenous bone grafts still remain the present golden

standard today for reconstructing bone defects. In fact, BMP-2 is currently the only FDA approved growth factor to be used as a bone graft substitute and has consolidated its position as a candidate to augment bone formation in spinal fusion surgeries (Cahill, Chi et al. 2009, Shimizu-Motohashi and Asakura 2014). However, now that BMPs have been in clinical practice for over a decade, controlled studies questioning their efficacy in treating bone defects have been presented (Hopkins 2016) and reports of their adverse effects have started to emerge. Recently James et al. reviewed that BMP-2 is linked to increased inflammatory complications, ectopic bone formation and impaired wound healing (James, LaChaud et al. 2016). Future studies should, not only be aimed at further elucidation of the specific mechanisms behind each cytokine during each phase of fracture healing, but also be focused on applying growth factors in conjunction with osteoconductive or osteoinductive materials and developing better delivery systems for bone growth enhancing factors to effectively treat the fracture with minimal adverse effects.

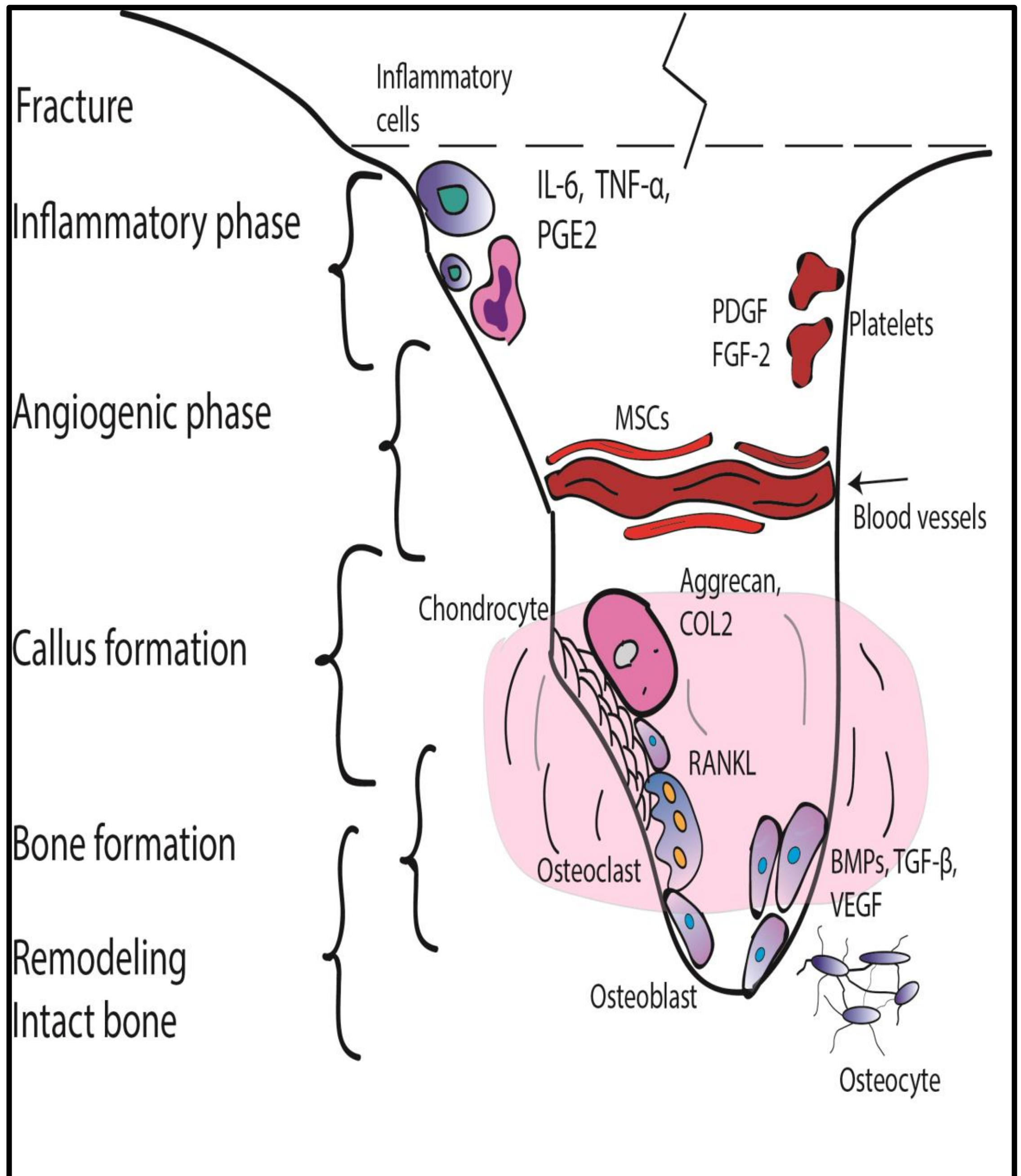

Figure 2: Illustration demonstrating the phases of endochondral fracture healing

2.1.4.3 Duchenne muscular dystrophy

Musculoskeletal disorders are a broad entity that includes a large amount of different conditions, symptoms and disabilities. Ranging from mild muscle strains to severe musculoskeletal atrophies, these pathological states often target both muscle and bone tissue. Duchenne muscular dystrophy (DMD) is a neuromusculoskeletal disorder, which is characterized by the degeneration of muscle fibers and their replacement with fibrous tissue. DMD is caused by mutations in the dystrophin gene, which results in the impaired form and function of the dystrophin protein. This leads to the dysfunctional attachment between muscle sarcolemmas and ECMs (Nowak and Davies 2004). Clinically, the disease manifests as continuous muscle weakness and impaired control of motor skills. As the disease progresses locomotion is restricted and patients are usually left immobilized by the age of 10 to 12. Ultimately, debilitated cardiac and respiratory functions result in death by the age of 20 to 25 (van Westering, Betts et al. 2015).

DMD also reduces bone quality. As the disease progresses, restrictions in ambulation in turn decrease loading signals in weight-bearing bone and decrease bone remodeling, which is assumed to be the primary factor leading to bone weakness (Hsu and Garcia-Ariz 1981). Fracture risk is also increased in DMD patients (McDonald, Kinali et al. 2002). Furthermore, lack of locomotion increases the rate of muscular atrophy, which in turn hinders the cross-talk between bone and muscle further aggravating the decrease in bone mineral mass. On the other hand some studies suggest that there might be also other mechanisms affecting bone mass in DMD. For example, young DMD patients have been reported to have a decreased BMD in the proximal femur even before the loss of ambulation (Larson and Henderson 2000), thus DMD may have a direct effect on bone quality. One explanation could be that inflammation and increased levels of inflammatory cytokines could have direct bone effects in DMD. Increased levels of certain interleukins (ILs) levels have been linked to osteoclastogenesis and increased bone turnover (Girasole, Passeri et al. 1994). In DMD, especially increased IL-6 levels have been reported, which alone may induce bone loss independent of the effects of immobilization (Rufo, Del Fattore et al. 2011).

At the present time, there is no ultimate panacea for DMD. Corticosteroids along with physical therapy are used to maintain muscular strength and to prolong the progression of the disease as well as increasing quality of life (Angelini and Peterle 2012). However, they can cause unwanted side-effects

such as increased adiposity, disruptions in normal metabolic pathways and even glucocorticoid-induced osteoporosis (Escolar, Hache et al. 2011). Therefore, alternative treatment options are needed. A dual treatment approach targeting both muscle and bone could be optimal in the treatment of DMD. First, preserved or even improved muscle mass and strength would help to maintain the patients' physical activity and mobility and prevent progression to dystrophic stage. Second, increased bone quality would reduce the fracture risk and support patient mobility. Ultimately, the treatment would aim to increase quality of life in patients and prolong the life expectancy of DMD patients.

2.2 Tissue crosstalk

2.2.1 Crosstalk between bone and skeletal muscle

Interactions between different musculoskeletal components have recently received a great deal of attention and latest research has highlighted that the interplay between bone and skeletal muscle extends well beyond biomechanical coupling. In 1892 Julius Wolff described that changes in bone form or function are followed by internal, cellular changes that alter the external conformation of bone. The mechanostat theory refined these findings and states that bone tissue adapts to mechanic force by inducing site-specific remodeling and adjusting its mass and architecture (Hattner, Epker et al. 1965, Frost 1998). The development of healthy bone is highly dependent on the contractions of adjacent skeletal and smooth muscle fibers (Sharir, Stern et al. 2011). Muscle forces regulate the shaping of bone and sustain the quality of bone microarchitecture by promoting bone remodeling. Conversely, muscle growth and myogenesis are also reliant on bone-derived stimuli (Bren-Mattison, Hausburg et al. 2011). Collectively, these factors provide a basis for bone and muscle to undergo constant crosstalk with each other to maintain proper homeostasis.

Muscle-forming myoblasts and bone-forming osteoblasts share the same progenitor cell and the commitment to a specific cell-line is dependent on a highly regulated network of intricate signaling pathways. Myoblast differentiation and myogenesis are dependent on several myogenic regulatory factors (MRFs) and myocyte enhancer factors (Mefs). The MyoD subfamily, belonging to the MRFs, consists of many factors such as MyoD, myogenin and myf5, all of which have been shown to be essential for skeletal muscle differentiation (Berkes and Tapscott 2005). The master regulator of skeletal myogenesis is

known as the mechanistic target of rapamycin (mTOR), a serine/threonine kinase that mediates protein synthesis and induces muscle fiber hypertrophy (Goodman, Hornberger et al. 2015). In addition, identification of the growth factor myostatin/growth differentiation factor (GDF)-8 has revolutionized current concepts concerning muscle regulation. Myostatin acts as a strong inhibitor of muscle cell proliferation and negative regulator of muscle mass. Myostatin inhibition has emerged as an attractive treatment approach for disorders of muscle wasting (Girgis, Mokbel et al. 2014).

Even though the mechanical communication between muscle and bone is an essential component in crosstalk, it is not the sole method of interaction. During the past few years, interest of musculoskeletal research communities has greatly increased towards the effects of secreted factors and molecular interactions between muscle and bone. Studies have suggested that agents secreted by bone cells could in fact mediate muscle formation. For example, bone-derived prostaglandin E2 and Wnt3a have been shown to promote myogenesis by accelerating myogenic differentiation (Mo, Romero-Suarez et al. 2012). On the other hand, FGF-23 has been found to cause cardiac muscle hypertrophy (Faul, Amaral et al. 2011) and OCN may have a direct effect on muscle strength (Fernandez-Real, Izquierdo et al. 2009). In addition, growth factors derived from skeletal muscle, such as FGF-2 and insulin-like growth factor (IGF)-1, have been shown to improve osteogenesis (Hamrick, McNeil et al. 2010). Other myokines, such as irisin, have also been suggested to have osteogenic potential as well (Colaianni, Cuscito et al. 2015, Kawao and Kaji 2015) (Figure 3).

There are also obvious clinical manifestations of muscle and bone crosstalk. One of the most relevant examples is the sarcopenia-immobility-osteopenia paradigm. Sarcopenia, the weakening of muscle mass and strength due to aging, has a strong correlation with high incidence of falls, poor balance and other factors that have a high contingency of leading to immobility. Not only is immobility pertained to muscular atrophy, but it is also strongly associated with decreased bone mass as the lack of mechanical impulses leads to unbalanced turnover in favor of bone resorption (Chen, Cameron et al. 2006). Additionally, weaker bones also predispose patients to fractures that again lead to even further immobility, and induces further deterioration of muscle quality, too. This vicious cycle clearly demonstrates the delicate relationship of bone and muscle cross-regulation. In another example, muscle flaps have been reported to enhance fracture healing, when mobilization and hence, mechanical loading, was not evident. This could be due to increased vascularization and the secretion of

osteogenic agents from the muscle (Davis, Griffin et al. 2015). On the other hand, muscle paralysis correlates with impaired fracture healing (Aliprantis, Stolina et al. 2012). Therefore, even though further research is needed to elucidate the mechanisms behind these findings, it can be concluded that crosstalk between bone and muscle tissue goes well beyond plain mechanical communication and involves direct molecular signals from one tissue to another.

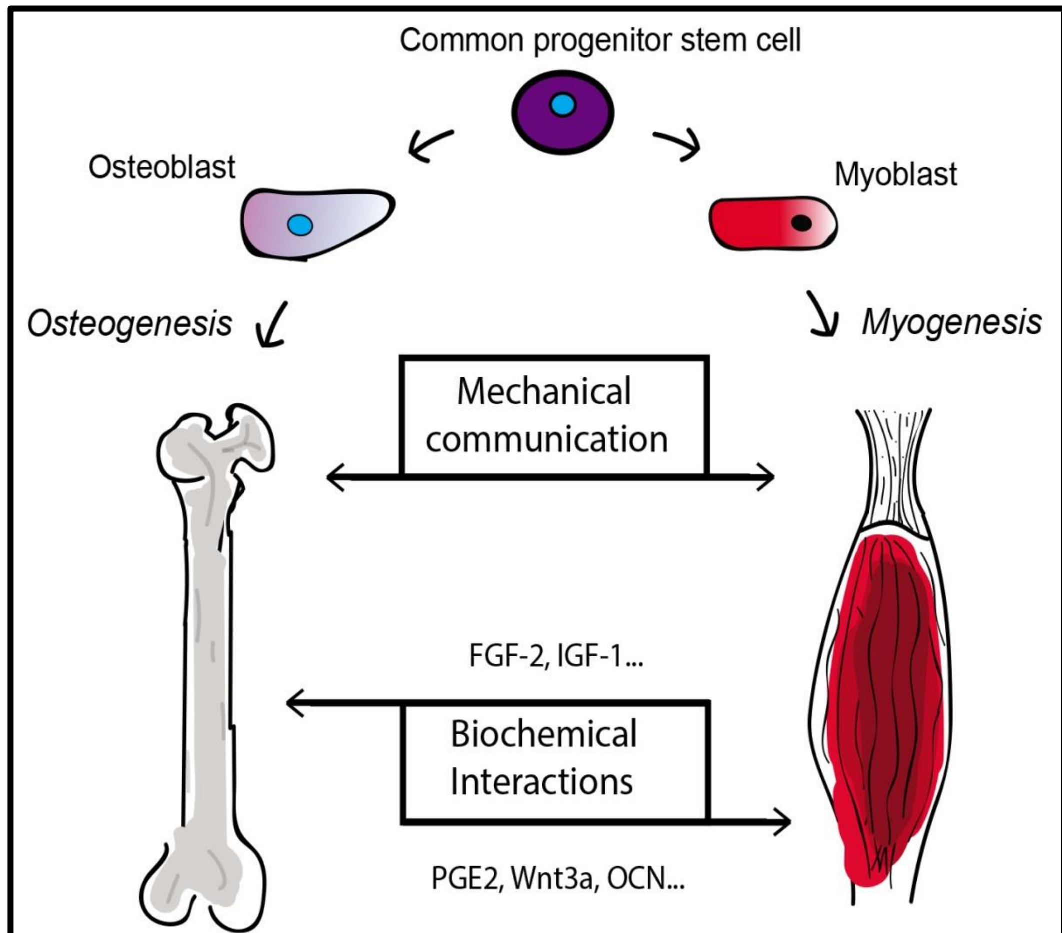

Figure 3: Illustration representing how crosstalk between bone and skeletal muscle goes well beyond mechanical interactions. Modified from (Brotto and Bonewald 2015)

2.2.2 Crosstalk between bone and adipose tissue

Bone and adipose cells interact with each other in numerous ways throughout their lifespan. Originating from the same common progenitor cells, osteoblasts and adipocytes engage in crosstalk during osteogenic-/adipogenic lineage commitment, cell differentiation and many physiologic events including lipid and energy metabolism. A strong relationship between fat mass and bone quality has been established, although the detailed mechanisms behind these changes are not clear (Shapses and Riedt 2006). As the prevalence of obesity and other metabolic disorders has alarmingly increased in humans, the interactions of these pathologic states and how they affect bone tissue has recently received more attention.

Adipose tissue mainly consists of fully matured adipocytes but also contains other cells including fibroblasts, adipose stromal/stem cells and preadipocytes. Adipocytes rise from MSCs that are recruited and then commit to the adipocyte lineage, known as the determination phase, after which they undergo terminal differentiation and develop into mature adipocytes. During adipocyte and osteoblast differentiation, these cells engage in reciprocal communication and their relationship is much more complex than an “either-or” paradigm (Beresford, Bennett et al. 1992). While adipocyte differentiation and formation of adipose tissue is regulated by numerous positive and negative molecular signals, peroxisome proliferator-activated receptor γ (PPARγ) has been identified as the key adipocyte differentiation factor (Chawla, Schwarz et al. 1994). Overexpression of PPARγ induces fibroblasts to differentiate into adipocytes (Tontonoz, Hu et al. 1994) and PPARγ knockout mice show decreased adipogenesis compared to controls (Jones, Barrick et al. 2005). In addition to adipogenesis, PPARγ is also required to maintain the differentiated state of adipocytes (Imai, Takakuwa et al. 2004).

Adipose tissue can be classified into white adipose tissue (WAT), brown adipose tissue (BAT), beige adipose tissue and bone marrow adipose tissue (BMAT). They differ in their locations and functions, so that WAT can be found throughout the body participating in energy metabolism and storing triglycerides. On the other hand BAT, which is found almost exclusively around the scapulae and neck, regulates thermogenesis. Brown adipocytes have a high number of mitochondria, numerous small lipid droplets and express uncoupling protein 1 (UCP-1) that is a fundamental regulator of heat formation in BAT (Argyropoulos and Harper 2002). White adipocytes in turn are usually larger in

size, contain one large lipid droplet, have less mitochondria and do not express UCP-1. Beige adipose tissue has similar properties compared to the other adipose tissue types in a way that beige adipose cells originate from WAT cells but gain the potential to express UCP-1 (Harms and Seale 2013). In addition to their expression markers, the origin, location and cellular characteristics, the fat depot also affects the properties of the adipose tissue. For example increased visceral fat is linked to insulin resistance and cardiovascular disease, whereas an increase in subcutaneous fat tissue is not (Rosen and MacDougald 2006). This is thought to be due to the differing mechanisms, which regulate adipose tissue metabolism, such as lipolysis, triglyceride storing and formation and secretion of different adipocyte-derived hormones (Lee, Wu et al. 2013). Bone marrow adipose tissue, a specific form of fat tissue confined to bone marrow compartments in long and axial bones, is a dynamic organ that is thought to mediate multiple metabolic processes, and has recently been associated with the development and progression of metabolic disorders, such as diabetes and insulin resistance (Zhang, McFarlane et al. 2012, Lee, Wu et al. 2013, Walji, Turecamo et al. 2016).

As stated earlier, the commitment and differentiation of adipocytes and osteoblasts share similar characteristics including allocated signaling pathways. Therefore, crosstalk between bone and adipose tissue is also evident. Changes in adipose tissue mass affect the expression of many hormones and alter the production of different adipokines such as leptin and adiponectin. Obesity is associated with increased leptin and decreased adiponectin levels. Leptin, a key regulator for energy metabolism, is a positive or negative regulator of bone mass depending whether the central or peripherical pathway is activated (Chen and Yang 2015). Adiponectin stimulates osteoblastogenesis and has an anabolic effect on bone remodeling (Mitsui, Gotoh et al. 2011). However, decreased adiponectin secretion has not been linked to decreased fracture risks or other positive effects on bone in clinical settings (Shapses and Sukumar 2012). In addition to the secretion of adipokines, accumulated fat tissue also affects bone metabolism in other ways. Obesity is associated with increased circulating levels of proinflammatory cytokines, such as numerous ILs and TNF-α. Studies have shown that these factors may enhance osteoclastogenesis by promoting their differentiation and recruitment (Chaplais, Thivel et al. 2015). In fact, obesity itself is a systemic inflammatory state (Monteiro and Azevedo 2010, Gregor and Hotamisligil 2011). Conversely, bone tissue secretes molecules that regulate adipocyte development and function. OCN has been demonstrated to regulate

adipocytes and to protect from obesity while BMP-4 has been reported to promote adipocyte differentiation (Ferron, Hinoi et al. 2008, Wei, Li et al. 2013).

The crosstalk of bone and adipose tissue has also clinical implications that have not yet been fully investigated. Obese people tend to have a higher BMD compared to normal-weight people, possibly due to increased mechanical loading, which is thought to protect obese people from osteoporotic fractures (Villareal, Apovian et al. 2005). However, obesity has been linked to a higher fracture risk at certain sites (Greco, Francomano et al. 2013). This is partly explained by the fact that obesity seems to decrease the volumetric BMD of cortical bone at some sites, which may result in weaker bone quality and increase fracture susceptibility. This could be due to a synergistic effect of dysfunctional adipokine signaling and impaired mechanotransduction at specific sites. Another possible explanation could be derived from the findings that obesity alters hormonal balances. Changes in the secretion of certain osteoinductive factors may disturb normal bone remodeling and lead to microachitectural degradation of bone tissue, which is a predisposing factor for bone fractures. As mentioned above, obesity is also associated with increased amounts of systemically circulating inflammatory cytokines, which can influence osteoclastogenesis and alter bone remodeling in the favor of resorption (Cao 2011). While obesity itself may have some beneficial effects on bone mass, it appears to negatively alter bone homeostasis, ultimately resulting in increased fracture risk.

2.3 Regulatory factors and their signaling pathways

2.3.1 TGF-β / BMP-signaling

The discovery of the TGF-β superfamily has brought detailed insight to our current knowledge on normal physiology as well as the development and progression of many different pathologic states. The TGF-β superfamily is a large group of different cytokines and growth factors that are structurally related to each other. They are known to regulate cell development, differentiation and proliferation in different tissue types (Table 1). These growth factors can be classified into smaller subdivisions, which include the TGF-β subgroup, the BMPs, the activin/inhibin group, the GDF subfamily and the Müllerian inhibiting substance (MIS). TGF-β binds to type I and type II receptors, which in turn triggers the phosphorylation of specific Smad proteins (Moustakas,

Souchelnytskyi et al. 2001). The Smads are a protein family responsible for intracellular signaling and are composed of smaller subfamilies, which are defined by their functions (Miyazawa, Shinozaki et al. 2002). These activated Smads then form heteromeric complexes with each other and then translocate into the nucleus to affect gene expression. TGF-β members can also initiate activation of non-Smad routes as well such as ERK, JNK and p38 MAPK pathways. (Shen, Li et al. 2014). In addition to their effects in many other tissues, TGF-β family members are essential for the development and growth of bone tissue as well. TGF-β1, one of the most abundant TGF-β cytokines found in the bone matrix, has been shown to induce migration of MSCs to bone resorption sites, which is decisive for osteoblast differentiation (Crane and Cao 2014). Mice deficient of TGF-β2 or TGF-β3 are subjected to numerous skeletal defects that especially affect the bones forming in the fore- and hindlimbs (Dunker and Krieglstein 2000).

BMPs have a myriad of functions in embryogenesis, the delivery of positional information and the formation of both hard and soft tissues (Urist 1965, ten Dijke, Korchynskyi et al. 2003). They promote bone formation through consecutive processes, starting from chemotaxis of progenitor cells and then differentiation of chondrocytes, cartilage formation, proliferation of MSCs, bone cell differentiation and bone mineralization. These growth factors also bind to type II receptors (activin type IIA and B receptors and BMP-specific BMP type II receptors), while activating different Smad molecules (Smads1, 5 and 8) differently than TGF-βs. Additionally, BMPs are also able to bind to BMP type I receptors with higher affinities than other TGF-β members, which usually have a high affinity towards BMP type II receptors. BMPs can also activate non-Smad-mediated pathways (Herpin and Cunningham 2007). The identification of BMPs and their further development into therapeutic agents have had an extensive impact on the current concepts in bone healing, and are today they are regularly applied to treat CSDs in orthopedic or maxillofacial surgery, where the reconstruction of bone is crucial to re-establish its proper form and function. So far over 20 BMP members have been described with most of them having osteoinductive or osteogenetic properties. Only BMP-3 has been shown to be a negative regulator of bone formation (Kokabu, Gamer et al. 2012). Recombinant human forms of BMP-2 and BMP-7 are used in clinical practice but their efficacy compared to autogenous bone grafts, the golden standard for bone grafting, has been questioned in recently published controlled studies (Ronga, Fagetti et al. 2013, Hopkins 2016)

Despite the fact that the numerous events related to TGF-β ligand signaling have been well documented, they do not take into account that the true function of each ligand is also highly dependent on the cellular context, in which the signaling takes place. Furthermore, some of these ligands competitively bind to the same receptors which results in the high-affinity ligand directly inhibiting the signaling pathway of the lower-affinity ligands. For example, activin A binds to ActRIIA with a high affinity, while BMP-7 binds to the same type II receptor with a lower affinity. This leads to the inhibition of the BMP-activated Smad1/5/8 pathway and solely the activin A-induced Smad2/3 pathway transduction is initiated. In the same context, high-affinity ligands can also antagonize other high-affinity factors. On the other hand, the superseded ligands can then bind to a different type I/II receptor. This demonstrates that the complexity of the TGFβ-signaling event is highly dependent on the cellular surroundings, concentrations of available ligands, amounts of competing and antagonizing factors and expression levels of the different type I and II receptors (Aykul and Martinez-Hackert 2016, Khalil, Dotimas et al. 2016). Therefore, results seen in a specific study setup or cell type may not always be reproducible in other experiments.

Name			*Source*
	Systemic function	Function in bone	
TGF-β1	Multifunctional effects	OB proliferation, Inhibits OB apoptosis	*(Janssens, ten Dijke et al. 2005)*
TGF-β2	Multifunctional effects	Induces OB proliferation	*(Erlebacher, Filvaroff et al. 1998)*
TGF-β3	Multifunctional effects	Osteoinductive	*(Ripamonti, Ramoshebi et al. 2008)*
Activin A	Stimulates FSH secretion	Induces OC differentiation	*(Fuller, Bayley et al. 2000)*
Activin B	Wound healing, Developing of Islets	Unknown	*(Bonomi, Brown et al. 2012)*
BMP-2		Osteogenic, induces OB differentiation	*(Cheng, Lou et al. 2001)*
BMP-3		Negative regulator of bone formation	*(Daluiski, Engstrand et al. 2001)*
BMP-4	Neural and lung development		*(Wang, Green et al. 2014)*
BMP-7	Kidney development, brown adipogenesis	Induces OB differentiation	*(Shen, Wei et al. 2010)*
GDF-11	Axial skeleton patterning		*(Lee and Lee 2013)*
GDF-1	Heart and blood vessel development		*(Karkera, Lee et al. 2007)*
GDF-8/ Myostatin	Negative regulator of skeletal muscle		*(McPherron, Lawler et al. 1997)*
MIS/AMH	Regulates folliculogenesis		*(Dewailly, Andersen et al. 2014)*

Table 1: A list of some known TGF-β superfamily members and their functions. OB Osteoblast, OC Osteoclast, FSH Follicle stimulating hormone.

2.3.2 Wnt / β-catenin

Wnt-signaling is best known as a complex signaling network, which is fundamental for correct embryonic development and maintenance of systemic homeostasis. Wnt members interact with specific receptors activating either the canonical, also known as the Wnt/β-catenin pathway or the non-canonical pathways (Komiya and Habas 2008). The canonical signaling pathway appears to more pertinent to bone tissue (Kobayashi, Uehara et al. 2015). The canonical signaling pathway is initiated when Wnt ligands bind to receptor complexes composed of frizzled seven-pass transmembrane receptors (FZD) and low-density lipoprotein receptor-related proteins (LRPs), mainly LRP5 and 6. This leads to the stabilization and accumulation of β-catenin as it escapes phosphorylation that would target its degradation in proteasomes. β-catenin then interacts with Lef1/Tcf transcription factors to regulate gene expression. The role of this pathway in bone metabolism has received a great deal of attention as numerous studies have showed that components of the Wnt canonical pathway are important factors in regulating the differentiation and function of bone cells. For example, β-catenin has been shown to stimulate MSC proliferation into osteoblasts and to inhibit osteoblast apoptosis (Baron and Kneissel 2013). Furthermore, aberrations in Wnt-signaling result in various clinical manifestations. For example, loss-of-function in LRP5 has been shown to cause osteoporosis pseudoglioma syndrome (OPPG) (Gong, Slee et al. 2001), and Wnt1 mutations induce early-onset osteoporosis and osteogenesis imperfecta (Laine, Joeng et al. 2013).

Sclerostin, a glycoprotein encoded by the *SOST* gene, is a Wnt-signaling antagonist, as it binds to LRP5/6 to prevent its interaction with Wnt ligands. This leads to the phosphorylation and subsequent degradation of β-catenin and, therefore sclerostin is a negative regulator of bone formation and osteoblast differentiation (Moester, Papapoulos et al. 2010). The recent finding that mature osteocytes can secrete sclerostin has significantly added our current knowledge on bone biology. It has been demonstrated to not only have an anti-anabolic effect by inhibiting the differentiation of osteoblast but also the capability to promote osteoclastogenesis. Indeed, SOST knock-out models exhibit increased

bone mass and accelerated fracture healing in numerous animal models (Li, Ominsky et al. 2008, Alzahrani, Rauch et al. 2016). These data demonstrate it to be an essential regulator of bone metabolism and highlights the role of osteocytes as orchestrators of bone remodeling. The use of monoclonal SOST antibodies have emerged as a potential therapeutic approach in order to increase bone mass and strength. Currently, romosozumab, a sclerostin antibody, is under clinical investigation for the treatment of osteoporosis (Cosman, Crittenden et al. 2016).

2.3.3 RANKL / OPG / RANK

The RANKL/OPG/RANK signaling pathway is an essential regulator of osteoclast differentiation and subsequently of bone turnover. Not only does it control the differentiation, activity and survival of osteoclasts, but the balance of the interplaying ligands acts as a marker in many pathological processes such as osteoporosis, osteoarthritis and periodontitis (Boyce and Xing 2007). RANKL is a soluble protein that belongs to the TNF superfamily. It is involved in many physiologic events, such as lactation and mediating inflammatory processes, but undoubtedly it is best known for its ability to stimulate osteoclast differentiation by regulating osteoclastogenesis and releasing immature progenitors into circulation. RANKL binds to its receptor, RANK, which leads to the recruitment of TRAFs. TRAFs attach to the cytoplasmic domain of RANK and activate the nuclear factor–κB (NF-κB), which translocates to the nucleus to induce the expression of a set of genes that promote osteoclast differentiation (Khosla 2001). Together with M-CSF, RANKL is a fundamental mediator of osteoclastogenesis and the activity of mature osteoclasts. Translational studies have shown that knockout mice lacking RANK are not able to produce osteoclasts (Li, Sarosi et al. 2000), and RANKL knockout mice exhibit severe osteopetrosis (Kong, Yoshida et al. 1999). Interestingly, cells of the osteoblast lineage are the major source of RANKL in bone to regulate the bone resorption. In contrast, OPG is a decoy receptor for RANKL produced mainly by osteoclastic cells, which prevents RANKL from binding to RANK receptor. This inhibits RANK signaling and, thus, regulates osteoclast-dependent bone remodeling. Over-expression of OPG leads to increased bone mass and BMD (Ominsky, Stolina et al. 2009), while OPG knockout mice develop early onset of osteoporosis (Bucay, Sarosi et al. 1998). In addition to different bone cells, RANKL and OPG are produced by other cell types that can also regulate bone metabolism. In periodontitis,

RANKL is mainly derived from B and T lymphocytes (Kawai, Matsuyama et al. 2006). The B cells have also been identified as a major source for OPG as B-cell deficiency (and thus loss of B-cell derived OPG) has been shown to induce osteoporosis in mice (Li, Toraldo et al. 2007). The RANKL/OPG ratio has been defined as the seesaw between the promotion and suppression of osteoclastic activity (Hofbauer and Schoppet 2004). Imbalances in this equilibrium can then tilt bone remodeling in favor of increased bone formation or enhanced bone resorption.

2.3.4 Parathyroid hormone

PTH is a polypeptide secreted by the chief cells of the parathyroid gland and is best known for its fundamental role in the accurate maintenance of calcium homeostasis and serum calcium levels. PTH binds to PTH receptors, mainly PTHr1, which results in the activation of several pathways. It seems that the protein kinase A pathway is essential for the bone anabolic effect, since the expression of the proto-oncogene c-fos is upregulated by this pathway (Aslan, Andersen et al. 2012). Interestingly, PTH is also able to initiate a catabolic response by inducing bone resorption. The response is heavily dependent of serum concentrations of PTH. A surge in PTH levels leads to an anabolic response while continuous infusion results in a negative balance of bone turnover (Frolik, Black et al. 2003). The human recombinant protein PTH 1-34, teriparatide, is the only FDA-approved bone anabolic agent for the treatment of osteoporosis, and it has been shown to effectively increase BMD, trabecular bone architecture and cortical bone thickness.

2.3.5 Insulin-like growth factors

IGFs are cytokines that are structurally related to insulin and participate in the modulation of normal physiology in numerous ways. This growth factor family consists of two secreted growth factors, IGF-1 and IGF-2 respectively, two IGF receptors and six IGF binding proteins (IGFBPs), which inhibit IGF actions by preventing them from interacting with IGF receptors. Bone tissue, along with muscle tissue and the liver, is an important source for IGFs as they are stored in their inactive forms but are then released in active form during bone remodeling. IGFs have been shown to regulate bone remodeling. Overexpression of IGF-1 in osteoblasts greatly enhances trabecular bone formation rate by increasing osteo-

blast activity (Zhao, Monier-Faugere et al. 2000) and systemically delivered IGF-1 accelerates fracture healing by improving endochondral ossification (Myers, Yan et al. 2012). Furthermore, IGFs are vital for the anabolic effect of PTH on bone (Tahimic, Wang et al. 2013). Further studies will address the question whether modulation of IGF activity could be used in the treatment of osteoporosis in the clinic.

2.3.6 Fibroblast growth factors

FGFs are a group of cytokines that regulate cellular communication during development, angiogenesis, wound healing and osteoblastogenesis by interacting with four different FGF receptors (FGFR). To date over 20 FGFs have been identified and, depending on the method of classification, they can be divided into canonical, intracellular and endocrine FGFs. FGF-8 is a major regulator of the expression of Core-binding factor alpha 1/Runt-related transcription factor 2(Cbfa-1/Runx2) but has also been suggested to promote osteoclastogenesis (Lin, Callon et al. 2009). FGF2 controls the lineage determination of MSCs to either of adipocytes and osteoblasts in favor of bone formation (Xiao, Sobue et al. 2010). Recently, the effects of FGF-23 on bone metabolism have received attention, because it has been shown to mediate renal phosphate intake and vitamin D metabolism and thus also bone metabolism (Quarles 2012). The roles of FGFRs in skeletal development have been well documented as many FGF receptor mutations manifest as clinical phenotypes such as dwarfism and craniosynostosis syndromes. For example, mutations in the gene encoding the FGFR3 results in achondroplasia, also known as short-limbed disproportionate dwarfism, and dysfunctions of FGFR2 induces Crouzon syndrome, a state characterized by craniosynostosis-induced facial deformities (Lin, Callon et al. 2009).

2.3.7 Platelet derived growth factors

PDGFs were first identified, and named accordingly, decades ago as dimeric cytokines originating from thrombocytes. They can enhance the proliferation of MSCs (Heldin, Westermark et al. 1979). Further research has revealed that PDGFs have essential roles in many physiological processes such as embryogenesis, angiogenesis, wound healing and development of the central nervous system. To date 5 PDGF ligands have been characterized, of which PDGF-BB is considered to be the universal PDGF (Hollinger, Hart et al. 2008) They exert

their actions by binding to specific PDGF receptors (PDGFR-a and b) and induce the intracellular phosphorylation of specific tyrosine kinases. PDGFs also participate in the regulation of bone growth by stimulating both osteo- and angiogenesis (Shah, Keppler et al. 2012). Clinical applications of PDGF are also being investigated, since in translational studies local and systemic administrations of PDGFs have been shown to accelerate fracture healing and enhance bone regeneration in CSD-animal models (Gothard, Smith et al. 2014).

2.3.8 Activins

2.3.8.1 *Classification and structure*

Activins are pleiotropic growth factors belonging to the TGF-β superfamily and were first identified as gonadal proteins that participate in the secretion of follicle stimulating hormone (FSH) (Ling, Ying et al. 1986, Vale, Rivier et al. 1986). The isolation and further characterization of these factors revealed them to participate not only in reproduction but also in regulation of systemic events and maintenance of homeostasis. Activins are classified according to their structural composition, which is based on the dimerization of different inhibin subunits linked together by disulphide bonds. To date, inhibin βA, βB, βC and βE (known as mature monomers) have been identified but notably βA and βB (coded by the genes *Inhba* and *Inhbb*, respectively) have been established as the building blocks for activins. The hetero- or homodimerization of these subunits gives rise to different activin molecules. Activin A is composed of two βA subunits, activin B is formed by two βB subunits, whereas activin AB consists of one βA and one βB subunit. The formation of activins is highly dependent on the dimerization of activin and inhibin proproteins and their further cleavage to form mature activin dimers named above. Other activins, such as activin C and E have also been identified, although their expression seems to be confined only to specific tissues, such as the liver, instead of having systemic effects. These proteins have been proposed to antagonize activin A signaling (Chabicovsky, Herkner et al. 2003, Mellor, Ball et al. 2003).

Ligands of TGF-β superfamily members share structural similarities such as the cysteine knot scaffold (Sun and Davies 1995). These scaffolds are composed of six cysteine residues and they are partly responsible for the high resilience and stability of TGF-β molecules. Crystallography studies have demonstrated that activins are composed of three structural domains; the "open

hand”-element, the “finger-like” domain and the “wrist” unit. The interaction between activin molecules and their corresponding type II receptors occurs in the finger region, whereas the interplay between activin molecules and activin type I receptors takes place in the wrist domain (Thompson, Woodruff et al. 2003). One theory suggests that the conformation of activins changes during the binding processes: at first activins are structurally tightly packed and closed, which allows them to bind only to type II receptors. After binding to a type II receptor, the conformation of the activin molecule changes as it extends and opens up. This could possibly expose more binding sites and allow for the phosphorylation of type I receptors (Greenwald, Vega et al. 2004).

2.3.8.2 *Receptors and signaling pathway*

Activins exert their actions by interacting with specific single-pass transmembrane receptors that activate specific serine/threonine kinases and their downstream signaling pathways eventually resulting in altered gene expression. These are known as activin type I receptors (ActRIs), or activin receptor-like kinases (ALKs) and activin type II receptors (ActRIIs). To date, seven ALKs have been identified, of which ALK 2 and 4 participate in activin signaling, while ALK 2, 3 and 6 are required for BMP-signaling (Nohe, Keating et al. 2004). Two ActRIIs, type IIA and IIB, have been characterized. They are structurally highly homologous and share similar signal transduction mechanisms but differ slightly in their binding affinities to different ligands and in their roles in developmental processes. (Oh, Yeo et al. 2002). Type II receptors have been shown to bind numerous growth factors other than activins such as myostatin (only ActRIIB) (Lee and McPherron 2001), inhibin A and B (Chapman, Bernard et al. 2002), BMP-2/6/7 (Macias-Silva, Hoodless et al. 1998, Ebisawa, Tada et al. 1999, Liu, Zhang et al. 2012) and GFD-5/11 (Nishitoh, Ichijo et al. 1996, Oh, Yeo et al. 2002). Structurally, activin receptors are composed of an extracellular, ligand-binding domain, a single transmembrane domain, and an intracellular serine-threonine kinase domain.

In addition to other TGF-β ligands, extracellular antagonists are also important regulators of activin ligand binding and receptor activity. Inhibins compete with activins for the same type II receptors, which prevents the recruitment of the type I receptor (Lewis, Gray et al. 2000). Follistatin, a natural antagonist for activin A, acts as an activin-binding protein that prevents activins from interacting with type II receptors and is a pivotal modulator of activin bio-

availability (Nakamura, Takio et al. 1990). Follistatin has at least two functional isomers and can act as a circulating factor, known as follistatin 315, or in its membrane-bound form, known as follistatin 288. Other membrane bound inhibitors of activins, known as BAMBIs or more specifically bone morphogenetic protein and activin embrane-bound inhibitors, have also been described (Luo, Hutley et al. 2012). Moreover, other TGF-β family members are able to bind to activin receptors and may act as competitive antagonists, adding another layer of complexity to this signaling environment.

The activin signaling pathway is initiated by activin binding to either the type IIA or type IIB receptor, which then triggers the recruitment and phosphorylation of the type I receptor (either ALK-4 or ALK-7) inducing its kinase activity (Figure 4). The phosphorylation of the type I receptor is required to activate the downstream signaling pathway (Attisano, Wrana et al. 1996). There are differences between activins and their affinities towards type I receptors. Activin B signals through ALK-7, whereas activin A uses ALK-4 as its signaling mediator (Harrison, Gray et al. 2003). The activation of activin receptors is responsible for initiating the Smad pathways. In activin signaling, receptor-regulated Smads (R-Smads) and common mediator Smads (co-Smads) are essential factors for proper signal transduction. Even though these Smads are structurally similar, they may differ in their co-factor recruitments and function (Schmierer, Schuster et al. 2003). Signal transduction through activin receptors results in phosphorylation of two R-Smads known as Smad2 and Smad3. They are then linked together with a specific co-Smad, Smad4, and this Smad-complex translocates to the nucleus, where it interacts with activin-responsive elements and transcriptional co-activators or co-repressors to target specific genes and to modulate their transcription (Table 2).

Competitive binding between activins and BMPs is also thought to be important in regulation of these pathways. The mechanisms behind these observations are not clear, but it seems that the specific interactions between the type II receptor and the type I receptors regulate the activation of the different Smad pathways. For example, activin A has been shown to antagonize the binding of BMP-6 and -9 to ActRIIA/B receptors and to inhibit the activation of the Smad1/5/8 pathway induced by these BMPs. On the other hand, activin A had no effect on the binding of BMP-2 (Olsen, Wader et al. 2015). ´This suggests that even though the activin signaling cascade initiated by the binding of activin A takes place, the simultaneous activation of BMP-2 induced signaling can also occur, another example of the complex nature of TGF-β ligand binding.

Soluble activin type IIA/B receptor fusion proteins (ActRIIA/B-Fc) have been produced, aimed at investigating the effects of activin receptor ligands on different animal models. These fusion proteins consist of two parts: the ectodomain of the human activin type II receptor and the Fc-chain of the human immunoglobulin G1 or mouse immunoglobulin G2, which therefore lack the signal transducing transmembrane and intracellular domains. These ActRIIA/B-Fc molecules function only as decoy receptors to harvest ligands that would normally bind to ActRIIA/B with a high affinity, subsequently inhibiting the activin signaling pathways. However, as these receptors are able to bind multiple ligands, these molecular tools cannot be used to differentiate the effects of each specific cytokine or growth factor but only the effects of the group of ligands binding to this specific receptor.

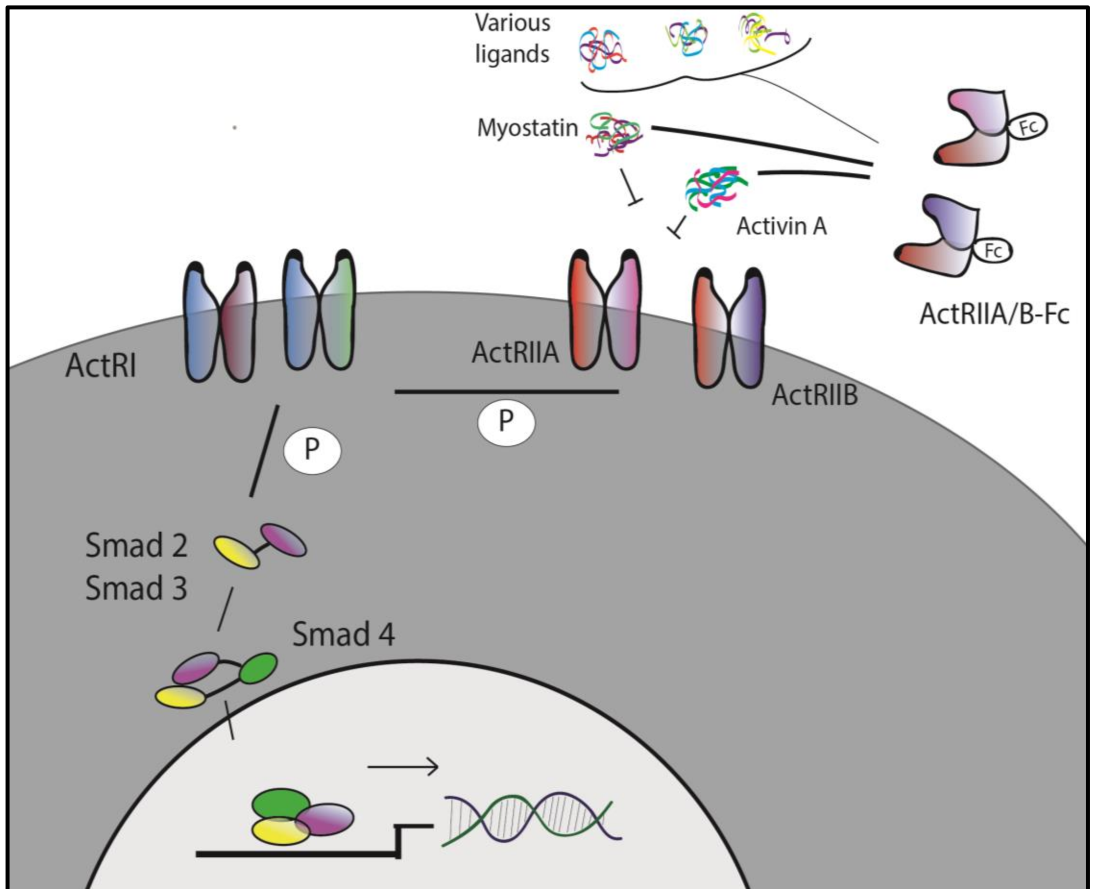

Figure 4: Simplified illustration demonstrating the activin signaling pathway. Activin A binds to either type IIA or IIB receptors, which triggers the activation of the type I receptor. This in turn phosphorylates the Smad2/3 pathway ultimately reaching the nucleus to modulate gene expression. ActRIIA/B fusion protein receptors are able to bind activin A and myostatin with a high affinity and a number of other ligands with lower affinities to inhibit them from activating their signaling pathways

Table 2: TGF-β ligands, their binding affinities towards activin type II receptors and the corresponding pathways they activate

Lig-and	*ActRIIA*	*ActRIIB*	*ALK1*	*ALK2*	*ALK3*	*ALK4*	*ALK5*	*ALK6*	*ALK7*	*SMAD 2,3*	*SMAD 1,5,8*
Activin A	+++	+++				X			(X)	X	
Activin B	+++	+++				X			X	X	
Myo-statin		++				X	X			X	
GDF11	+++	++			X	X	X			X	X
BMP-2	+	+			X			X			X
BMP-3		+									X
BMP-4	++	++			X			X			X
BMP-6	+	++		X	X			X			X
BMP-7	+++	+		X				X			X
BMP-9	+	++	X								X
BMP-10	++	++	X								X

2.3.8.3 Roles in normal and pathophysiology

Activins are referred to as pleiotropic growth factors as they have systemic roles in maintaining homeostasis. They are important regulators of the development and functioning of many tissue and cell types. Activin A was first identified to stimulate the secretion of follicle stimulating hormone (FSH) through the hypothalamus-pituitary gland axis, which regulates numerous phases and events in the reproductive system (Vale, Rivier et al. 1988). Further studies revealed activin A to be crucial in the regulation of ovarian cell functions, folliculogenesis and the proliferation of testicular cells (Ethier and Findlay 2001). The expression of activins in different tissue types demonstrate that activin signaling takes place through autocrine, paracrine and endocrine mechanisms (Xia and Schneyer 2009). Indeed, activins have been shown to be expressed in numerous

extragonadal tissue, types such as bone, bone marrow, the pituitary gland, the pancreas and the lungs. The expression and overexpression of activins have been identified in numerous diseases, which suggests them to be useful markers of different pathologic states.

Activin A expression is induced during acute inflammation and it has been established as an important regulator of these events (de Kretser, O'Hehir et al. 2012). However, its role is dependent on the phase of inflammatory cascade. It seems that activin A is able to activate macrophages in the early stages of inflammation to secrete essential inflammatory mediators, such as ILs and TNF-α. As the macrophages are stimulated and the inflammation progresses into a more active state, the effects of activin A are inhibitory in terms of impeding the maturation of inflammatory markers and promoting the expression of IL receptor antagonists (Hedger and de Kretser 2013). Therefore, depending on the conditions, activins can be regarded as pro- or anti-inflammatory. Activin A expression has been shown to be elevated in many diseases, which are characterized by systemic states of inflammation, including chronic obstructive pulmonary disease and inflammatory bowel diseases (Sideras, Apostolou et al. 2013). These findings suggest activins to be key modulators of phases of inflammation, which can partly help explain the pathogenesis behind many inflammatory disorders.

Due to globally increasing prevalence of obesity and other metabolic disorders, the relationship between activins and metabolic processes has also been recently investigated. Activins and their receptors are highly expressed in adipose tissue. Activin A has been shown to regulate adipocyte formation by increasing adipocyte proliferation (Zaragosi, Wdziekonski et al. 2010). However, when adipocytes start to differentiate, activin A levels robustly fall suggesting that activin A is a negative regulator of adipocyte differentiation. In addition, treatment with a specific activin A-antibody suppressed adipocyte proliferation. Obesity is also known to induce metabolic ramifications. Different types of diabetes are characterized by the destruction or dysfunction of pancreatic islet cells, impaired glucose metabolism and insulin resistance. This in turn can lead to serious complications affecting the cardiovascular and nervous system. Activins, especially activin B, have been shown to promote the formation of pancreatic islets (Yamaoka, Idehara et al. 1998) and improve the transdifferentiation of alpha cells to beta cells (Andrzejewski, Brown et al. 2015). Furthermore, activin A has also been suggested to directly decrease tissue sensitivity to insulin, which results in increased blood glucose levels (Hashimoto and Funaba 2011),

while transgenic mice expressing mutant, dysfunctional forms of ActRIIB result in islet hypoplasia and reduced glucose tolerance (Yamaoka, Idehara et al. 1998). Conversely, ActRIIB-Fc administration worsens glucose clearance in diabetic mice (Wang, Guo et al. 2015), while systemic activin levels have been reported to be unaltered in patients with type II diabetes (Weigert, Neumeier et al. 2009). It is plausible to speculate that the effects of activins on glucose homeostasis and insulin production, as well as the mechanisms behind the altered glucose metabolism, could be at least in part due to alterations in other tissues than pancreas. Current concepts suggest that the development of metabolic disorders is highly dependent on liver function, too. While it seems that activins are critical regulators of the liver homeostasis (Ungerleider, Bonomi et al. 2013), further studies are needed to determine, whether activin signaling in the liver modulates metabolic perturbations of obesity and diabetes.

Recent observations have shown that activins have distinct roles in the pathophysiology of different cancer types. The incidence rates of specific cancers have grown during the past few decades and significant efforts are on-going to develop new treatment methods in all fields of oncologic research in basic, translational and clinical studies. Due to the variance in disturbances of the genetic code behind each neoplasm, each malignancy has unique traits regarding biological behavior, and treatment approaches should be specified to target each specific cancer type. The development and progression of cancer is dependent on a myriad of different factors and under favorable conditions, cancer cells are able to promote angiogenesis, spread to metastasize and evade cell growth suppressors (Hanahan and Weinberg 2011). The roles of activins in cancer development and progression have not yet been clearly established. Studies have suggested that activins both promote and suppress malignancies depending on the context. For example, activins inhibit the progression of breast, pancreatic and prostate cancer (Ungefroren, Schniewind et al. 2007, Katik, Mackenzie-Kludas et al. 2009). One hypothesis is that, under certain conditions, activins have anti-angiogenic properties that restrict the invasion of malignant cells to surrounding tissues (Antsiferova and Werner 2012). On the other hand, activins can also have proliferative, mitogenic effects on tumor cells in tissues, where activins are important regulators of normal tissue growth, such as the testis and endometrium, but possibly also in other cancer types, such as lung cancers and esophageal carcinomas (Leto 2010). These varying effects can be partially explained by the wide spectrum of different signaling pathways regulating the cellular activity of cancers. For example in pancreatic carcinomas, Smad2 and 4 are mutated, which

impairs the activin signaling pathway, suggesting an essential role for activins in suppressing carcinogenesis (Tsuchida, Nakatani et al. 2009). In oral squamous cell carcinoma, overexpression of activin A has been linked to tumor progression and increased patient morbidity (Chang, Kao et al. 2010). Therefore, activin levels could be used as novel diagnostic biomarkers in the pathogenesis and treatment of certain malignancies.

Bone matrix is an abundant storage of activins, especially activin A, and recent research has aimed to explain their roles in bone metabolism. The true role of activins and their actions on regulating the functions of different bone cells are yet unclear, and some discrepancies still remain over whether activins are positive or negative regulators of bone remodeling. Activins appear to have an inhibitory effect on osteoblast differentiation as most *in vitro* studies indeed show activins to reduce the mineralization capacity of osteoblasts and negatively regulate bone formation (Eijken, Swagemakers et al. 2007, Alves, Eijken et al. 2013). In addition, the majority of reports have suggested that activin A to enhances bone resorption and has a synergistic effect with RANKL on osteoclast differentiation (Sakai, Eto et al. 1993, Fuller, Bayley et al. 2000). However, discrepancies in the role of activins in bone arise when shifting from *in vitro* studies to *in vivo* studies. Some animal studies have shown that injections of activin A improve bone mass and strength in rats and mice, proposing activins to have pro-osteogenic properties.

These controversies are most likely due to unexplained, systemic effects of activins that require further investigations and are likely to be highly dependent of the study setup in question. It is possible that activins might have roles in both bone formation and resorption and the desired effect might be dependent on activin levels, hormonal status and/or the cellular context (Hirotani, Ohtsuka-Isoya et al. 2002).

In the study by Sakai et al., the authors demonstrated that intramuscular injections of recombinant activin A increased bone mass and strength of the lumbar vertebrae in ovariectomized (OVX) rats (Sakai, Fujita et al. 2000). This suggests that activins are anabolic regulators of bone mass. A curious observation was noted as the anabolic effects of activin were actually negatively dose-dependent, that is, lower concentrations of activin A had a more beneficial effect on bone mass than higher concentrations. Furthermore, the authors only reported the effects on the 4th lumbar vertebrae and not on long bones. There was also no data presented on the serum levels of bone turnover markers or other biomarkers or on molecular analyses, nor was any convincing mechanism proposed for the

putative bone anabolic effect of activin A. In another study, Hirotani and colleagues reported that topical infusion of activin A with bone grafts increased bone mass (Hirotani, Ohtsuka-Isoya et al. 2002). Even though there was a slight increase in woven bone formation surrounding the transplanted grafts, the authors also noted a significant increase in Tra(c)p/Acp5-positive osteoclast in the grafted bone. This partly supports the notion that activins are promoters of osteoclast-dependent bone resorption and that the development of woven bone could be similar to the mechanisms, by which activin A enhances BMP-induced bone formation (Ogawa, Schmidt et al. 1992)

Similarly, the effects of activins on regulating fracture healing, where both bone formation and remodeling are required, have also been controversial. In 1998, Nagamine et al. published an immunohistochemical study, where they displayed that activins and activin receptors to be expressed in the different cells participating in fracture healing (Nagamine, Imamura et al. 1998). Further, Sakai and colleagues reported that activin A injections to the fracture site improved fracture healing in a rat fibula fracture model (Sakai, Miwa et al. 1999), suggesting activin A to be a pro-osteogenic molecule that enhances bone repair. However, the authors only reported results of the first three weeks of fracture healing, while the late phases of bone healing in rats can occur up to four to six weeks after the fracture takes place. Therefore, the study setup was not ideal because the report did not feature the effects of bone formation inside the callus. It is possible that, due to the known proinflammatory functions of activin, the given injections indeed did promote the initial phases of fracture healing reinforcing the hypothesis that the effects of activin on fracture healing are dependent on the timing of the administration. On the other hand activin, A administration could also potentially promote angiogenesis or cartilage formation, which would accelerate fracture healing during the first weeks.

The recent reports of the use of soluble activin type II fusion protein receptors have demonstrated that activins/activin receptor ligands are predominantly negative regulators of bone formation. These studies have aptly demonstrated that inhibition of activins with ActRIIA/B-Fc has great anabolic and anti-resorptive effects on bone tissue in different animal models including healthy mice (Pearsall, Canalis et al. 2008), ORX mice (Koncarevic, Cornwall-Brady et al. 2010) as well as in different transgenic mouse models (Bialek, Parkington et al. 2014, DiGirolamo, Singhal et al. 2015). Especially the work by Bialek and colleagues thoroughly and convincingly dedicated the potency of ActRIIB-Fc as an anabolic bone agent. Not only did they report that treatment with ActRIIB-Fc

increases essential parameters of bone formation such as mineral apposition rate and bone formation rate, but that enhanced bone mass also occurred in *MSTN* $^{-/-}$ and *BMP-3* $^{-/-}$ mice. This indicated that myostatin or BMP-3 were not involved in the regulation of bone formation via ActRIIB. This further suggests that these effects are mainly due to the inhibition of activin A. These findings reinforce the concept of activins being negative regulators of bone formation.

Recently, Fowler and colleagues presented convincing data that activins are negative regulators of osteoclast differentiation and function in bone marrow macrophage cultures (Fowler, Kamalakar et al. 2015), in contrast to whole bone marrow cultures, where activin A has shown to be a potent enhancer of osteoclast formation (Gaddy-Kurten, Coker et al. 2002). In this report, the authors concluded that activin A suppressed the differentiation and bone resorption of osteoclasts, while decreasing their motility. The authors stated that their results directly question the current knowledge regarding activin A action and bone. While this requires further addressing and provides valuable information on the biological basis of activins, the findings do not seem to overpower the convincing and encouraging results from the *in vivo* studies. However, this report does challenge whether the positive bone effects are indeed solely derived from the actions of activins or whether other ligands are responsible as well. Most likely the explanations behind these discrepancies are due to the complex signaling entity of TGF-β family members. While it may be that activin A can have negative and positive effects on some phases of bone remodeling, the *in vivo* or *ex vivo* effects are context-dependent and the heterogeneity of these models can produce differing outcomes.

Collectively, the results provided by these reports have initiated the emerging of the inhibition of activin ligands as a novel approach for targeting musculoskeletal diseases. However, these reports have also left unanswered questions regarding the specific mechanisms that regulate these effects, and the systemic actions these ligands may have, which are then blocked by the fusion receptors. Therefore, due to the inadequate state of the current literature, we set out to investigate the effects of a soluble ActRIIB-Fc on bone formation and body composition in several animal models.

3 AIMS OF THE STUDY

Despite recent advances in contemporary treatment methods, musculoskeletal disorders are still a significant burden for public health. Systemic approaches may alleviate some of the signs and symptoms of these disorders but adverse effects can cancel out some of the beneficial responses. Remedial approaches for bone disorders are particularly complex to develop as the delicate relationship of coupling between bone formation and resorption has a tendency to normalize after pharmaceutical interventions. Therefore we hypothesized that the inhibition of the activin signaling pathway could have an anabolic effect on bone homeostasis as well as improve other parameters associated with musculoskeletal disorders.

In order to investigate the effects of a soluble activin type IIB receptor-Fc protein on bone formation and body composition in different animal models, the aims for the presented studies were as follows:

1) Does the use of ActRIIB-Fc affect bone mass and strength in a muscle dystrophy (mdx) mouse model? (*Study I*)

2) Does physical exercise module the effects of ActRIIB-Fc treatment on bone tissue? (*Study I*)

3) Does ActRIIB-Fc treatment promote osteogenesis and improve biomechanical strength of the fracture callus in a closed tibial fracture mouse model? (*Study II*)

4) Can treatment with ActRIIB-Fc prevent ovariectomy-induced bone loss in OVX mice? (*Study III*)

5) Does ActRIIB-Fc treatment prevent the negative metabolic changes resulting from OVX? (*Study III*)

4 MATERIALS AND METHODS

4.1 Ethics statement

The treatment of the used animals was in strict accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocols used in the making of this thesis were approved by the National Animal Experiment Board (Permit Number: ESLH-2009-08528/Ym-23 for *Study I* and ESAVI-11044/04.10.07/2014 for *Studies II, III*).

4.2 Production of soluble activin type IIB receptor

The soluble ActRIIB-Fc used in these studies was kindly provided by Dr. Olli Ritvos. The production of this protein was based on the fusion of the ActRIIB-part with the Fc-chain. The ectodomain of human ActRIIB was amplified by PCR from a plasmid containing the human ActRIIB sequence. The Fc domain was then produced from a human IgG1-Fc domain with a COOH-terminal His6 tag, which was amplified by PCR from the plgPlus expression plasmid. These products were subcloned into vectors, sequenced and fused before cloning them into the expression vector. Finally, the expression vector was transfected into Chinese hamster ovary (CHO) cells, which were grown in a suspension culture.

4.3 Animals and experimental designs

Animals were kept in cages under standard laboratory conditions (temperature 22°C, light from 6:00AM to 6:00PM). Water and food pellets [Soya-free in *Studies II, III (*RM3E, special diets services, UK)] were available ad libitum, excluding fasting periods before euthanization and glucose- and insulin tolerance tests in *Study III*. Animals that developed rashes or post-operative complications were omitted from the studies (n=3 in *Study II*, n=4 in *Study III*.) No unexpected or adverse effects that were considered to be caused by ActRIIB-Fc injections were noted.

Study I

In this experiment six to seven week old male muscle dystrophy (mdx) mice from C57Bl/10ScSnJ background were used (Jackson laboratory, Bar Harbor, Maine, USA). 32 mice were randomly assigned to either 1) phosphate-buffered saline (PBS) control group, 2) ActRIIB-Fc group, 3) PBS running group or 4)

ActRIIB-Fc running group. All animals received an intraperitoneal PBS or ActRIIB-Fc (5mg/kg dose) injection once a week. In the running groups, running wheels were placed inside the cages. Voluntary wheel running was chosen as the mode of exercise for the mice, since it has been shown to benefit mdx mice in terms of muscle growth and bone health (Call, McKeehen et al. 2010, Ma, Turpeinen et al. 2011). At the end of the experiment, mice were euthanized by CO_2 and cervical dislocation, after which tissue samples were harvested.

Study II

For this experiment 10-12 week old C57Bl/6 male mice were evenly divided into four groups. All of the animals were subjected to a closed, diaphyseal tibial fracture and its surgical treatment, and were then administered with intraperitoneal injections of PBS or ActRIIB-Fc (5mg/kg) once a week for two or four weeks. The mice were then euthanized on either day 15 or day 29 by CO_2 overdose and cervical dislocation, after which samples were collected.

Study III

In this study, seven to nine-week old C57Bl/6 female mice were subjected to an OVX or sham surgical procedure. These mice then received intraperitoneal injections of either PBS or ActRIIB-Fc (5mg/kg) once a week for 7 weeks. Magnetic resonance imaging (MRI) was used to assess body composition during weeks 1, 5 and 8. Glucose- and insulin tolerance tests (GTT and ITT) were performed eight days and one day, respectively, before the mice were killed. Calcein and demeclocyclin injections were administered intraperitoneally ten and three days before euthanization, respectively. Mice were euthanized by CO_2 overdose and cervical dislocation after which blood and tissue samples were gathered.

4.4 Surgical procedures

Study II

Subcutaneous injections of buprenorphine (0.05mg/kg) and carprofen (5mg/kg) were administered under isoflurane anesthesia (250-400ml/min 2.5%) and aseptic surgical conditions. A vertical incision was then made over the patella of the right hind limb. The patellar tendon was then incised vertically to expose the proximal head of the tibia. A 25-gauge needle was used to drill a hole through the cortical bone above the tibial tuberosity. A stainless-steel rod (0.2mm diame-

ter) was then inserted into the intramedullary canal reaching the distal end of the tibia. After wound closure, a standardized, closed diaphyseal tibial fracture was performed as reported before (Bonnarens and Einhorn 1984, Hiltunen, Vuorio et al. 1993). The right hind leg of the animal was placed on two resting pins so that the blunt blade of the guillotine-like apparatus struck the anterolateral surface of the lower midshaft of the tibia when a weight of 220 g attached to a spring was dropped from a height of 300 mm. This resulted in a closed fracture induced by three-point bending. Post-operatively the animals were housed in individual cages, closely monitored and received injections of buprenorphine and carprofen for the first two days.

Figure 5: Insertion of steel rod into the intramedullary canal

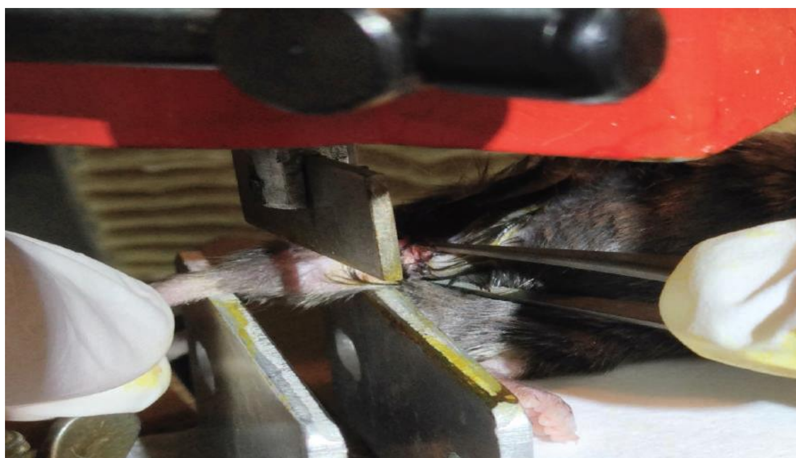

Figure 6: Blunt blade of the guillotine-like apparatus inducing a closed, diaphyseal fracture

Study III

Subcutaneous injections of carprofen (5 mg/kg) and buprenorphine (0.05 mg/kg) were given under isoflurane anesthesia (250-400 ml/min 2.5%) and aseptic surgical conditions. A midline incision was done in the mid-dorsum of the mouse. The cutaneous and subcutaneous tissues were then gently separated laterally from the underlying muscle tissue. Then a small incision was done over the muscle layer to expose the ovary. The ovary was then gently raised and removed from the surrounding fat tissue by cauterization. The ovarian horn was then released back into the peritoneum. A self-resorbing suture was placed over the muscle layer and a non-resorbing suture was placed on the skin. The contralateral side was operated the same way. The sham procedure was done as above, except that after raising the ovaries from their surroundings, they were gently put back into place and left intact. The mice were administered with post-operative injections of carprofen, and monitored regularly for the next 2-3 days.

4.5 Body weight measuring

During all the experiments, the animals were weighed with a high quality scale that was calibrated accordingly before use with a 200.00g weight. The animals were weighed before each surgical procedure, each injection, each MRI scan and euthanization.

4.6 Voluntary wheel running

For the running groups, custom-made running wheels were placed into the cages, and they were available for use excluding 24 hours before euthanization of the mice. As voluntary wheel running may benefit both muscle and bone tissue (Ma, Turpeinen et al. 2011) in mdx mice, it was chosen as the modality of exercise for this experiment. The running distance was recorded 24 hours daily.

4.7 Gathering of tissue samples

In *Study I* the hind leg muscles were carefully excised, weighed and stored immediately after the animal was killed. The femurs, tibias and lumbar vertebrae were also gathered and stored in 3.7% formaldehyde and subsequently in 70% EtOH.

In *Study II* the calluses were extracted by removing the fractured tibia from its' proximal and distal joints and stored in either 3.7% formaldehyde and 70% EtOH, wrapped in PBS-soaked gauzes and tin foil and stored in -20°C, or snap-frozen and stored in -70°C.

In *Study III* the liver, the brown adipose tissue depots from under the scapulae, the retroperitoneal, gonadal, the mesenteric and inguinal white adipose depots were excised with precision, weighed and stored accordingly. Serum samples were gathered via exsanguination by cardiac puncture. The uteri were also collected and weighed immediately to confirm the success of the OVX procedure. Both femurs and tibias from the hind legs were gathered for bone analyses as described above.

4.8 Micro-computer tomography (μCT) analysis

X-ray microcomputer tomography (Skyscan 1070, Kontich, Belgium) was used to image and analyze the bone structure of the cortical and trabecular bone in all of the studies. Scanning was done with identical settings which included a voxel resolution of 5.33μm, X-ray potential of 70kVp, a current of 200μA and an integration time of 3900ms. The samples were placed in transparent, plastic cylinders and wrapped in paraffin to ensure immobility and moisture during the scanning process. The object rotated in 0.45° throughout the scanning for a total revolution of 182.45°. After scanning the images were reconstructed (Nrecon 1.4, Skyscan) with identical settings. Misalignment of the samples was less than 3 in all the samples, ring artifacts reduction was set to 11, beam hardening correction to 95% and the intensity gap was set to 0.017-0.13. The regions of interests (ROI) were drawn (CTan 1.4.4, SkyScan) and the results were then quantified and analyzed. BMD values were calibrated by using two phantoms (calcium hydroxyapatite discs with densities of 0.25 g/cm^3 and 0.75 g/cm^3, respectively) during the scanning phase.

In *Studies I* and *III*, the starting points off the ROI for the trabecular bone were 2.4mm proximally of the distal femoral growth plate with a new ROI drawn every 0.2mm for a total length of 3 mm and for the cortical bone 12 mm proximally of the distal growth plate for a total length of 2 mm. In the 2nd lumbar vertebrae the starting point for the ROI extended 3 mm distally of the cranial growth plate for a total length of 3 mm covering the body of the vertebra excluding its cortical borders.

In *Study II*, the corresponding ROIs were chosen for locations where the fracture line was distinctly evident inside the callus and the cortex of the tibial bone was not intact. The ROI surrounded the newly formed trabecular bone excluding the borders of the callus and the previously existing cortical bone.

4.9 Histological and histomorphometrical analyses

For histological analyses, bone samples from study I and II were fixed in formalin, decalcified in EDTA (*Studies I, III*) or formic acid (*Study II*), embedded in paraffin and then sectioned using a microtome. 5 µm sections were then fixed on to transparent slides for staining. The sections were then deparaffinized, rehydrated and stained with either hematoxylin and eosin (*Studies I, II, III*) or Tra(c)p/Acp5 stain (*Studies I, III*).

For histomorphometry (*Study III*), the tibiae were removed, fixed with 3.7% formalin in PBS, dehydrated in 70% EtOH and embedded in methyl metacrylate (Sigma-Aldrich). Sagittal sections (4 µm) were cut using a Leica RM2255 microtome and then Von Kossa, Toluidine blue and Tra(c)p/Acp5-staining was performed on the sections with standard protocols.
The slides were then analyzed using an Osteomeasure-histomorphometry work station (Osteometrics, USA).

For *Studies I* and *III*, the analyzed area of the femur was defined as 800 µm x 1000-1200 µm starting 200-400 µm proximally from the distal growth plate excluding the cortical bone borders. The trabecular islands and the Tra(c)p/Acp5-stained areas were drawn and quantified. For *study II*, the region of analysis consisted of the callus without the periosteum, fracture ends of the cortical bones or the bone marrow cavities. The areas of cartilage and woven bone were quantified of the two week old calluses and the amount of lamellar bone and trabecular numbers were analyzed from the four week group, respectively.

In *Study III* the histomorphometrical analysis of the tibiae was done on a standard sampling site, 200 µm below the growth plate on an area of 1.17 mm^2. Quantitative measurements were performed semiautomatically under the Osteomeasure image analyzer according to standardized protocols (Dempster, Compston et al. 2013).

For other histological analyses in *Study III*, the WAT samples were fixed in formalin, embedded in paraffin, sectioned and fixed on to slides before staining with hematoxylin and eosin to assess adipocyte morphology. The liver samples were handled as above for hematoxylin and eosin staining and frozen liver samples were prepared and stained with Oil-Red-O-staining to measure liver adiposity.

4.10 Biomechanical analyses

In *Studies I* and *II*, the biomechanical properties of the femurs, tibias and calluses were assessed using a three-point bending test using a biomechanical testing device (Mecmesin, West Sussex, UK for *Study I*, and Lloyd Instruments LRX, Lloyd Instruments Ltd., Fareham, UK for *Study II*). The bones were positioned horizontally and centered on two support pins (span of 9mm) and middle part of the bone or callus was subjected to a vertical force at a speed of 4.5mm/minute and sampling rate of 10Hz until complete separation of the bone fragments. The biomechanical device was connected to a monitoring recorder which converted the measured data into a load-displacement curve in real time. Maximum force and deformation values and the break force and deformation values were read from the highest point of the load-deformation curve and from the failure point, respectively. The stiffness was calculated from the slope of the curve.

4.11 Magnetic resonance imaging (MRI)

An MRI (EchoMRI-700, Echo Medical Systems, Houston, TX, USA) was used to measure the body composition of the animals in *Study III*. Calibration of the machine according to the manufacturer's instructions was done before imaging. The mobility of the animals was restricted by the use of a transparent cylinder tube during scanning. The fat and lean mass were calculated and adjusted to the body weight of each individual animal.

4.12 Glucose- and insulin tolerance tests

In *Study III*, a glucose and insulin tolerance test (GTT and ITT, respectively) were performed on the animals to evaluate changes in glucose and insulin metabolism. For GTT, the animals were allowed to fast for four hours and were then administered an individual dose of 5% glucose solution (1g/kg) i.p. The blood glucose levels were measured immediately before injection and 20, 40, 60 and 90 minutes after the injection using Precision Xtra glucose monitoring device (Abbott Diabetes Care, Abbott Park, Illinois, USA). For ITT, the subjects were allowed to fast for one hour and were then injected i.p. with human insulin (1 IU/kg) (Actrapid, Novo Nordisk, Bagsvaed, Denmark). The blood glucose levels were measured right before injection and at 20, 40 and 60 minutes afterwards.

4.13 Serum samples

In *Study III*, blood samples were collected via exsanguination by cardiac puncture and samples were centrifuged to separate the serum from the blood clot. Serum was then collected and stored at -80C°. Serum levels of C-terminal telopeptide (CTX) and N-terminal type I procollagen (P1NP) were analyzed by ValiRx laboratory, Finland with RatLaps (CTX-1) EIA and Rat/Mouse P1NP EIA assay kits, respectively. Serum insulin levels were measured using an ultrasensitive mouse insulin ELISA-kit (Mercodia, Uppsala, Sweden) by the simultaneous sandwich technique.

4.14 Quantitative real-time PCR

In *Studies I* and *III*, RNA was gathered from the right femur by performing an osteotomy to the distal and proximal ends of the bone and briefly centrifuging it to remove the bone marrow. In *Study II*, the RNA was gathered by performing an osteotomy and separating the callus from the surrounding tibial bone. For RNA isolation, the samples were pulverized under liquid nitrogen and homogenized in sample buffer. The samples were then treated with DNAase treatment followed by RNA clean up using RNeasy mini kit (Qiagen, Hilden, Germany). The cDNA was then synthesized from 1µg of RNA with SensiFAST probekit (Bioline, UK) before performing quantitative real-time PCR using iQ SYBR Green Supermix (Bio-Rad laboratories, USA). The relative mRNA expression levels were then quantified using the $2^{-\Delta\Delta CT}$ method and the results were expressed as fold values compared to the housekeeping gene *β-actin.* The primers used are given in Table 3.

Table 3. List of primer sequences used.

Primer	Forward sequence	Reverse sequence
β-actin	CGTGGGCCGCCCTAGGCACCA	TTGGCCTTAGGGTTCAGGGGG
Runx2	GCCCAGGCGTATTTCAGA	TGCCTGGCTCTTCTTACTGAG
Alp	ACTCAGGGCAATGAGGTCAC	CACCCGAGTGGTAGTCACAA
Sp7	GTCCTCTCTGCTTGAGGAAG	GGGCTGAAAGGTCAGCGTAT
Col1a1	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
Opg	ACCCAGAAACTGGTCATCAGC	CTGCAATACACACACTCATCACT
Rankl	TGAAGACACACTACCTGACTCCTG	CCACAATGTGTTGCAGTTCC
Ctsk	AGGCATTGACTCTGAAGATGCT	TCCCCACAGGAATCTCTCTG

Tracp	CGTCTCTGCACAGATTGCAT	AAGCGCAAACGGTAGTAAGG
Dmp-1	TTGGGATGCGATTCCTCTAC	GGTTTTGACCTTGTGGGAAA
Opn	ATCTGGGTGCAGGCTGTAA	CCCGGTGAAAGTGACTGATT
Dkk-1	GACAACTACCAGCCCTACCC	GATCTGTACACCTCCGACGC
Sost	GCAGCTGTACTCGGACACATC	TCCTGAGAACAACCAGACCA
Ppar-γ	GGGGGTGATATGTTTGAACTTG	AAGACAACGGACAAATCACCA
Fapb4	GCCCTTTCATAAACTCTTGTGG	GAAAACGAGATGGTGACAAGC

4.15 Statistical analyses

All results were subjected to statistical analyses done with IBM SPSS Statistics v.20 (IBM, NY, USA). Results are expressed as mean values and standard deviations (±). Results were considered statistically significant when the corresponding p-value was less than 0.05.

In *Study I,* a 2x2 analysis of variance (ANOVA) was used to test the effects of the treatment, exercise and the interactive effect between the groups. The unpaired, two-tailed student's T-test was used as a post-hoc test to assess the statistical significance when comparing individual groups to each other.

In *Study II* the unpaired, two-tailed student's T-test used to assess the statistical significance of the values between PBS and ActRIIB-Fc treated mice in both the two week and the four week groups.

In *Study III,* a 2x2 analysis of variance (ANOVA) was used to test the effects of the treatment method, surgical procedure and the interactive effect between the groups. Tukey's test or Tukey-Kramer's test was then used as post-hoc tests when comparing individual groups to each other. Single comparisons were made using two-tailed Student's t tests.

5 RESULTS

5.1 Effects on bone

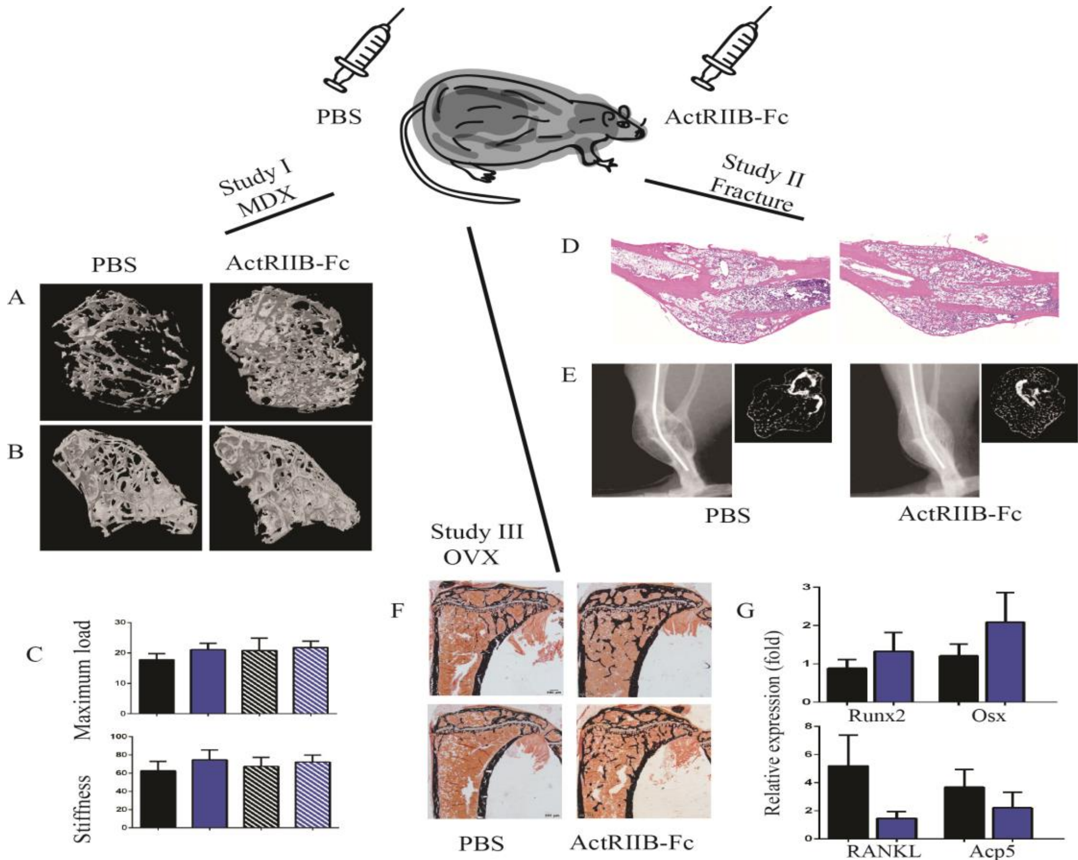

Figure 7: Study I: ActRIIB-Fc treatment increased bone mass and quality in (A) the distal femur and (B) the lumbar vertebrae of mdx mice. Enhanced bone mass translated into slightly improved bone strength in biomechanical testing (C) (black =PBS, blue = ActRIIB-Fc, solid color = sedentary groups, stripes = running groups.) Study II: ActRIIB-Fc improved (D) trabecular number and (E) callus calcification resulting in enhanced fracture healing. Study III: ActRIIB-Fc treatment robustly increased bone mass in SHAM mice (F, top row) and ameliorated OVX-induced bone loss (F, bottom row). Gene expression analysis showed ActRIIB-Fc treatment improved expression of (G, top row) osteoblast marker genes and decreased expression of genes related to (G, bottom row) osteoclast function (black = PBS OVX, blue = ActRIIB-Fc OVX).

Microcomputer tomography
In *Study I*, µCT analyses revealed that ActRIIB-Fc treatment greatly improved bone mass in both long bones and the axial skeleton (Figure 7). In the distal femur, trabecular bone volume per tissue volume (BV/TV) increased by over 80% in ActRIIB-Fc-treated mice compared to non-running PBS-treated controls. In addition, trabecular numbers (Tb.N) increased by over 70%, trabecular separation (Tb.Sp) decreased by 22% and volumetric bone mineral density (vBMD) improved by 52%. The effects on cortical bone parameters were more modest as cortical thickness (Ct. Th) improved by 14% and cross-sectional bone area was increased by 10%. Analogous results were seen in the ActRIIB-Fc-treated running mice as well. However, there were no significant differences between sedentary and running ActRIIB-Fc-treated mice suggesting that physical exercise combined with ActRIIB-Fc did not induce a combinatorial or synergistic effect on bone mass. In the 2nd lumbar vertebrae a more modest effect was seen as ActRIIB-Fc treatment increased trabecular BV/TV by 20% and Tb.N by 30% in the non-running mice compared to PBS controls. Running itself also slightly improved these parameters. As noted in the femur analysis, running and ActRIIB-Fc did not have a combined effect in increasing bone mass or BMD.

In *Study II*, µCT analyses demonstrated ActRIIB-Fc treatment to accelerate callus mineralization and enhance fracture healing. At two weeks, minor improvements in trabecular BV/TV and vBMD were noted in ActRIIB-Fc-treated mice, but these were not statistically significant. However, at four weeks the changes in trabecular BV/TV and Tb.N were prominent as these parameters increased by 60% and 55%, respectively, compared to PBS-treated controls. Additionally, vBMD increased by 55% and structural model index (SMI) decreased by 16%, suggesting a better trabecular structure of the newly formed callus.

In *Study III*, µCT results indicated OVX to induce an expected decrease in bone mass in the femur, as well as the 2nd lumbar vertebrae as BV/TV decreased by 39% and 31%, and Tb.N by 28% and 23%, respectively, compared to PBS-treated sham controls. ActRIIB-Fc treatment ameliorated these changes as femoral BV/TV increased by 220% and femoral Tb.N by 180% compared to PBS-treated OVX mice. Furthermore ActRIIB-Fc treatment also induced robust increases in the same parameters in SHAM mice as well. As noted in *Study I*, the effects of ActRIIB-Fc on cortical bone compared to trabecular bone parameters were much more modest in both sham and OVX mice. In the 2nd lumbar

vertebrae the observations were similar to the femoral analysis as ActRIIB-Fc attenuated bone loss by increasing BV/TV by 60% and Tb.N by 40% compared to sham-treated OVX mice. Concurrent changes were noted in sham mice as well.

Collectively these results suggested that ActRIIB-Fc treatment enhanced bone mass and quality in weight bearing long bones and the axial skeleton, and improved callus mineralization especially by increasing trabecular bone volume, but also mildly affecting the cortical bone as well.

Histology and histomorphometry

In *Study I*, the histologically assessed structural parameters were analogous to those observed in the µCT analysis as BV/TV and Tb.N were both improved due to ActRIIB-Fc treatment. In addition, the mice administered with ActRIIB-Fc had lower osteoclast numbers per bone perimeter (N.Oc/B.Pm), suggesting a suppressive effect on osteoclast-dependent bone resorption.

In *Study II,* the histological structures of the calluses were analyzed at two and four weeks. At two weeks, a significant increase in the amount of woven bone (WoBV/TV) and cartilage (CgV/TV) was noticed in ActRIIB-Fc-treated mice compared to the PBS controls suggesting that ActRIIB-Fc treatment enhanced callus formation. At the four-week time point, ActRIIB-Fc treatment resulted in robust increases of BV/TV (+44%) and Tb.N (+84%), while Tb.Sp decreased by -51% compared to the control calluses, which suggests a potent, anabolic effect on bone formation and fracture healing.

In *Study III*, histomorphometrical analyses were done to assess the effects of ActRIIB-Fc treatment on the structural and dynamic parameters of bone remodeling in OVX and sham mice. ActRIIB-Fc treatment mainly had an impact on the osteoclast parameters as osteoclast surface per bone surface (Oc.S/BS) decreased by 26% and 32% and N.Oc/B.Pm by 26% and 29% when compared to their corresponding PBS-treated sham and OVX-group, respectively. Trends in anabolic parameters were seen as well as ActRIIB-Fc treatment increased the mineralizing surface per bone surface (MS/BS) and bone formation rate per bone surface (BFR/BS). Histological analysis confirmed the reduced N.Oc/B.Pm. Results of both histological and histomorphometrical structural analyses were in concordance with µCT results as ActRIIB-Fc-treated mice had 130% and 200% higher BV/TV in sham and OVX mice, respectively, compared to their corresponding control groups.

Biomechanical testing
In *Study I*, ActRIIB-Fc resulted in mild increases in stiffness and bone strength compared to the non-running PBS controls, but there were no significant differences between the running groups. It seems that in mdx mouse model the robust increases in bone volume did not translate into stronger bones of the same magnitude.

In *Study II,* there were no differences in biomechanical properties between the ActRIIB-Fc-treated and control calluses at two weeks. However, at four weeks ActRIIB-Fc treatment significantly increased the maximum load by 45% and stiffness by 65%, suggesting that ActRIIB-Fc enhanced the strength of the callus compared to PBS controls during the later stages of fracture healing.

These results suggest ActRIIB-Fc treatment to increase bone strength and stiffness, especially during callus healing, but it also mildly improves biomechanical parameters in mature bones.

Gene expression analysis
In *Study II*, the calluses were analyzed at two weeks to measure the differences in gene expression levels of those genes that have been shown to regulate fracture healing. There were 2.5 and 5-fold increases in expression levels of essential osteogenic marker genes *Osx* and *Runx2*, respectively. This suggests that ActRIIB-Fc treatment enhanced osteoblast proliferation and differentiation. In addition *Alp1* expression increased by 75%, demonstrating improved osteoblast function. Additionally, the expression levels of important osteoclast markers were significantly lower (*Ctsk* -94% and *Tra(c)p/Acp5* -85%, respectively) indicating decreased bone resorption within the callus.

In *Study III*, we observed OVX to induce a strong increase in the expression of characteristic osteoclast markers, including 5.5-fold increase in *RANKL* and 3.5-fold increase in *Tra(c)p/Acp5* compared to sham controls This verified that OVX-induced estrogen deficiency remarkably promotes bone resorption. ActRIIB-Fc treatment significantly lowered the expression of these markers in OVX mice, and similar trends were seen in sham mice as well. Furthermore, we noticed ActRIIB-Fc to increase the expression of osteoblast markers *Runx2, Osx, Col1A1* and *Alp1*, similarly to *Study II.* In addition, ActRIIB-Fc-treated mice had higher expression levels of mineralization markers *DMP-1* and *OPN.*

Collectively, these data demonstrate that ActRIIB-Fc treatment preserved a beneficial effect on bone cells by promoting osteoblast development and function and by suppressing osteoclast activity. Therefore, ActRIIB-Fc ap-

pears to have a dual effect on bone uncoupling the bone resorption and formation by inducing an anabolic response in osteoblasts and an anti-resorptive effect in osteoclasts.

Serum markers

In *Study III,* the serum markers for P1NP and CTX were analyzed to assess differences in biomarker levels, which reflect osteoblast and osteoclast activity. ActRIIB-Fc treatment did not have a significant effect on these levels compared to their corresponding PBS-treated groups. A small decrease in P1NP levels of OVX-operated sham mice were noted compared to the PBS sham group.

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5.2 Effects on body composition

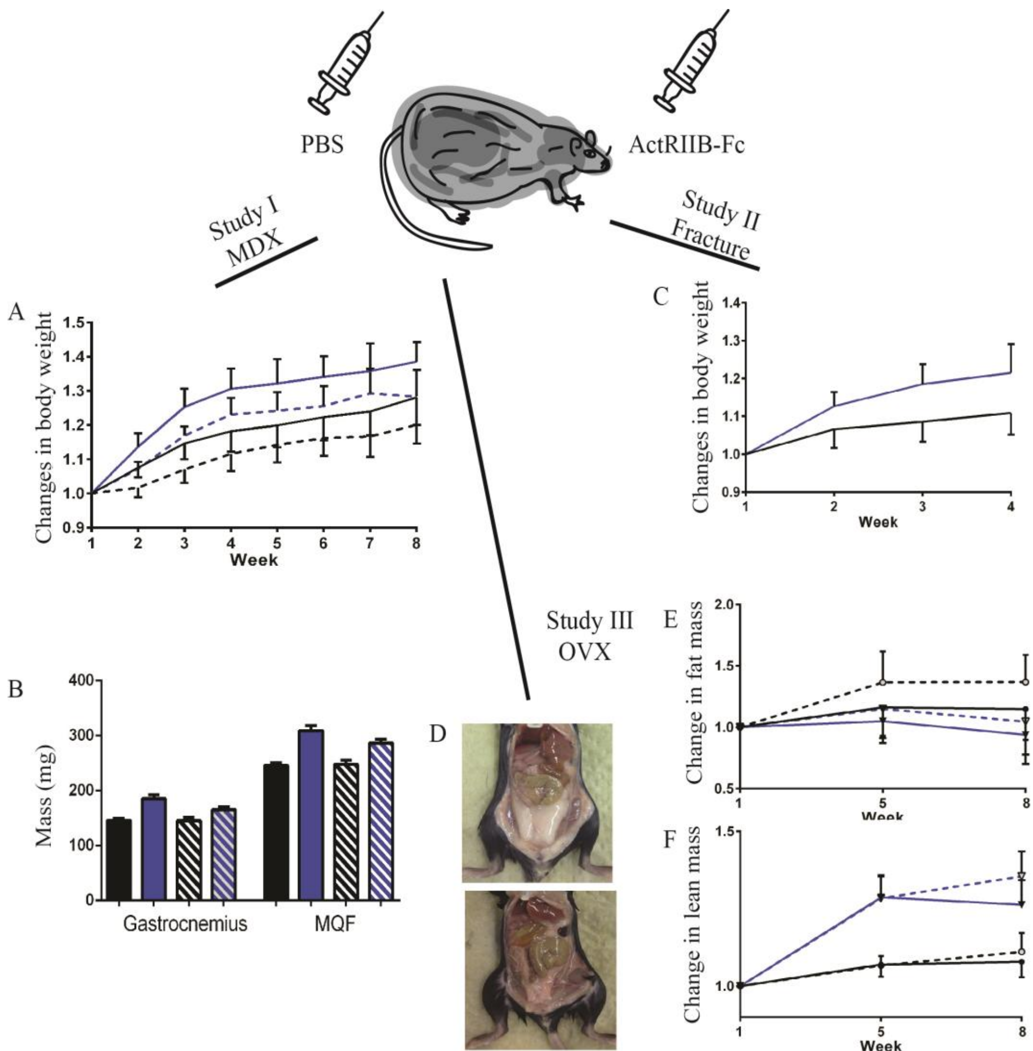

Figure 8: Study I: ActRIIB-Fc treatment increased (A) body mass throughout the experiment and increased (B) hind leg muscle masses in mdx mice (black = PBS, blue = ActRIIB-Fc, solid color/line = sedentary groups, stripes/dotted line = running groups.) Study II: ActRIIB-Fc-treated mice (C) gained more weight than PBS mice throughout the experiment. Study III: (D) Gonadal fat pads of PBS (top) and ActRIIB-Fc (bottom) treated OVX-mice. ActRIIB-Fc reduced (E) fat accumulation in both sham and OVX mice as well as greatly increased (F) lean mass in these mice during the experiment (black = PBS, blue = ActRIIB-Fc, solid line = sham, dotted line = OVX.)

Body weight and muscle mass

In *Study I*, ActRIIB-Fc treatment induced robust changes in body mass in both active and sedentary mice. As myostatin inhibition results in advanced muscular hypertrophy, the gain in weight was most likely due to increased muscle mass. This was confirmed by weighing the masses of different hind leg muscles. ActRIIB-Fc treatment induced muscular hypertrophy in numerous muscles, such as gastrocnemius, quadriceps femoris and tibialis anterior. Running decreased total body weight gained during the experiment compared to their corresponding PBS- and ActRIIB-Fc-treated sedentary groups, and this was most likely due to decreased fat mass. In addition, running itself also mildly increased muscle mass compared to non-running PBS controls. Similar to the bone analyses, combination of running and ActRIIB-Fc did not result in an additive or synergistic effect on muscle mass.

In *Study II,* the changes in body weight were similar to those noted in the previous experiment. ActRIIB-Fc-treated mice gained more weight throughout the experiment compared to PBS controls. The muscle depots were not weighed, but based on other reports it is plausible to speculate that most of the gained body weight was indeed due to increased muscle mass. As the mice didn't lose weight during the experiment, it suggests that the mice could tolerate the surgical procedure performed on them.

In *Study III*, ActRIIB-Fc treatment greatly induced a gain in body mass throughout the experiment, similar to the previous studies. MRI-analysis revealed that this was due to increased muscle mass induced by ActRIIB-Fc treatment in both OVX and sham mice. No differences were noted in the PBS-treated OVX and sham mice, suggesting that the OVX procedure did not have an acute effect on lean mass composition (Figure 8).

*Wheel running **

During *Study I* each mouse from PBS-R vs ActRIIB-Fc-R groups ran a total of 4100±1400m vs 2600±1900m per day during the first week, 6000±1700m vs 3200±2400m during the second week ($p<0.05$), 6100±1700m vs 3700±2200m during the third week ($p<0.05$), 6100±1400m vs 4500±1800m during the fourth week, 5700±2000m vs 4400±2000m during the fifth week, 5800±1700m vs 5300±1800m during the sixth week and 4600±1300m vs 4200±1900m during the 7th week, respectively. Treatment with ActRIIB-Fc decreased running activi-

ty in mice during the first weeks of the study, but the differences evened out towards the end of the experiment.
***** *reproduced with the permission of Juha Hulmi et al.2013***

Fat mass
In *Study IIII*, MRI-analyses were performed to assess the accumulation of systemic adipose tissue during the experiment. OVX is known to cause intra-abdominal fat accumulation, which was confirmed in our experiment. It was evident that OVX increased systemic fat mass by 37% during the experiment compared to 17% increase in sham controls (both from baseline). ActRIIB-Fc treatment prevented this fat accumulation in OVX mice as fat mass only increased by 5% from baseline. In addition, a similar trend was noticed in ActRIIB-Fc-treated sham mice since their fat mass decreased by 6% from baseline. Specific fat pads were also weighed. The administration of ActRIIB-Fc decreased the weight of the gonadal fat pad by 42% and mesenteric fat pad by 25% in OVX mice compared to PBS-treated OVX group. These effects on specific fat depots weights were not observed in sham mice.

5.2 Effects on metabolic parameters

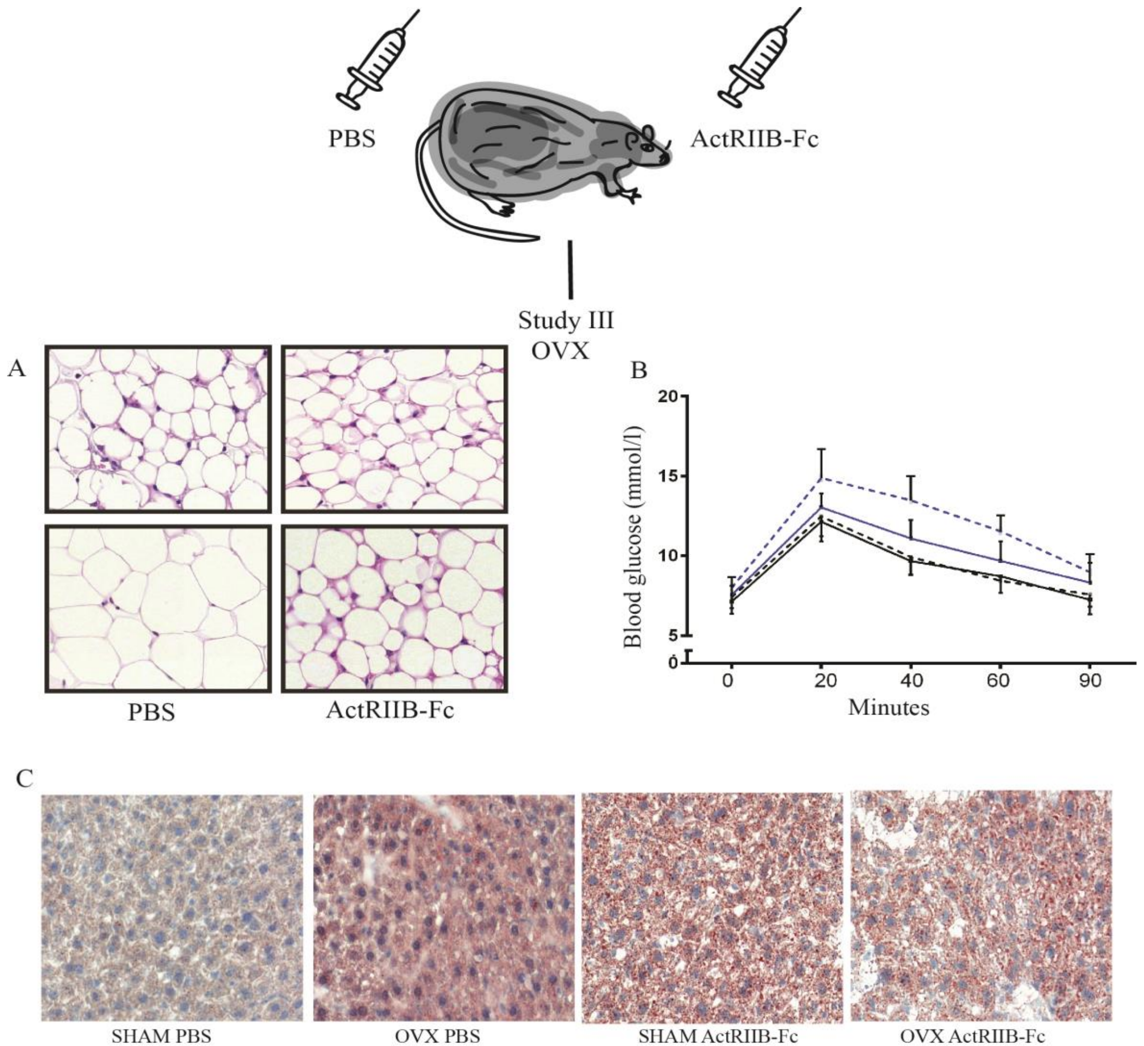

Figure 9: ActRIIB-Fc treatment (A, top row) slightly decreased adipocyte size in sham mice and (B, bottom row) protected from OVX-induced adipocyte hypertrophy. Unexpectedly ActRIIB-Fc-treated mice had (B) impaired glucose clearance in OVX mice and a similar trend was seen in sham mice as well (black = PBS, blue = ActRIIB-Fc, solid line = sham, dotted line = OVX.) ActRIIB-Fc treatment induced (C) hepatic steatosis as seen by red-stained adipocyte infiltration.

Adipocyte size

As expected, OVX induced adipocyte hypertrophy as measured from the gonadal fat pad adipocytes. ActRIIB-Fc treatment protected from these changes showing 42% smaller adipocytes compared to PBS-treated OVX mice (Figure 9). Adipocytes were also slightly smaller in ActRIIB-Fc-treated sham mice. Additionally, ActRIIB-Fc treatment greatly decreased the amount of large adipocytes (>100µm diameter) in OVX mice. A similar, but milder effect was observed also in the sham mice as well.

Glucose tolerance

Glucose tolerance test was performed to assess the effects of ActRIIB-Fc on glucose clearance in sham and OVX mice. There were no changes in glucose clearance between PBS-treated sham and OVX mice suggesting that in this experimental setup OVX did not induce a glucose intolerance. Surprisingly, ActRIIB-Fc on the other hand induced significantly higher blood glucose values throughout the different time points in OVX mice, and also in some time points in sham mice as well. The area under the curve was quantified, which verified that treatment with ActRIIB-Fc impaired glucose clearance in OVX mice. An analogous trend was seen in sham mice. These data demonstrated ActRIIB-Fc to reduce glucose tolerance.

Insulin metabolism

Insulin tolerance test was performed and fasting insulin levels were measured to evaluate the effects of ActRIIB-Fc treatment on insulin metabolism. Differences between the groups in ITT were noted only in the late time points, as OVX induced mild insulin resistance regardless of ActRIIB-Fc or PBS treatment. The blood glucose values of PBS- and ActRIIB-Fc-treated shammice followed a typical trajectory. These results suggested that ActRIIB-Fc did not affect insulin resistance. To investigate whether treatment with ActRIIB-Fc affected insulin secretion, the fasting insulin levels were measured. There were no differences in insulin levels between the groups. These data suggestActRIIB-Fc does not affect the efficacy or secretion of insulin.

Liver adiposity

Liver weights were slightly increased in ActRIIB-Fc-treated mice compared to their corresponding PBS-treated sham and OVX mice. Hematoxylin and eosin staining showed no major differences in the morphology of the hepatocytes be-

tween the groups, while Oil-Red-O-staining revealed robust adipocyte infiltration in both ActRIIB-Fc-treated sham and OVX groups. This was confirmed by the quantification of the adipocyte droplets suggesting ActRIIB-Fc to induce hepatic steatosis.

6 DISCUSSION

Despite the fact that activins were first identified thirty years ago, their pleiotropic nature in regulating numerous physiologic processes has remained unclear to this day. The experimental and clinical evidence of activins maintaining physiologic homeostasis and modulating the progression of many pathological states highlight their importance as multifunctional growth factors. Activin receptor ligand inhibition has emerged as a treatment approach for multiple pathologic conditions including musculoskeletal and metabolic disorders. Still, unresolved discrepancies and adverse side-effects require further clarification before progressing into further clinical trials. In this series of translational studies we were able to successfully answer most of our specific aims. In *Study I,* we were able to show that in addition to improving muscle mass, treatment with ActRIIB-Fc improved bone mass and strength in mdx mice. Moreover, the physical exercise combined with ActRIIB-Fc treatment did not induce a combinatorial or synergistic effect compared to ActRIIB-Fc treatment alone. Together these data suggest ActRIIB-Fc ligand inhibition to be an attractive treatment approach to musculoskeletal disorders, where mobility is limited. In *Study II,* we demonstrated ActRIIB-Fc to enhance callus mineralization and biomechanical strength, which suggests that ActRIIB-Fc accelerates fracture healing. In *Study III,* we displayed ActRIIB to ameliorate bone loss induced by OVX but also robustly improve bone mass in sham mice as well. In addition, we showed ActRIIB-Fc to protect from systemic fat accumulation and to prevent adipocyte hypertrophy. However, administration of ActRIIB-Fc did not prevent all the negative metabolic changes resulting from OVX. It worsened glucose clearance and inflicted hepatic steatosis also in healthy mice.

The concept of treating musculoskeletal disorders with activin receptor fusion proteins is almost single-handedly derived from the observation that ActRIIB-Fc binds myostatin and inhibits its functions, which result in a robust increase in muscle mass. The observation of activin inhibition to enhance bone mass was secondary. There are numerous studies available in which the effectiveness of ActRIIB-Fc on muscle growth has been investigated. Some discrepancies remain regarding the ideal dose of ActRIIB-Fc have remained. In our series of studies, the mice were administered with a 5 mg/kg dose of ActRIIB-Fc, which was based on the observation that there were no differences in gained muscle mass between a weekly 5mg/kg and 10mg/kg dose (Hulmi, Oliveira et al. 2013). Additionally, the choice was justified by aiming to give a physiologi-

cal dose of ActRIIB-Fc, that is, a minimal dose to induce the muscle effects. On the other hand, in most of the other studies a 10 mg/kg dose was used and in some reports the administration was done twice weekly based on the half-life and circulating ActRIIA/B-Fc levels (Fajardo, Manoharan et al. 2010). Our results of increased muscle depot weights were similar and analogous to other reports where the ActRIIB-Fc dose was 10mg/kg (Pistilli, Bogdanovich et al. 2011). This supports the notion that a larger dose does not necessarily result in a linear increase in muscle size, and may be explained by the fact that once a certain threshold level of myostatin/all circulating myostatin is inhibited, higher ActRIIB-Fc dose does not translate into higher skeletal muscle hypertrophy.

The concept of ActRIIB inhibition increasing muscle mass is well-documented, while the effect of myostatin and activin inhibition on the quality of the newly formed muscle remains debated. Conflicting reports have been published, where the effects of ActRIIB-Fc on muscle strength have been beneficial (Akpan, Goncalves et al. 2009, Chiu, Peekhaus et al. 2013) or inconsequential (Hoogaars, Mouisel et al. 2012). Based on these studies ActRIIB-Fc treatment can either improve energy supplies (Bechir, Pecchi et al. 2016) or trigger muscle fatigability (Relizani, Mouisel et al. 2014). These discrepancies in the results are likely dependent on the method used to measure muscle strength or endurance as significant differences have been observed in the results depending on the used methodology (Aartsma-Rus and van Putten 2014). In our study, the increased muscle mass induced by ActRIIB-Fc was not associated with higher grip strength (data not shown), suggesting impaired quality of the newly formed muscle as suggested before (Relizani, Mouisel et al. 2014). In addition to the modality to measure muscle strength or the fusion protein used, the observation may also be due to the mdx phenotype, since ActRIIB-Fc does not correct the functional defect in the muscle fibers, but merely increases muscle mass (Faber, Hall et al. 2014). Whether ActRIIB-Fc does improve muscle strength in normal mice or humans remains to be determined. Moreover there may also be differences between the fusion proteins. Our ActRIIB-Fc was produced in-house compared to the commercial product from Acceleron, which has been used in other studies.

To our knowledge, there are no other reports published, which investigate the effects of ActRIIB-Fc on bone tissue in sedentary and running mdx mice. Therefore, our data is novel, but also supports previous publications of ActRIIB-Fc having pro-osteogenic impact. The beneficial effects of ActRIIB-Fc treatment on bone were seen both in the long bones and the axial skeleton. In

addition to increasing bone volume, BMD was increased and biomechanical parameters improved demonstrating that ActRIIB-Fc treatment increased bone mass and strength in mdx mice. These findings set forth ActRIIB-Fc as a therapeutic candidate for treating musculoskeletal disorders.

It was somewhat surprising that we did not notice a combinatorial or a synergistic effect of exercise and ActRIIB-Fc. This could be due to the analyzed bone area as muscle strain has been suggested to have a greater stimulative effect on the femur compared to the proximal tibia (Montgomery, Pennington et al. 2005). Mechanotransduction has been demonstrated to be an essential modulator of bone development and growth (Duncan and Turner 1995, Robling and Turner 2009) and, therefore, a dual effect in our study was expected. Hamrick et al. reported that forced physical exercise in myostatin-deficient mice resulted in a stronger effect on bone than in sedentary mice (Hamrick, Samaddar et al. 2006). The disparities to our results could be due to the animal background (mdx mice vs transgenic *MSTN*-deficient mice) as their mice had increased muscle mass postnatally throughout their lifespan, which could result in a more powerful mechanotransduction effect on bones. Mdx mice have also been reported to have decreased bone mass due to unknown mechanism, which can modulate response to exercise (Nakagaki and Camilli 2012). Alternatively, the choice of exercise method (voluntary wheel running vs forced physical exercise), or the fact that in our study ActRIIB-Fc-treated mice ran slightly less compared to their corresponding PBS-treated controls, could also have attenuated the combined effects of ActRIIB-Fc treatment and running bone. Moreover, ActRIIB-Fc treatment inhibits many other ligands in addition myostatin, which could modify the bone response to exercise. Our results suggest that exercise is not required for the full anabolic effect of ActRIIB-Fc and thus ActRIIB-Fc treatment could be an attractive treatment approach for musculoskeletal diseases in which mobility is also limited.

To date there has been one randomized, placebo-controlled clinical trial which investigated the effects of ACE-031, the human ActRIIB-Fc, in the treatment of young DMD patients (Campbell, McMillan et al. 2016). The authors reported results similar to those of previous *in vivo* studies. Lean mass and BMD density of axial skeleton, as measured by dual-energy X-ray absorptiometry (DXA), were both significantly increased in a dose-dependent manner. In addition there was a tendency of ACE-031 to decrease systemic fat mass compared to the placebo group in a dose-dependent manner as well. Patients receiving ACE-031 also improved their six-minute walk test distance. Collectively, these

results were mostly in agreement with those from translational studies and ActRIIB-Fc treatment enhanced parameters that are known to be impaired in DMD. However, despite these beneficial effects, the study was interrupted due to the development of spontaneous epistaxis and telangiectasias in over 50% of the subjects in the group receiving the higher ACE-031 dose. The authors reported no changes in hematological markers, and suggested that these changes could be due to the impaired signaling of BMP-9 and BMP-10 as they have been shown to regulate angiogenesis (David, Mallet et al. 2007). Preclinical models have also suggested mdx mice to have decreased vascular density and impaired angiogenesis and further disruption of angiogenic signaling in patients with DMD could result in spontaneous bleeding (Shimizu-Motohashi and Asakura 2014). Further studies are warranted to elucidate the effects of inhibiting ActRIIB ligands on both angiogenesis, as well as blood clotting markers such as thrombocyte functions. Even though at this time ACE-031 is not undergoing further clinical development, this study provided auspicious results regarding myostatin inhibition and its beneficial effects in DMD patients. In order to further develop the myostatin/activin signaling pathway as a therapeutic option for DMD, the specific roles of each of the receptor ligands need to be thoroughly evaluated in order to elucidate the mechanisms of the possible side-effects.

In our second study, we were able to convincingly show ActRIIB-Fc treatment to enhance fracture healing as assessed by different imaging, histological, biomechanical and molecular methods. There are no previous publications where fractures have been treated with ActRIIB-Fc. owever, Morse et al. reported that ActRIIA-Fc treatment augmented callus formation in rats (Morse, Cheng et al. 2015). In this study ActRIIA-Fc treatment did indeed increase the bone volume within the callus, but this did not translate into enhanced biomechanical strength of the same magnitude. The authors suggested that this was due to impaired remodeling of the new trabeculae, resulting in their unfavorable structure. However, the authors presented limited analysis on the effects of ActRIIA-Fc on bone structure and bone cell functions making the comparison to our study and challenging. Additionally, the authors did not further account for their findings of bone volume per tissue volume or tissue mineral density of ActRIIA-Fc-treated rats not differing from PBS controls. These observations would be a more credible explanation to the fact that ActRIIA-Fc treatment did not enhance callus strength. Moreover, the differences between our findings could be due to the choice of animal, fracture location, choice of activin receptor fusion protein or its dosage. Indeed, as ActRIIA-Fc is not able to bind myostatin, it is plausible

to suggest that myostatin could also participate in fracture healing based on these results. Recent publications have suggested that the specific inhibition of myostatin using a recombinant myostatin propeptide enhanced bone regeneration (Hamrick, Arounleut et al. 2010) and treatment with myostatin decreased fracture callus bone volume (Elkasrawy, Immel et al. 2012). Furthermore, it is interesting to speculate the role of muscle tissue on fracture healing as discussed earlier. Increased muscle mass could enhance the secretion of myokines with known osteogenic roles (Colaianni, Cuscito et al. 2015, Kawao and Kaji 2015). All in all, these reports suggest that the inhibition of activin A and myostatin could be a more effective treatment option for fracture healing compared to inhibition of activin A alone.

In another recent study, the effects of activin receptor blockade with Bimagrumab (BYM338) was investigated in a rat fibula osteotomy model (Tanko, Goldhahn et al. 2016). The authors reported that treatment with BYM338 did not improve mature callus size, BMD or biomechanical parameters and concluded that activin receptor blockade does not have a major impact on fracture healing. These assertions require further addressing. The controversial results could be due to differences in the study setup (mouse vs rat, tibia vs fibula, closed fracture vs osteotomy), but also the method of ActRII-inhibition could partly explain these findings. Bimagrumab is a human monoclonal antibody that mainly binds to activin type IIB receptors, but also to type IIA receptors with a smaller affinity, which result in the inhibition of ActRIIA/B ligand binding to the receptors (Lach-Trifilieff, Minetti et al. 2014). Bimagrumab binds to the extracellular portion of activin type II receptors, and thus, inhibits all signaling that is normally initiated through ligand binding including myostatin, activin A and different BMPs. ActRIIB-Fc on the other hand binds to these corresponding ligands extracellularly, and does not interact with ActRIIA/B itself. The binding affinity of our ActRIIB-Fc fusion protein towards BMPs is low and we propose that most of the observed effects are due to the inhibition of myostatin and activin A while the osteoinductive effects of the BMPs are unaltered. In addition to BMP type II receptors, BMPs exert their actions by binding to ActIIA/B as well. Therefore, Bimagrumab could partly inhibit BMP-dependent signaling. Indeed BMPs play essential roles in proper fracture healing and Bimagrumab treatment could partly cancel out these effects. Indeed, *in vitro* studies have suggested that BMP-2 initiated events are completely abolished only when both BMPRII and ActRIIB are blocked (Liu, Zhang et al. 2012). As BMPS play essential roles in proper fracture healing, the inhibition of both BMP

and activin/myostatin signaling via ActRIIA/B by Bimagrumab could result in neutral effect on bone cells and thus explain the lack of effect of Bimagrumab on fracture healing.

The effects of ActRIIB-Fc on OVX and sham mice were in concordance with our hypothesis. It was interesting to note that instead of ActRIIB-Fc treatment only restoring bone mass to its original levels in OVX mice, they were robustly surpassed, verifying ActRIIB-Fc to be a potent anabolic agent regardless of the surgical procedure done to the mice. Similar to the results in *Study I,* bone mass increased in both long bone and the axial skeleton. There are no other reports available regarding ActRIIB-Fc treatment on OVX mice but Pearsall et al. published a study where they assessed the effects of ActRIIA-Fc treatment on control and OVX mice (Pearsall, Canalis et al. 2008). Even though their administration took place twice a week, with a higher dose and for a longer duration, our results were comparable when analyzing both the long bones and the axial skeleton at similar time points. They did not perform histomorphometric analyses on OVX mice but in control, healthy mice they were able to verify the anabolic effects of ActRIIA-Fc, such as prominent increases in mineral apposition rate and bone formation rate. Interestingly, these parameters seemed to decrease the longer the study went on. In contrast, our histomorphometric analysis on dynamic parameters suggested the changes in bone mass were mostly due to a robust anti-resorptive effect on osteoclast number and function, even though some trends in osteoblast-derived anabolic parameters were noted, too. Unfortunately, we did not perform these analyses on bones from earlier time points so we can only speculate the strongest anabolic effects of ActRIIB-Fc to take place during the first weeks of administration. Indeed, it is likely that the anabolic effects on bone formation took place earlier in the study, and by the time the animals were euthanized and samples collected, the bone remodeling cycle had adapted to a new balance, hence, reflecting only modest changes in the dynamic parameters of the histomorphometric analysis. The non-significant changes in serum biomarker levels also support this notion. Alternatively, the anabolic mechanism may be different from that of for example PTH-induced bone formation. The available data on effects of ActRIIB-Fc treatment on gene expression is also limited. In *Study III,* we demonstrated OVX to induce a strong increase in the expression of important osteoclast markers, such as *RANKL* and *Tra(c)p/Acp5*. ActRIIB-Fc treatment significantly suppressed the expression of these genes, suggesting an anti-resorptive effect in agreement with our histomorphometric results. Significant increases in the expression of some osteo-

blast- and mineralization markers were noted as well, which are in concordance with our histomorphometric data, and supports the notion that ActRIIB-Fc also induces anabolic effects.

The role of activins in adipocyte development and function are not entirely clear. Reports regarding the accumulation of fat tissue during ActRIIB-Fc administration have been investigated in different study setups with somewhat inconsistent results. Akpan et al. demonstrated ActRIIB treatment decreased fat mass in mice fed with either normal chow or a high-fat diet (Akpan, Goncalves et al. 2009), and Koncarevic and colleagues demonstrated that ActRIIB-Fc-treated mice were protected from the fat gain induced by ORX (Koncarevic, Cornwall-Brady et al. 2010). On the other hand, results of ActRIIB-Fc treatment not ameliorating fat gain in previously obese mice have also been reported (McPherron, Guo et al. 2012). Therefore, the effects of postnatal administration of ActRIIB-Fc on adiposity are not entirely clear. In our study, OVX induced an increase in systemic fat mass compared to PBS-treated sham controls. ActRIIB-Fc significantly reduced this accumulation and our results suggest ActRIIB-Fc to act as an anti-adipogenic agent especially in states, where subjects are prone to fat accumulation. This concept endorses the hypothesis that activins are essential regulators of adipocyte precursors, while they have smaller roles in the modulation of already cumulated fat depots. Furthermore, treatment with ActRIIB-Fc protected from OVX-induced adipocyte hypertrophy and also decreased adipocyte size in sham mice. The mechanisms behind these effects have not been investigated thoroughly. One hypothesis is that increased lean mass increases energy expenditure and fatty acid phosphorylation and thus could oppose fat gain as shown recently in ActRIIB-Fc-treated-mice (Bechir, Pecchi et al. 2016) as well as myostatin-deficient mice (Choi, Yablonka-Reuveni et al. 2011).This is supported by the fact that increased skeletal muscle mass enhances insulin-mediated glucose uptake. However, as discussed below, in our model ActRIIB-Fc actually impaired glucose tolerance. Another plausible explanation is the fact that ActRIIB-Fc has been shown to increase UCP-1 expression and induce the “browning” of WAT by activating brown adipogenesis (Fournier, Murray et al. 2012).UCP-1 is an essential regulator of proper thermogenesis and energy consumption, and it has been targeted as treatment approach to counter obesity (Kajimura and Saito 2014). The notion that myostatin is a major factor regulating adipose tissue mass and function is supported by the studies showing that *MSTN* $^{-/-}$ mice have lower triglyceride content in WAT and increased expression levels of *UCP-1* mRNA. Thys, myostatin inhibition could be used as a

novel strategy to induce BAT formation (Zhang, McFarlane et al. 2012). On the other hand, activin A has also been proposed to regulate adipocyte proliferation and differentiation (Zaragosi, Wdziekonski et al. 2010). As adipocyte hypertrophy can be induced by inflammatory responses, inhibition of activin signaling with ActRIIB-Fc may attenuate these changes in adipocyte morphology via its anti-inflammatory effects. Unfortunately we were not able to perform specific molecular analyses on our WAT samples to investigate the effects of ActRIIB-Fc on adipocyte function or browning under our experimental conditions. Further studies are warranted to elucidate the functions of activins and other ActRIIB-Fc ligands regarding adipocytes.

Our findings on the effects of ActRIIB-Fc treatment on glucose metabolism were somewhat unexpected. Our hypothesis was that increased muscle and lean mass together with decreased systemic adiposity would result in enhanced insulin sensitivity and improved glucose tolerance. Surprisingly, treatment of OVX mice with ActRIIB-Fc led to impaired glucose clearance, and a similar trend was noticed in ActRIIB-Fc-treated sham mice, too. An analogous result was noted in a recent publication, demonstrating that treatment with ActRIIB-Fc worsens glucose tolerance in mice with pharmacologically-induced diabetes (Wang, Guo et al. 2015). As activins are important regulators of beta cell development in pancreas, activin inhibition could lead to impaired beta cell functions. Interestingly, ActRIIB-Fc treatment had no effect on insulin resistance, as shown in ITT, nor fasting insulin levels in the serum. Therefore, ActRIIB ligand inhibition could either impair glucose-stimulated insulin secretion or affect the expression levels of glucose transporters or by other means increase endogenous glucose production. Another unexpected observation was the induction of hepatic steatosis due to ActRIIB-Fc treatment. Liver fattening has been associated with increased hepatic gluconeogenesis (Sunny, Parks et al. 2011) and may partly explain the observed changes in glucose tolerance. These findings were not in agreement with our hypotheses, and side-effects of this magnitude could diminish some of the attractiveness of ActRIIB-Fc as a treatment method for metabolic disorders. Further studies are indicated to further elucidate the molecular mechanisms underlying these findings.

A number of different experimental approaches have been used to address the effects of the inhibition of ActRIIA/B signaling. An ideal, systematic approach would be to use transgenic homozygous ActRIIA/B-Fc-deficient mice (known as *Acvr2(b)*$^{-/-}$). However, increased perinatal lethality in subsets and developmental issues (including cleft palate, anteroposterior axis defects and

abnormalities in reproduction) have prevented them from being an attractive model to be used in translational studies (Matzuk, Kumar et al. 1995, Oh and Li 1997). On the other hand transgenic models of myostatin-deficient mice (*MSTN* $^{-/-}$) as well as inhibin A-knockout (*Inhba* $^{-/-}$) mice have been used, and they can help to elucidate the specific mechanisms of these growth factors in cellular development and function. Recent studies have relied on the postnatal inhibition of ActRIIA/B ligands by other means. As explained thoroughly before, ActRIIA/B-Fc fusion proteins exert their actions by binding to ActRIIA/B ligands and trapping them. These trap receptors leave the original, extracellular portions of the ActRIIA/B intact. However, these trap receptors bind multiple ligands, and specific actions of each ligand cannot be differentiated and specified with the use of the fusion proteins. Specific monoclonal anti-antibodies for myostatin (Camporez, Petersen et al. 2016) and activin A (Murata, Saito et al. 1996) have also been used in different study setups. However, there have been discrepancies on their optimum dosage, as well as variability in their efficacy between studies (Lach-Trifilieff, Minetti et al. 2014). Furthermore, the identification and synthesis of the inhibitory cores of prodomain regions of these growth factors (Ohsawa, Takayama et al. 2015) has also emerged as an intriguing approach for activin signaling inhibition, since they seem to effectively interact with specifically-targeted ligands compared to the broad-spectrum functionality of receptor fusion proteins (Chen, Walton et al. 2015).

Similar to the execution and setup of numerous other studies, this series of translational studies also had its limitations. In our first study, the limitations were mostly due to the fact that we started our collaboration at a late stage of the animal experiment. This meant that the study setup was not ideal for examining the effects of ActRIIB-Fc on bone tissue of mdx mice. We were not able to perform dynamic histomorphometrical analysis to assess how bone formation was affected nor were we able to assess serum levels of biomarkers associated or mRNA expression of genes with bone remodeling. Furthermore, the reliability of using mdx mice as a preclinical model to examine changes in muscle and bone tissue has lately been questioned. For example, the phenotypical severity of muscle fibrosis and loss of function in the hind limbs is less than that of humans and the inflammatory profiles contributing to the muscle weakening are also different between the human and animal model.

In our second study, the limitations were mostly confined to the stability of the fracture callus and the exact reproducibility of the fracture. To stabilize the fractured tibia, we inserted a thin, steel rod in to the intramedullary canal to

ensure the immobility of the fracture ends during the experiment. However, due to the thinness of the rod, some mobility of the fragments was allowed and fully stabilized fracture ends were not always met. This resulted in a slight malposition of the callus and the fracture healing may have been slightly different between some individuals. In the future, placement of a larger rod, such as a 26g syringe needle, in the intramedullary canal to provide further stabilization of the fractured ends could prevent this. Furthermore, the two-week old calluses were extremely fragile and some samples were lost while removing the intramedullary rod from the callus. The study could have also had a later time point of six to eight weeks to evaluate the late remodeling of the fracture callus.

Finally, in our third study some mice that were assigned to the sham groups had different levels of systemic adiposity at the beginning of the study compared to the other mice used. This could alter the metabolic profile of these mice, hence, some of the results including some fat pad weights are not necessarily comparable. In addition, more comprehensive methods could have been used to assess the metabolic findings. For example, in order to get a better understanding of nature of glucose and insulin metabolism in these mice, the use of glucose- and hyperinsulinemic-euglycemic clamps could have been indicated. These were left for future studies as we didn't want to induce too much stress and strain on the mice that could have influenced the bone phenotype. Furthermore, due to the unexpected technical failure of one of our -80°C freezers, we lost a great amount of the gathered samples that would have been extremely useful in analyzing the effects of ActRIIB-Fc on different adipose depots. Finally, as it cannot be highlighted enough, the use of our ActRIIB-Fc only allows for the exploration of the combined effects of inhibiting activin type II receptor ligands, and not the specific role of each growth factor and cytokine.

With our results we demonstrate that ActRIIB-Fc ligands play essential roles in bone formation and body composition in clinically relevant animal models. We were able to answer many questions that were left open from prior studies, and elucidate some of the mechanisms by which ActRIIB-Fc alters bone remodeling, and to confirm majority of our hypotheses. However, our results also gave directions for further study setups. To elucidate the true functions of each ActRIIB-Fc ligand on different tissue types, a different method of ligand inhibition is required. Additionally, the findings of reduced glucose tolerance and liver fattening require further clarifications, as these are adverse effects that could diminish the attractiveness of targeting ActRIIB ligands as a therapeutic approach. Finally, molecular mechanisms behind our findings need to be studied

in more detail. Even though we were able to identify some of the mechanisms underlying the effects of ActRIIB-Fc on bone mass, detailed studies on the roles of specific growth factors in different settings would also provide us with relevant information how these ligands affect different cell and tissue types. The future studies could provide the essential information required to further develop therapeutic agents targeting activin receptor ligands in order to treat disorders which involve bone, muscle and fat tissue.

7 CONCLUSIONS

Based on the presented translational studies, the following conclusions can be made:

1. Treatment with ActRIIB-Fc robustly increased bone mass in healthy, muscle dystrophic and estrogen deficient mice and accelerated closed fracture healing through both anabolic and antiresorptive mechanisms.

2. Biomechanical strengths of mature bones and fracture calluses were also improved due to ActRIIB-Fc.

3. Running combined with ActRIIB-Fc did not induce a synergistic or combinatorial effect on bone in muscle dystrophic mice compared to ActRIIB-Fc treatment alone.

4. ActRIIB-Fc-treated mice had increased muscle mass compared to control mice, while this did not necessarily translate into enhanced muscle strength.

5. ActRIIB-Fc treatment protected mice from systemic fat accumulation and prevents adipocyte hypertrophy in estrogen-deficient mice, while similar but more modest effects were seen in control mice.

6. Mice treated with ActRIIB-Fc had impaired glucose clearance and hepatic steatosis, but ActRIIB-Fc did not affect insulin resistance in control or estrogen-deficient mice.

7. Further studies are warranted to elucidate the specific mechanisms behind the perturbed metabolic responses, as well as for some of the musculoskeletal effects

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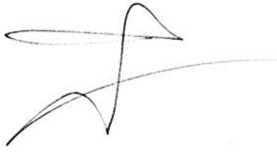

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Treatment with soluble activin type IIB-receptor improves bone mass and strength in a mouse model of Duchenne muscular dystrophy

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Abstract

Background: Inhibition of activin/myostatin pathway has emerged as a novel approach to increase muscle mass and bone strength. Duchenne muscular dystrophy (DMD) is a neuromuscular disorder that leads to progressive muscle degeneration and also high incidence of fractures. The aim of our study was to test whether inhibition of activin receptor IIB ligands with or without exercise could improve bone strength in the mdx mouse model for DMD.

Methods: Thirty-two mdx mice were divided to running and non-running groups and to receive either PBS control or soluble activin type IIB-receptor (ActRIIB-Fc) once weekly for 7 weeks.

Results: Treatment of mdx mice with ActRIIB-Fc resulted in significantly increased body and muscle weights in both sedentary and exercising mice. Femoral μCT analysis showed increased bone volume and trabecular number (BV/TV +80%, Tb.N +70%, $P < 0.05$) in both ActRIIB-Fc treated groups. Running also resulted in increased bone volume and trabecular number in PBS-treated mice. However, there was no significant difference in trabecular bone structure or volumetric bone mineral density between the ActRIIB-Fc and ActRIIB-Fc-R indicating that running did not further improve bone structure in ActRIIB-Fc-treated mice. ActRIIB-Fc increased bone mass also in vertebrae (BV/TV +20%, Tb.N +30%, $P < 0.05$) but the effects were more modest. The number of osteoclasts was decreased in histological analysis and the expression of several osteoblast marker genes was increased in ActRIIB-Fc treated mice suggesting decreased bone resorption and increased bone formation in these mice. Increased bone mass in femurs translated into enhanced bone strength in biomechanical testing as the maximum force and stiffness were significantly elevated in ActRIIB-Fc-treated mice.

Conclusions: Our results indicate that treatment of mdx mice with the soluble ActRIIB-Fc results in a robust increase in bone mass, without any additive effect by voluntary running. Thus ActRIIB-Fc could be an attractive option in the treatment of musculoskeletal disorders.

Keywords: Bone μCT, Bone-muscle interactions, TGF-βs, Animal models, Exercise

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Background

Inhibition of the activin/myostatin pathway has recently emerged as a potential therapeutic approach for the treatment of osteoporosis. Activins are a group of multifunctional growth factors belonging to the TGF-β superfamily that play multiple roles in many physiological and systemic processes such as secretion of follistatin-stimulating hormone, wound healing, morphogenesis, and tooth formation [1–3]. Myostatin in turn inhibits muscle development and its inhibition leads to increased muscle mass [2, 3]. Activins and myostatin signal via activin type IIA or type IIB receptors [4]. The role of activins in bone physiology remained unclear until recent studies indicated that inhibition of activin receptor ligands leads to increased bone mass [5, 6]. In these experiments, soluble activin receptor-Fc fusion proteins were used as decoy receptors harvesting and thus inhibiting their ligands including activin A and myostatin. These studies suggested that inhibition of activin pathway could be a promising therapeutic target for metabolic bone diseases [7]. The use of a soluble activin type IIA-receptor has been shown to increase bone mass in several in vivo [5, 6, 8] models. The effects of soluble activin type IIB-receptor (ActRIIB-Fc) on bone metabolism have also been studied recently [9–12]. Furthermore, exercise, in addition to its various other health benefits, may have positive effects on bones of young individuals [13, 14]. Previously, the effects of exercise have also been investigated in a model of increased body mass through increased fat, not muscle mass [15]. In that model aerobic exercise does not further increase bone strength when compared to increased body mass alone suggesting interaction between physical activity and increased body mass. However, increasing body weight through muscle mass in combination with exercise has not been investigated before.

Duchenne muscular dystrophy (DMD) is a neuromuscular disease that is caused by a single mutation in the dystrophin gene. Patients suffering from this disorder are left immobilized and eventually are often deceased around the age of 20 [16]. Duchenne muscular dystrophy is also known to lead to secondary osteoporosis and increased fracture risk, at least in part due to the reduced mobility of DMD patients [16, 17]. By improving both muscle and bone function and strength, patient mobility could be prolonged and quality of life increased. The effects of exercise on bones in DMD as well as the interaction of the physical activity with ActRIIB-Fc ligand blocking are not known.

Muscle and bone tissues interact in multiple ways and muscle tissue can directly induce bone formation locally via several different molecular pathways [18, 19]. Further, muscle and body masses per se can also indirectly effect bone [20]. There is also reciprocal signaling from bone to muscle [21]. Myostatin and activins are inhibitors of muscle growth and their inhibition could have therapeutic implications in frailty and other diseases with muscle wasting [4, 22]. Intriguingly, ActRIIB-Fc can block both activin A as well as myostatin [23], that could potentially be very beneficial in conditions, such as frailty and DMD, which involve both muscle and bone [9].

Based on the previous data, we hypothesized that inhibition of the activin/myostatin pathway could provide a novel dual-effect treatment approach for musculoskeletal conditions improving both the muscle and bone properties. We aimed to answer the following questions: 1) does inhibition of activin receptor ligands with the use of a soluble activin type IIB-receptor (ActRIIB-Fc) affect bone volume and quality in a muscle dystrophy (mdx) mouse model [24] parallel to changes in muscle mass and 2) is there an interaction between ActRIIB-Fc treatment and low-intensity aerobic exercise.

Methods

Animals

In this experiment 6- to 7 week-old muscle dystrophy (mdx) male mice from a C57Bl/10ScSnJ background were used (Jackson Laboratory, Bar Harbor, Maine, USA). The mice were housed in standard laboratory conditions (temperature 22°C, light from 8:00 AM to 8:00 PM) and had free access to tap water and food pellets (R36, 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labfor, Stockholm Sweden).

Experimental design

Thirty-two male mice were evenly divided into four groups: 1) PBS control group, 2) ActRIIB-Fc group, 3) PBS running group (PBS-R) and 4) ActRIIB-Fc running group (ActRIIB-Fc-R). PBS or ActRIIB-Fc was injected intraperitoneally once a week with a 5mg/kg dose. The chosen exercise modality was voluntary wheel running. To allow the treatment to take effect, the running wheels were locked during the first injection day and the following day preventing mice from exercising. On the last two days, the mice did not have access to running wheels so that the possible acute exercise effects would not affect the outcome. During the experiments all conditions were standardized. Mice were euthanized by cervical dislocation at the end of the experiment, after which tissue samples were harvested. One specimen from the ActRIIB-Fc-R group was moved to the ActRIIB-Fc group due to no voluntary running activity.

Production of soluble ActRIIB-Fc

The ActRIIB-Fc protein used in the present study is similar, but not identical to the one originally generated by Se-Jin Lee [25]. The in house production of this recombinant protein has been described earlier [26].

Shortly, the protein contains the ectodomain of human ActRIIB linked to IgG1-Fc and was expressed in Chinese hamster ovary (CHO) cells grown in a suspension culture.

Voluntary wheel running

As studies have shown that voluntary wheel running may benefit mdx mice in terms of muscle properties [27] and can also have positive effects on murine bones [15], it was chosen as the exercise modality for this study. The mice had free access to custom-made running wheels and the total running distance was recorded 24 h daily.

Muscle mass and body weight measurement

The mice were weighed once every seven days to measure their body weight. After euthanization of the animals, gastrocnemius and quadriceps femoris muscles were collected and weighed immediately after dissection.

Micro-computed tomography (μCT) analysis

X-Ray Micro-computed tomography of the distal femur and second lumbar vertebrae were done with SkyScan 1070 μCT scanner (SkyScan, Kontich, Belgium) to assess the microarchitecture of trabecular bone. Femur and lumbar vertebrae samples were placed in to plastic tubes and sealed with paraffin to minimize sample mobility. Scanning parameters included a voxel resolution of 5.33 μm, X-ray potential of 70kVp, current of 200uA and an integration time of 3900ms. The object rotated in 0.45° steps throughout the scanning for a total revolution of 182.45°.

Reconstruction of the scanned images was done (Nrecon 1.4, Skyscan) with identical settings (misalignment < 3, ring artifacts reduction 11, beam hardening correction 95%, and intensity gap 0.017–0.13). The regions of interest were drawn blindly (CTan 1.4.4, SkyScan). The corresponding starting points for the trabecular analysis were 120 layers (2.4mm) proximally of the distal femoral growth plate and 150 layers (3mm) distally of the cranial growth plate of the 2nd lumbar vertebrae. The regions of interest (ROIs) were extended 150 layers (3mm) with a new ROI drawn every 10 layers (0.2mm). For the cortical analysis the ROI was drawn around the cortical bone of the femoral diaphysis, 600 layers (12mm) proximally of the distal growth plate, ranging 100 layers (2mm) in the proximal-distal plane. Finally the 3D, 2D and bone mineral density results were converted into numerical data for statistical analyses.

Histological analysis

Femur samples were formalin-fixed, decalcified in EDTA, embedded in paraffin and sectioned using a microtome. The sectioned slides were deparaffinized, rehydrated and stained by either Hematoxylin & eosin or a tartrate resistant acid phosphatase (TRACP) stain and then counterstained with Mayer's hematoxylin according to the manufacturer's instructions. The trabecular bone architecture and osteoclasts were then analyzed blindly using Osteomeasure-histomorphometry work station (Osteometrics, USA). The analyzed area was defined as 800μm x 1200μm starting from 400μm proximally to the distal growth plate excluding the cortical bone borders. The region of interest was analyzed from three slices and the mean of each parameter was used as the corresponding value.

Quantitative real-time PCR

To analyze the molecular effects of ActRIIB-Fc on bone C57Bl/6 F mice were administered with either PBS or ActRIIB-Fc (5mg/kg) once a week for 8 weeks ($n = 6$-7 per group). RNA was extracted from the right femur by performing an osteotomy to the distal and proximal ends of the bone and briefly centrifuging it to remove the bone marrow. The RNA was isolated using RNeasy minikit (Qiagen, Germany). The cDNA was synthesized from 1μg of RNA with SensiFAST probekit (Bioline, UK) before performing quantitative real-time PCR using iQ SYBR Green Supermix (Bio-Rad laboratories, USA). The relative mRNA expression levels were then quantified using the 2-ΔΔCT method. β-actin was used as the internal control.

Primer	Forward sequence	Reverse sequence
β-actin	CGTGGGCCGCCCTAGGCACCA	TTGGCCTTAGGGTTCAGGGGG
Runx2	GCCCAGGCGTATTTCAGA	TGCCTGGCTCTTCTTACTGAG
Col1a1	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
Opn	ATCTGGGTGCAGGCTGTAA	CCCGGTGAAAGTGACTGATT
Dmp-1	TTGGGATGCGATTCCTCTAC	GGTTTTGACCTTGTGGGAAA
Sost	GCAGCTGTACTCGGACACATC	TCCTGAGAACAACCAGACCA
Dkk-1	GACAACTACCAGCCCTACCC	GATCTGTACACCTCCGACGC
Rankl	TGAAGACACACTACCTGACTCCTG	CCACAATGTGTTGCAGTTCC
Opg	ACCCAGAAACTGGTCATCAGC	CTGCAATACACACACTCATCACT
TrAcp5	CGTCTCTGCACAGATTGCAT	AAGCGCAAACGGTAGTAAGG
Ctsk	AGGCATTGACTCTGAAGATGCT	TCCCCACAGGAATCTCTCTG

Testing of mechanical properties

The mechanical properties of the femur and tibia were determined by a three-point bending test using biomechanical testing device (Mecmesin, West Sussex, UK). The femur was positioned horizontally with the anterior surface upward, centered on the supports (span = 9mm); and the middle point of the femoral and tibial shaft were vertically compressed at a constant speed of 4.5mm/min, and data was collected at a sampling rate of 10Hz until failure. The measured data was converted to a load-displacement curve in a

monitoring recorder linked to the tester. The maximum force and deformation and the break force and deformation were read respectively from the highest point of the load-deformation curve and from the failure point. Stiffness was calculated as the slope of the curve, which was fitted in the linear part of the load-deformation curve.

Statistical analyses

Two-way analysis of variance (2x2 ANOVA) was used for statistical evaluation and Student's *T*-test as a post hoc test with the statistical significance set to $p < 0.05$.

Results

Treatment with soluble ActRIIB-Fc increases body weight and muscle mass

This is a follow-up study of Hulmi et al. 2013 and the muscle and body weight results have been published previously [28, 29]. In brief, our results show that the muscle mass increased in the ActRIIB-Fc treated groups. A significant change in the masses of both the gastrocnemius muscles and quadriceps femoris muscles was noted when comparing ActRIIB-Fc and PBS groups to each other. The running ActRIIB-Fc-R mice also developed larger muscle mass compared to the running PBS-R group but changes were more modest compared to the non-running groups.

As expected both active and sedentary ActRIIB-Fc treated mice also had a significant increase in body weight compared to their respective PBS controls. However, both running groups gained less weight during the experiment and weighed less also at the end compared to non-runners, mainly due to decreased fat mass as published earlier [15]. Therefore at the end of the experiment the bodyweight was equal in the ActRIIB-Fc-R and PBS groups. The final mean weights for the mice per group were as follows: PBS 30.3 ± 0.8g, ActRIIB-Fc 34.0 ± 1.9g, PBS-R 29.7 ± 1.7g and ActRIIB-Fc-R 31.6 ± 2.3g.

ActRIIB-Fc increases bone volume and bone mineral density in appendicular skeleton

The μCT analysis of the distal femur showed a remarkable increase in bone mass in the ActRIIB-Fc-treated mice (ActRIIB-Fc effect ANOVA: $p < 0.001$) and this effect of ActRIIB-Fc was seen in both running and sedentary mice (Fig. 1). Bone volume and trabecular number (Fig. 1c-d) were increased in ActRIIB-Fc treated sedentary mice by over 80% and over 70% ($p < 0.001$), respectively. Volumetric bone mineral density (vBMD) (Fig. 1f) increased in the ActRIIB-Fc treatment group and the separation (Fig. 1e) between trabeculae also decreased (ActRIIB-Fc effect $p < 0.001$). These results demonstrate larger, more numerous trabeculae that are located more closely to each other resulting in a more dense tissue compared to PBS controls, when activin receptor ligand signaling is inhibited. Running alone had little effect on bone volume and trabecular number in PBS-treated mice. In addition, there was no significant difference in trabecular bone structure or vBMD between the ActRIIB-Fc and ActRIIB-Fc-R groups indicating that running did not further improve bone architecture in ActRIIB-Fc-treated mice (ANOVA ActRIIB-Fc x running interaction effect $p = 0.434$). The cortical bone analysis of the femoral mid-shaft that ActRIIB-Fc treatment, with and without running, increased cortical thickness by 14% ($p < 0.05$ in both) when comparing to PBS controls (Fig. 1g). Running alone also resulted in a 10% increase ($p < 0.05$ compared to sedentary PBS group) in cortical thickness. Mean total cross-sectional bone area was also increased by 10% by ActRIIB-Fc treatment and running (Fig. 1h). However, there was no additional effect on either cortical thickness or area by the combination of ActRIIB-Fc and running (ANOVA ActRIIB-Fc x running interaction effect $p = 0.436$ and $p = 0.421$, respectively).

ActRIIB-Fc treatment results in increased bone mass also in axial skeleton

We also wanted to assess whether the treatment with ActRIIB-Fc only affected weight bearing long bones or if the axial skeleton was involved as well. For this purpose we performed μCT analysis on the second lumbar vertebrae.

The ActRIIB-Fc treatment-induced changes in the vertebrae were similar but more modest compared to the distal femur (Fig. 2). Bone volume (Fig. 2c) was increased by 20% and trabecular number (Fig. 2d) by 30% in the ActRIIB-Fc-treated mice compared to PBS group ($p < 0.001$). Similar to the femur analysis, bone mineral density (Fig. 2e) was also increased and trabecular separation (Fig. 2f) decreased (ActRIIB-Fc effect $p < 0.05$). Running seemed to induce an increase in trabecular bone volume and vBMD in vertebrae when comparing PBS-R to the PBS-controls (running effect $p < 0.05$). As noted previously for femur, the combined effect of ActRIIB-Fc and running on vertebral bone did not significantly differ from the effect of ActRIIB-Fc treatment alone.

ActRIIB-Fc decreases the number of osteoclasts on trabecular bone

Histological analysis of distal femur confirmed our findings in μCT analyses of increased bone volume per tissue volume and trabecular number in the ActRIIB-Fc-treated mice (Fig. 3). Interestingly, a decrease in osteoclast number (N.Oc/B.Pm) was also observed (Fig. 3h). This suggests that in addition to its published effects on bone formation ActRIIB-Fc treatment suppresses osteoclast differentiation and therefore inhibits bone resorption.

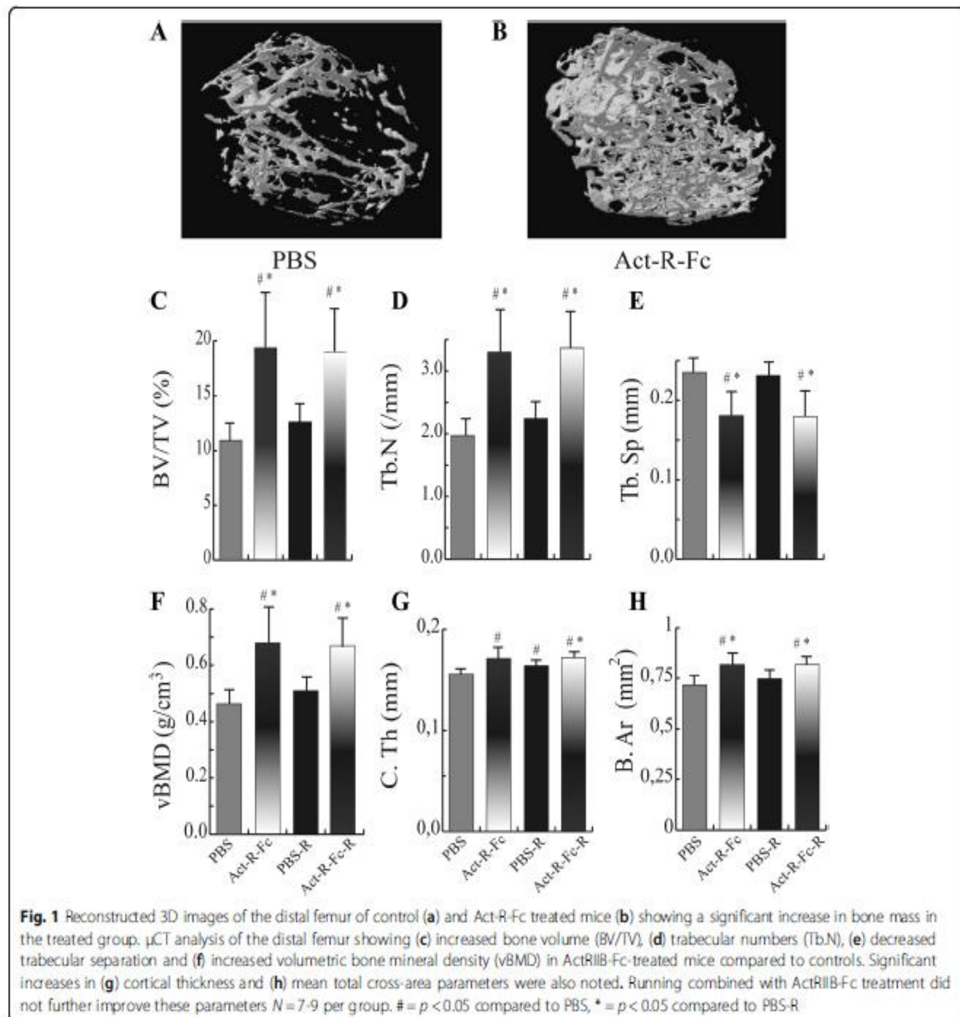

Fig. 1 Reconstructed 3D images of the distal femur of control (**a**) and Act-R-Fc treated mice (**b**) showing a significant increase in bone mass in the treated group. μCT analysis of the distal femur showing (**c**) increased bone volume (BV/TV), (**d**) trabecular numbers (Tb.N), (**e**) decreased trabecular separation and (**f**) increased volumetric bone mineral density (vBMD) in ActRIIB-Fc-treated mice compared to controls. Significant increases in (**g**) cortical thickness and (**h**) mean total cross-area parameters were also noted. Running combined with ActRIIB-Fc treatment did not further improve these parameters $N = 7$-9 per group. # = $p < 0.05$ compared to PBS, * = $p < 0.05$ compared to PBS-R

ActRIIB-Fc treatment increases expression of osteoblast markers and decreases expression of osteoclast markers

To further investigate the mechanisms behind these effects we treated C57Bl mice with ActRIIB-Fc or vehicle, collected bone samples and measured the expression of essential osteoblast and osteoclast marker genes. qPCR analysis (Fig. 4) revealed increased expression of characteristic osteoblast markers (Col1A1 + 80% $p = 0.05$, OPN +55% $p < 0.05$) and osteocyte marker (DMP-1 + 125% $p < 0.01$) as well as decreased expression of RANKL (-44% $p < 0.01$) a regulator of bone resorption. There was also a trend of decreased TRAP expression (-33% $p = 0.1$) in the treated group. These results suggest that ActRIIB-Fc simultaneously increases osteoblast induced bone formation and decreases bone resorption.

Treatment with ActRIIB-Fc improves mechanical strength

To test whether treatment with ActRIIB-Fc would also improve the mechanical properties of long bones, we performed a three point bending test on femurs and tibias. There was a significant increase in stiffness and bone strength in the ActRIIB-Fc-treated compared to the PBS group in sedentary mice (Fig. 5). Running alone resulted in a trend of stiffer and stronger bones but this did not appear to be statistically significant. In addition, voluntary running did not further improve bone biomechanical properties in the running ActRIIB-Fc-treated mice.

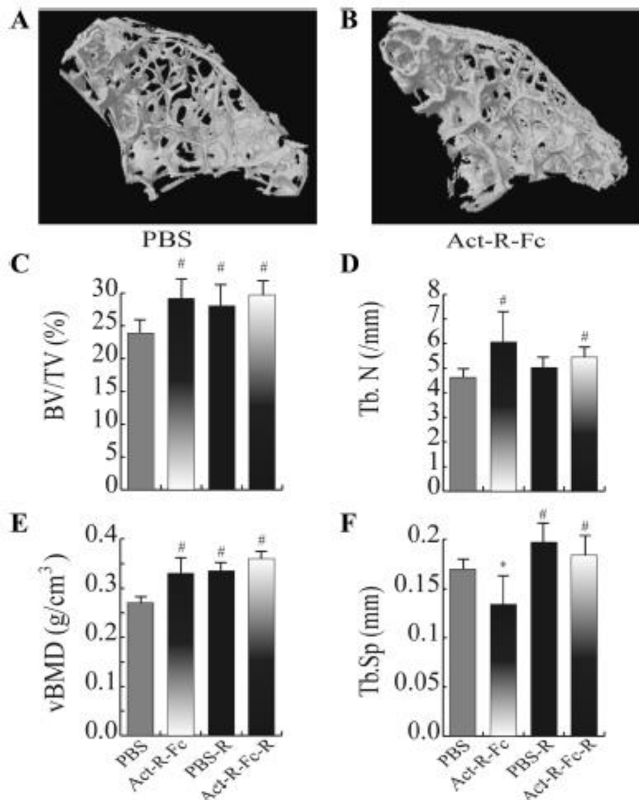

Fig. 2 Reconstructed 3D images of the 2nd lumbar vertebrae also showing a noticeable difference in bone mass between the control (**a**) and treatment group (**b**). Similar to the distal femur, (**c**) bone volume (BV/TV), (**d**) trabecular number (Tb.N) and (**e**) volumetric bone mineral density (vBMD) were increased while (**f**) trabecular separation (Tb. Sp) was decreased in ActRIIB-Fc-treated mice compared to controls. ActRIIB-Fc and running did not result in further beneficial effect. # = $p < 0.05$ compared to PBS, * = $p < 0.05$ compared to PBS-R

To further explore the effects of ActRIIB-Fc treatment on bone tissue, the results were then adjusted with body mass acting as the covariate. After adjustment minor changes could only be seen in the μCT analysis of the vertebrae that did not affect the interpretation of the results. Therefore the differences between the body weights of the mice did not seem to significantly affect the results of our analyses. The datasets analyzed during the current study are available from the corresponding author upon request.

Discussion

In this study we evaluated the effects of inhibition of ActRIIB ligands on bone and muscle tissue using a soluble activin type IIB-receptor in an mdx mouse model. We also aimed to test whether voluntary physical activity combined with ActRIIB-Fc treatment would have an effect on these tissues. Our results indeed confirm our hypotheses. First, we were able to show a significant increase in bone mass in ActRIIB-Fc treated mice compared to PBS treated control mice. Second, our findings demonstrate that ActRIIB-Fc affects both appendicular and axial bone mass and beneficially modifies biomechanical properties of long bones. Finally, although exercise alone had some positive effects on bone structure, combination of running exercise with ActRIIB-Fc did not further increase bone mass or strength compared to ActRIIB-Fc treatment alone.

Our μCT results of the distal femur showed that the ActRIIB-Fc treatment resulted in increased vBMD, number of bone trabeculae and increased bone volume. The separation of trabeculae was also decreased. Our findings are consistent with previous reports demonstrating that treatment with either ActRIIA-Fc [6, 8] or ActRIIB-Fc [9, 10] resulted in increased bone volume. Increased BMD in trabecular bone also suggests that at least part of the effect could be derived from decreased bone resorption, possibly due to slower bone turnover and prolonged secondary mineralization and filling of resorption

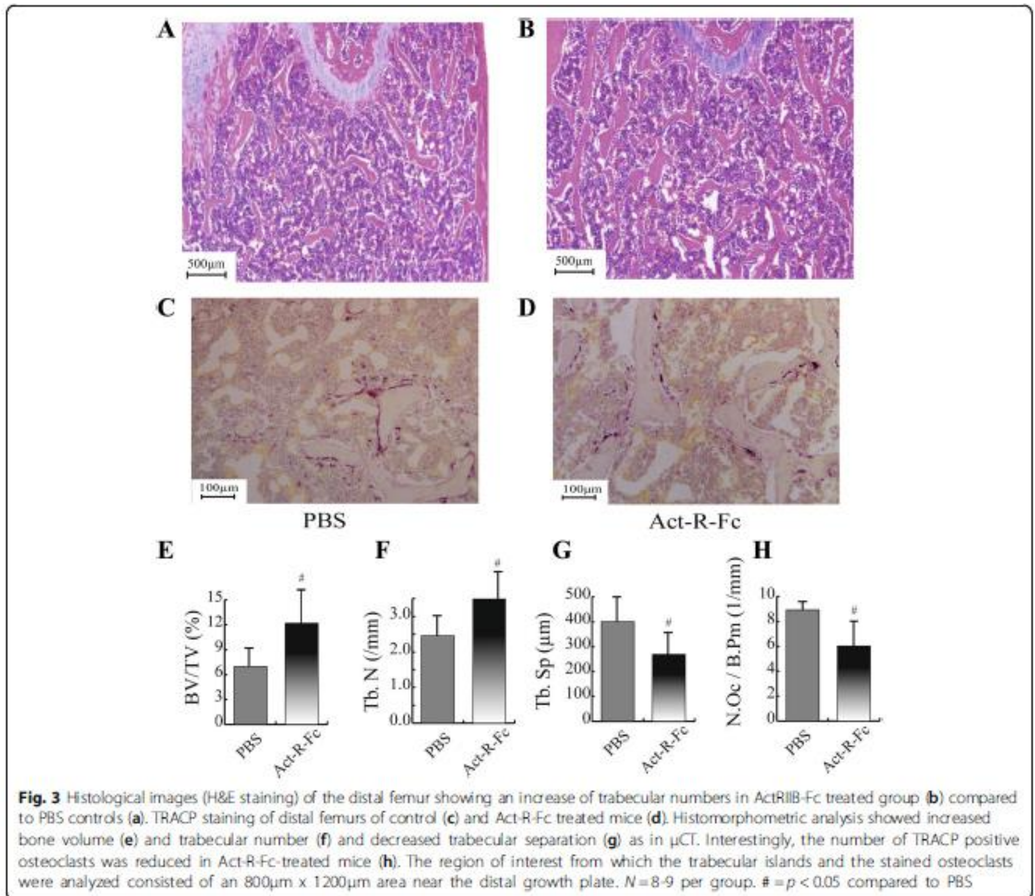

Fig. 3 Histological images (H&E staining) of the distal femur showing an increase of trabecular numbers in ActRIIB-Fc treated group (**b**) compared to PBS controls (**a**). TRACP staining of distal femurs of control (**c**) and Act-R-Fc treated mice (**d**). Histomorphometric analysis showed increased bone volume (**e**) and trabecular number (**f**) and decreased trabecular separation (**g**) as in µCT. Interestingly, the number of TRACP positive osteoclasts was reduced in Act-R-Fc-treated mice (**h**). The region of interest from which the trabecular islands and the stained osteoclasts were analyzed consisted of an 800µm x 1200µm area near the distal growth plate. $N = 8$-9 per group. # = $p < 0.05$ compared to PBS

spaces. However, it was interesting to note that the differences between ActRIIB-Fc and ActRIIB-Fc-R groups were miniscule showing that physical activity did not have a noticeable further effect on trabecular bone in the murine femur in this model. ActRIIB-Fc-R mice ran significantly less especially in the beginning of the study as previously reported [26], which could at least in part explain the lack of additional effect of running on bone mass or strength in the ActRIIB-Fc-R group. The non-significant change between PBS and PBS running group in bone mineral density was also surprising suggesting that physical activity in a form of voluntary running does not greatly affect bone quality in long bones in young mdx mice.

In humans, weight bearing exercise has positive effects on the bones of young individuals and voluntary running may have positive effects on murine bones [30]. However, as often rather high intensity/volume of exercise is needed for positive effects on bone, our training modality might have been of too low intensity to induce more robust effects on bone mass [14]. In contrast to our results, Hamrick et al. suggested that the combination of exercise and increased muscle mass in myostatin-deficient mice has a much greater effect on bone strength than exercise or muscle mass alone [31]. This could be explained by the fact that the myostatin-deficient mice used in their study had increased lean mass postnatally and this increased contractile forces induced by locomotion and resulted in a more powerful mechanotransduction effect on bones. In addition, their exercise regime based on force exercise was most likely more intense compared to our method of voluntary exercise. Finally, as Hamrick et al. analyzed the radius, they stated that their effects could be partly explained

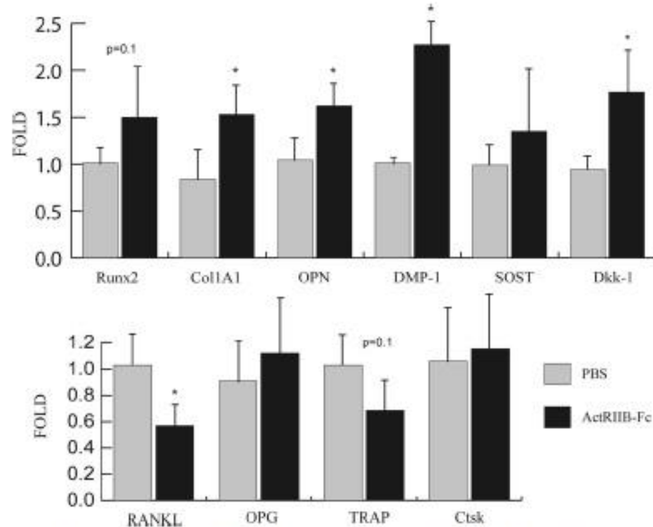

Fig. 4 qPCR analyses show changes in the expression of key markers for bone metabolism. Abbreviations in order: Runt related transcription factor 2, Type I collagen, Osteopontin, Dentin matrix protein 1, Sclerostin, Dickkopf-related protein 1, Receptor activator of nuclear factor kappa-B ligand, Osteoprotegerin, Tartrate resistant acid phosphatase, Cathepsin K. $N = 6\text{-}7$ per group. * = $p \leq 0.05$ compared to PBS group

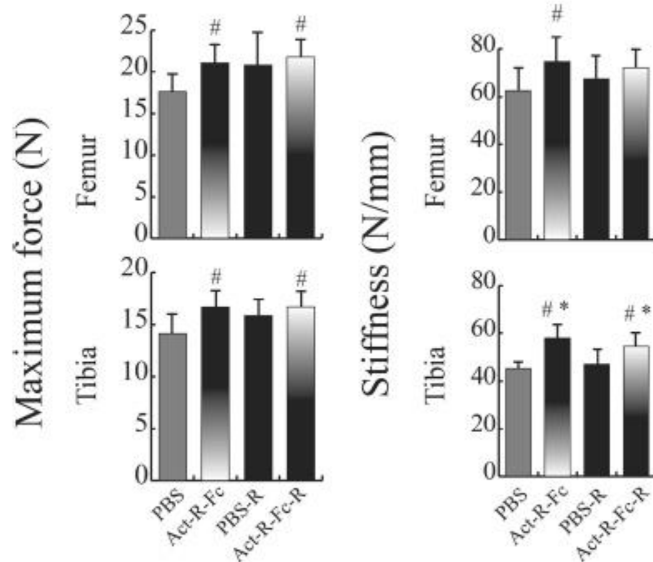

Fig. 5 Biomechanical testing of femurs and tibias. A significant increase in both maximum force and stiffness was measured in the Act-R-Fc treated mice compared to the control group. However there was no statistical significance between the ActRIIB-Fc-treated and control running groups. $N = 7\text{-}9$ per group. # = $p < 0.05$ compared to PBS, * = $p < 0.05$ compared to PBS-R

by the difference in the load induced by the curvature of the bone they analyzed.

The effect of ActRIIB-Fc treatment was also seen in second lumbar vertebrae but it was more modest than in distal femur analysis. Bone volume was increased by 22% in vertebrae compared to 83% in the femur. Our results on lumbar vertebrae are consistent with the article published by Bialek et al., in which they used normal C57Bl mice [9]. As in femur, voluntary physical activity combined with ActRIIB-Fc treatment did not have a synergistic effect in vertebrae. Trabecular numbers increased only marginally and the increases in bone volume and volumetric bone mineral density were not statistically significant.

Improved cortical geometry in long bones also translated into significantly improved biomechanical properties as both the maximal failure load as well as stiffness increased in the femurs and tibias of ActRIIB-Fc treated mice. Bialek et al [9] also reported that ActRIIB-Fc treatment increased bone strength when compared to vehicle group but found this only in the L4 vertebrae. In our study voluntary running did not have statistically significant effect on bone strength although there was a positive trend in both failure load and stiffness. This lack of effect could be due to the relatively small sample size.

Histological analysis of the distal femur comparing PBS and ActRIIB-Fc groups was done to assess the effect of blockage of ActRIIB-ligands on osteoclast parameters. Histological analysis confirmed the increase in bone volume and trabecular number as was noticed in μCT. However, we also found a significant decrease in the number of osteoclasts per bone perimeter in the ActRIIB-Fc treated animals. This suggests a suppression of osteoclast differentiation and subsequently bone resorption that has not previously been reported with ActRIIB-Fc. As discussed above, this finding is in agreement with the increased trabecular vBMD in the distal femur observed with μCT. Activin A has been shown to induce osteoclast differentiation in vitro and in vivo [32–35], although some reports suggest a negative effect on survival and motility of mature osteoclasts [36]. The decreased osteoclast number in our study supports the role for activin A to induce osteoclast differentiation, although we cannot exclude the possible effect of other ligands binding to ActRIIB-Fc. In addition, ActRIIB ligand inhibition could also affect the osteoblast-dependent regulation of osteoclastogenesis. Previous studies have shown that blocking of activin receptor ligands results in increased bone formation translating into increased bone mass. Unfortunately, the simultaneous signaling and metabolic analyses of muscle tissues performed in this study [26] and the limited number of animals available prevented us from using fluorochromes to measure bone formation in mice. However, based on the very robust increase in trabecular bone volume, gene expression discussed below and the previously published data, it is very likely that ActRIIB-Fc molecule used in our study also induced an increase in bone formation.

Our results also provide novel data regarding the molecular mechanism behind the effects of ActRIIB-Fc on bone growth. We were able to show that ActRIIB-Fc induces an increase in osteoblast and osteocyte gene markers suggesting that ActRIIB-Fc indeed also has an anabolic effect on bone. Furthermore we were able to confirm our hypothesis of ActRIIB-Fc acting as a suppressor of osteoclast activity as the expression of osteoclast markers decreased noticeably. Interestingly, expression of DKK-1 and sclerostin, key markers for negative regulation of WNT signaling, also significantly increased. This could be due to negative feedback loop induced by increased bone mass and/or reflect enhanced Wnt signaling. Alternatively, the concomitantly increased osteocyte number could also contribute to the increased expression of DKK1 and sclerostin.

Based on the present experiment, the molecular basis for the effects of ActRIIB-Fc treatment and running on bone tissue, independent of increased body and muscle mass, remain unclear. If ActRIIB-Fc and running have independent signaling pathways for bone adaption, one would have expected an additive effect. However, investigating this interaction is encumbered by the marked effect of running on body mass, and the inextricable link between the body and skeletal size. On the other hand, adjusting the data to body weight did not significantly alter the results on bone structure or strength. Considering the effectiveness of the interventions in isolation, the results indicated a clear and robust skeletal effect by ActRIIB-Fc. However, judging by the PBS groups the effects of the running intervention on skeletal mass were much more modest, and could not be observed in the femur analysis. Clearly, from the two interventions applied in the present study, ActRIIB-Fc was more effective, as we expected. Therefore our study provides promising evidence that ActRIIB-Fc could be applied as a therapeutic agent in musculoskeletal disorders where physical activity is limited.

It is also notable that as soluble activin receptors also bind other growth factors in addition to activin A [23, 37]. It remains unclear which is/are the main effectors inducing the observed alterations. Activin A suppresses bone formation and induces resorption and is therefore the primary suspect for direct bone effects of ActRIIB-Fc, but inhibition of other growth factors may also contribute. Most likely the majority of the effect on bone is derived from direct regulation of bone cell functions. However, due to the known muscle-bone interactions it is also possible that some of the positive effects on bone could stem from simultaneous increase in muscle mass either via increased body mass or by direct

molecular cross talk between muscle and bone tissues. As adjusting for the body mass did not alter the results the effect would likely be direct signaling between the tissues. Further molecular studies are needed to clarify this question.

Conclusions

In conclusion, we show here that inhibition of activin receptor ligand signaling using ActRIIB-Fc positively affects bone mass and quality and subsequently bone mechanical strength, without further positive effect by physical exercise. Soluble ActRIIB-Fc could provide an intriguing approach for the treatment of coexisting muscle and bone loss in many diseases, aging and in injuries.

Abbreviations

ActRIIB-Fc: Activin type IIB receptor fusion protein; BV/TV: Bone volume per tissue volume; Col1a1: Collagen type I; DMD: Duchenne muscular dystrophy; Mdx: Dystrophic mouse with point mutation in the dystrophin gene; N.Oc/B.Pm: Number of osteoclasts per bone perimeter; OPN: Osteopontin; PBS: Phosphate buffer solution; RANKL: Receptor activator of nuclear factor kappa-B ligand; Tb.N: Trabecular number; TGF-β: Transforming growth factor β; Trap: Tartrate-resistant acid phosphatase; vBMD: Volumetric bone mineral density; μCT: Micro-computed tomography

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Availability of data and materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' contributions

JJH, HK, TP, RK, KH and OR participated in the designing of the study. JJH, HM and AP participated in the study conduct. Data was collected by HM, TP and JJH. TP and HM analyzed the data. TP, HM, RK, JJH, TR, OR were involved in interpreting the results. TP drafted the manuscript. The content of the manuscript was revised by TP, TR, JJH, HM, HK, OR, AP, KH and RK. Finally TP, TR, JJH, HM, HK, OR, AP, KH and RK approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The treatment of the animals was in strict accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocol was approved by the National Animal Experiment Board (Permit Number: ESLH-2009-08528/Ym-23).

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RESEARCH ARTICLE

Soluble activin type IIB receptor improves fracture healing in a closed tibial fracture mouse model

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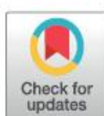

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Abstract

Fractures still present a significant burden to patients due to pain and periods of unproductivity. Numerous growth factors have been identified to regulate bone remodeling. However, to date, only the bone morphogenetic proteins (BMPs) are used to enhance fracture healing in clinical settings. Activins are pleiotropic growth factors belonging to the TGF-β superfamily. We and others have recently shown that treatment with recombinant fusion proteins of activin receptors greatly increases bone mass in different animal models by trapping activins and other ligands thus inhibiting their signaling pathways. However, their effects on fracture healing are less known. Twelve-week old male C57Bl mice were subjected to a standardized, closed tibial fracture model. Animals were divided into control and treatment groups and were administered either PBS control or a soluble activin type IIB receptor (ActRIIB-Fc) intraperitoneally once a week for a duration of two or four weeks. There were no significant differences between the groups at two weeks but we observed a significant increase in callus mineralization in ActRIIB-Fc-treated animals by microcomputed tomography imaging at four weeks. Bone volume per tissue volume was 60%, trabecular number 55% and bone mineral density 60% higher in the 4-week calluses of the ActRIIB-Fc-treated mice ($p<0.05$ in all). Biomechanical strength of 4-week calluses was also significantly improved by ActRIIB-Fc treatment as stiffness increased by 64% and maximum force by 45% ($p<0.05$) compared to the PBS-injected controls. These results demonstrate that ActRIIB-Fc treatment significantly improves healing of closed long bone fractures. Our findings support the previous reports of activin receptors increasing bone mass but also demonstrate a novel approach for using ActRIIB-Fc to enhance fracture healing.

Introduction

Fracture healing is a complex process consisting of multiple events, which happen both simultaneously and in succession, aiming to restore the initial form and function of the fractured bone. This process is strictly regulated by numerous different cytokines, growth factors, proteases and

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angiogenic factors [1–3]. Recent advances in this research field have also highlighted the importance of muscle-bone cross-talk in the formation of new bone during fracture healing [4].

Numerous growth factors and signaling pathways have been identified to regulate bone remodeling. Many of these growth factors have also been shown to participate in different phases of fracture healing. Therefore, they have also emerged as potential therapeutic agents [5]. Most notable ones include members of the Transforming Growth Factor β (TGF-β) superfamily. Specific members of this superfamily, such as Bone Morphogenetic Proteins (BMPs), have shown auspicious results in different *in vivo* models in enhancing bone formation, although their efficacy has been questioned in recent clinical trials [6–9]. Furthermore, due to the lack of long-term control studies and high costs, they are yet to rival autogenous bone grafts in clinical practice, which are still conceived as the golden standard in reconstructing bone defects [10].

Activins are pleiotropic growth factors belonging to the TGF-β superfamily, which have also been linked to different pathophysiological processes [11–14]. Activin type II receptors, which initiate the activin signaling cascade consisting of activin type I and II receptors as well as receptor-regulated/common-mediator Smads, have recently been identified as novel therapeutic approaches to increase bone mass [15]. Recombinant fusion proteins of these receptors, ActRIIA-Fc and ActRIIB-Fc, function by trapping numerous ligands, including activins, and thus inhibit the ligand functions. We and others have recently shown that treatment with these activin inhibitors greatly increases bone mass systematically in different animal models [16–18]. Additionally loss of type II BMP-receptor has been reported to increase osteoblast activity and lead to increased bone mass [19].Recent report also shows that treatment with ActRIIA-Fc promotes callus formation in a closed femoral fracture model in rats [20]. Another study, however, suggested that the administration of Bimagrumab, an anti-ActRII antibody that directly inhibits the ActRII receptor instead of trapping its ligands, does not have a major impact on fracture healing [21]. Therefore further clarification of the effects of activin signaling pathways on fracture healing is needed.

We set out to investigate the effects of ActRIIB-Fc on fracture healing using a standardized, closed, diaphyseal tibial fracture mouse model. We hypothesized that inhibition of activin type IIB receptor ligands using ActRIIB-Fc would improve callus mineralization and increase bone formation resulting in accelerated fracture healing.

Materials and methods

Animals

In this experiment 12–14 week old male C57Bl/6 mice were used (Harlan Laboratories B.V, Netherlands). The animals were housed individually in cages under standard laboratory conditions (temperature 22˚C, light from 6:00AM to 6:00PM.) Water and soy-free food pellets were available ad libitum, excluding a four-hour fasting period before euthanization. Two animals were sacrificed by CO_2 asphyxia followed by cervical dislocation due to post-operative complications and three animals developed a severe rash behind the back of the neck during the experiment. This was observed in both PBS- and ActRIIB-Fc-treated mice and was most likely related to the C57BL/6 background of the animals. These animals were sacrificed by CO_2 asphyxia followed by cervical dislocation and omitted from the analyses. Fractures that did not meet the standard criteria were also excluded. Altogether 70 calluses were analyzed.

Surgical procedure

Under isoflurane anesthesia (250-400ml/min 2.5%) and aseptic surgical conditions, injections of buprenorfin (0.05mg/kg) and carprofen (5mg/kg) were administered subcutaneously. A

vertical incision was made over the patellar region of the right hind leg. This was then followed by a vertical incision through the patellar tendon exposing the proximal head of the tibia. A 25-gauge needle was used to drill a hole through the cortical bone above the tibial tuberosity. A sterile Ø0.2mm stainless-steel rod was then inserted into the tibial intramedullary canal reaching the distal end of the tibia. The wound was then closed with two non-absorbable simple interrupted sutures and an anti-septic (Betadine, Takeda) was applied locally on top of the closed wounds. Standardized, closed diaphyseal tibial fractures were then performed using a fracture apparatus as previously reported [22, 23].

After the fracture was produced, the mice were closely monitored and placed on heating beds to maintain standard body temperature. Upon waking up the animals were placed in individual cages and were allowed a post-operative healing time of three to four days with administration of postoperative analgesic injections of buprenorfin and carprofen for the first two days.

Study design

The mice were divided into two groups and were given either PBS or ActRIIB-Fc prepared in PBS (5mg/kg) intraperitoneally (i.p.) once a week. Bodyweights were recorded before every injection. Animals were sacrificed either on day 15 (two-week time point) or 29 (four-week time point) by CO2 overdose and cervical dislocation followed by sample collection.

Production of ActRIIB-Fc

The production of the ActRIIB-Fc protein used in this study has been reported earlier [24, 25]. Briefly, the growth factor receptor consists of the ectodomain of human ActRIIB-Fc joined with IgG1-Fc. It was then expressed in Chinese hamster ovary (CHO) cells grown in a suspension culture.

Preparation of tibia samples

The right hind legs were gathered and prepared for microcomputer tomography and histological analyses, biomechanical analyses or RNA analyses. For the first set of analyses, fracture calluses were stored in 3.8% formaldehyde for a period of 24 hours. The samples were then rinsed with PBS and stored in 70% EtOH in a dark room with a temperature of 8˚C. The muscles and tendons were then removed while preserving the callus. The metal rod was removed from the proximal end of the tibia before micro computed tomography imaging (n = 6–8 per group). For the biomechanical analyses the calluses were gathered and the muscles, tendons and the intramedullary rod were removed immediately after animal euthanization. The bones were then separately wrapped in PBS-soaked gauzes and tin foil and then stored in -20˚C. The samples were thawed right before biomechanical testing. (n = 5–8 per group). For the RNA analyses, fracture calluses were extracted, removed of the attached muscle soft tissue, snap frozen in liquid nitrogen and stored in -80˚C. (n = 7–8 per group).

Measurement of gene expression

For total RNA isolation, the calluses were pulverized under liquid nitrogen, homogenized in TriSure reagent (Bioline) after which RNA was extracted. The samples were treated with DNAase treatment followed by RNA clean up using RNeasy mini kit (Qiagen, Germany). The cDNA was then synthesized from 1μg of total RNA with the SensiFAST probekit (Bioline, UK). Quantitative real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad laboratories, USA). The relative mRNA expression levels were then analyzed and quantified

using the 2-ΔΔCT method. The expression levels were normalized to β-actin, which was used as the internal control. Primer sequences are available upon request.

Micro-computed tomography (μCT) analysis

X-ray micro-computed tomography of the fractured area was done using SkyScan 1070 μCT Scanner (SkyScan, Kontich, Belgium) to assess the structure of the callus using the following settings: voxel resolution of 5.33 um, X-ray potential of 70kVp, current of 200uA and an integration time of 3900ms. The object was rotated in 0.45˚ steps throughout the scanning for a total revolution of 182.45˚. Reconstruction of the scanned images (Nrecon 1.4, Skyscan) was then done with identical settings (misalignment < 3, ring artifacts reduction 11, beam hardening correction 95%, and intensity gap 0.014–0.13). The corresponding region of interest (ROI) was drawn by the outlines of the callus (CTan 1.4.4, SkyScaN). The first and last set points of the ROI were chosen as where the fracture line was distinctly evident and the cortex of the tibial bone was not intact. The threshold values were chosen by binarization and threshold delineation so the analysis only accounted for the newly formed mineralized tissue and not the preexisting cortical bone. Bone mineral density values were calibrated using two phantoms (calcium hydroxyapatite discs with densities of 0.25g/cm3 and 0.75g/cm3 respectively) during the scanning phase. The results were then quantified and analyzed (Batman, Skyscan).

Histological analysis

After μCT imaging the samples were decalcified in formic acid, embedded in paraffin and then sectioned using a microtome along the sagittal plane of the middle of the callus. The sections were then deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) according to the standard protocols. Histological analyses to assess bone parameters were measured blinded for the treatment using Osteomeasure–system (Osteometrics, USA). The region of interest consisted of the callus without the periosteum, fracture ends of cortical bone or the bone marrow cavities. In the two-week samples the amount of cartilage and woven bone was analyzed and in the four-week samples the amount of trabecular bone inside the callus was assessed. Woven bone was differentiated from trabecular bone by the structure of the trabecular islands and the amount of cellularity within the trabeculae. Cartilage area was determined by the presence of prehypertrophic and hypertrophic chondrocytes based on the H&E staining.

Immunohistochemical staining

4μm sections were cut from the paraffin-embedded samples for immunohistochemical analyses. Sections were pre-incubated with 3% bovine serum albumin (BSA) prior to the primary antibody reaction. Immunohistochemical staining was performed using the following primary antibodies: Cathepsin K (AF9210, Acris, Germany), Runx2 (D1H7 #8486, Cell Signaling Technology, USA), P-Smad1 (Ab73211, Abcam, UK) and P-Smad2 (Ab188334, Abcam, UK). The secondary antibody: Poly-HRP-Anti-Rabbit IgG (Immunologic, Netherlands). 3–3'Diaminobenzidine (DAB) was used as the chromogen and sections were counterstained with Mayer's hematoxylin. Antibody-positive cells were then quantified blinded from a representable area ($1mm^2$) inside the callus of each sample.

Testing of biomechanical properties

A three-point-bending test was performed to evaluate biomechanical strength of the fracture calluses using a universal testing machine (Lloyd Instruments LRX, Lloyd Instruments Ltd.,

Fareham, Uk). The fractured tibias were placed in a lateral position and the proximal and distal ends were fixed to two in-house support pins (span = 9mm) to ensure sample stability during the procedure. The anterolateral surface of the middle part of the callus was subjected to vertical compression at a constant velocity (4.5mm/min) until breaking of the callus occurred. The measured data was converted into a load-displacement graph in real-time and the numerical values for the maximum force, stiffness and Young's modulus of bending were then analyzed.

Statistical analyses

All of the analyses were subjected to statistical evaluation and are shown as mean and standard deviations of the values (±). Student's t-test as was used to assess statistical significance where the p value was set to 0.05. All statistical analyses were done with IBM SPSS Statistics v.20 (IBM, USA).

Ethics statement

This study protocol including all the procedures was approved by the National Animal Experiment Board ELLA (license: ESAVI/11044/04.10.07/2014). All animal experiments were performed strictly according to the approved protocol.

Results

ActRIIB-Fc treatment augments body weight gain during fracture healing

The body mass increased significantly more in ActRIIB-Fc-treated mice compared to PBS controls during the experiment (Table 1) ActRIIB-Fc treatment induced a 12.6% increase in the body weight during the first week vs 6.6% increase in PBS groups ($p<0.001$) in relation to baseline and a 18.8% increase during the second week vs 8.6% increase in PBS groups ($p<0.001$) when compared to baseline weight (Table 1). At the third and fourth week the changes were more modest as shown in Table 1.

ActRIIB-Fc robustly accelerates fracture healing and callus mineralization

Radiographic images indicated no major differences between the fracture callus opacity in the two week groups but at four weeks the degree of callus maturation was greatly improved due to ActRIIB-Fc treatment (Fig 1A–1D). Cross-sectional µCT images demonstrated a greater amount of newly formed trabeculae inside the callus. A µCT analysis was done to assess the trabecular bone structure and bone mineral density in the calluses. At the two-week time point there were only minor differences between the PBS- and ActRIIB-Fc-treated groups. Slight increases in bone volume per tissue volume (BV/TV), trabecular numbers (Tb.N) and volumetric bone mineral density (vBMD) were observed in ActRIIB-Fc-treated mice but these were not statistically significant (Fig 1E–1G). However, at four weeks these differences were

Table 1. Changes in body weight compared to the start of the experiment (%).

	1 week	2 weeks	3 weeks	4 weeks
PBS	6.6 ± 4.9	8.6 ± 5.3	10.9 ± 5.7	11.5 ± 6.3
ActRIIB-Fc	12.7 ± 3.7 ***	18.5 ± 5.2 *****	21.5 ± 7.6 ***	24.7 ± 7.5 ***

Mean ± SD *** = p<0.001 vs PBS group

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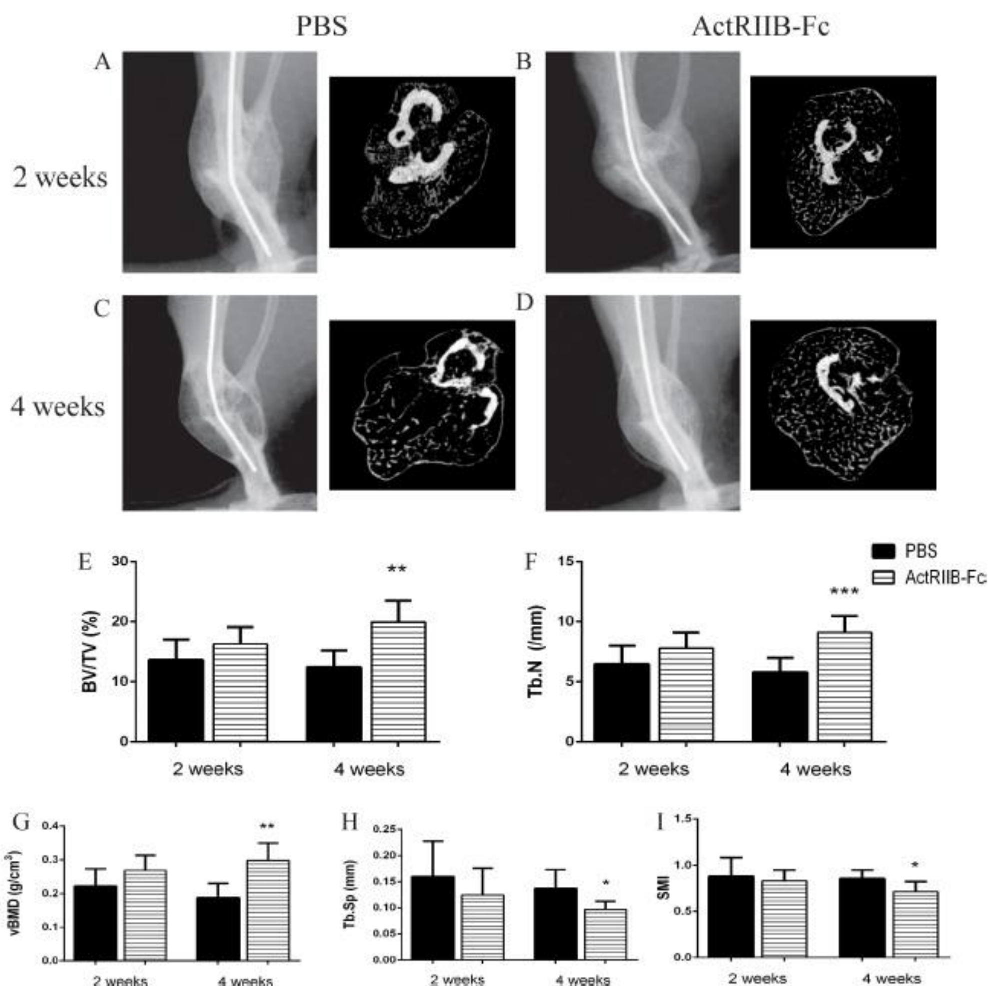

Fig 1. ActRIIB-Fc robustly accelerated fracture healing and callus mineralization. (A-D) Representative radiographic and micro-computed cross-sectional images of the fracture callus of the PBS- and ActRIIB-Fc-treated mice at two and four weeks. There were no significant differences in bone structure between the groups at the two-week time point. (E-I) At four weeks, ActRIIB-Fc treatment resulted in greater increases bone volume/tissue volume, trabecular numbers and volumetric bone mineral density as well as decreased trabecular separation and structural model index-* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. n = 8 for PBS 2 weeks, 7 for ActRIIB-Fc 2 weeks, 8 for PBS 4 weeks and 6 for ActRIIB-Fc 4 weeks.

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very prominent. ActRIIB-Fc treatment resulted in a greater bone volume per tissue volume BV/TV (+60%, $p<0.01$), Tb.N (+55%, $p<0.001$) and vBMD (+55%, $p<0.01$) compared to PBS control group. A clear decrease in trabecular separation (Tb.Sp) of the ActRIIB-Fc calluses was noted as well (-29%, $p<0.05$.) (Fig 1H) A difference in the structural model index (SMI) difference was also seen (-16%, $p<0.05$) in the ActRIIB-Fc-treated mice suggesting a better trabecular structure of the newly formed callus (Fig 1I).

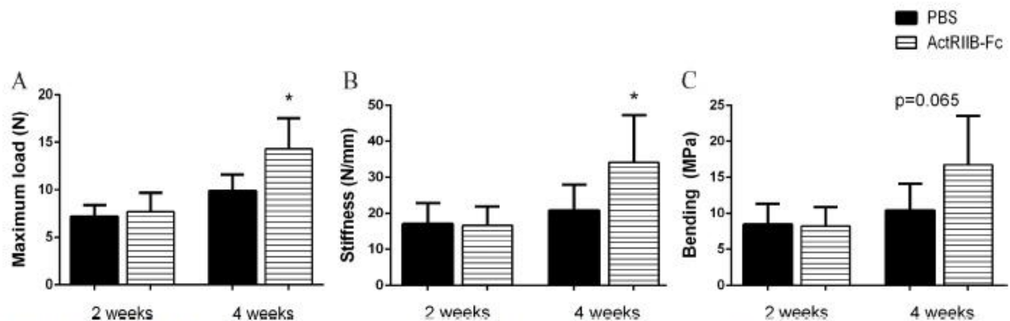

Fig 2. Treatment with ActRIIB-Fc improves mechanical strength of calluses. ActRIIB-Fc increases callus strength in the four week groups compared to PBS controls in terms of (A) maximum load, (B) stiffness and (C) bending strength. No significant changes were noted between the two week groups. * = $p<0.05$ n = 6 for PBS 2 weeks, 8 for ActRIIB-Fc 2 weeks, 5 for PBS 4 weeks and 7 for ActRIIB-Fc 4 weeks.

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Treatment with ActRIIB-Fc improved the callus mechanical strength

The three-point-bending test was used to assess the changes in biomechanical properties of the calluses between the control and treatment groups (Fig 2). There were no significant differences in the biomechanical strength of the calluses between PBS and ActRIIB-Fc groups at two weeks. At the four-week time point ActRIIB-Fc treatment resulted in improved bone strength as maximum load increased by over 45% ($p<0.05$) (Fig 2A) and stiffness by 65% ($p<0.05$) (Fig 2B). A trend in Young's Modulus of bending was noticed as it improved by 61% ($p = 0.065$) (Fig 2C) compared to PBS controls. These results demonstrate that ActRIIB-Fc treatment enhances the strength of the callus compared to PBS controls.

Histological analysis of the calluses

Histological analysis indicated significant differences in callus structure and composition in both the two- and four-week time points between ActRIIB-Fc-treated and PBS groups. At two weeks ActRIIB-Fc treatment resulted in larger amount of woven bone (WoBV/TV +38%, $p<0.01$) and cartilage (CgV/TV +106%, $p<0.05$) (Fig 3E–3G.) The combined woven bone and cartilage volume increased greatly (MdV/TV +47% $p<0.01$) due to ActRIIB-Fc. Similarly to the two-week time point, at four weeks ActRIIB-Fc treatment increased the amount of trabecular bone (BV/TV +44%, $p<0.01$ and Tb.N + 84%, $p<0.001$) while Tb. Sp was significantly decreased (-51%, $p<0.001$) (Fig 3H–3J) compared to the control group suggesting that ActRIIB-Fc treatment resulted in elevated numbers of larger trabeculae which are more densely adjacent to each other (Fig 3H–3J).

Increased expression of osteogenic marker genes in ActRIIB-Fc-treated calluses at two weeks

Quantitative real-time PCR analysis was done to assess the expression levels of specific gene markers that are known to be expressed during fracture healing (Fig 4). ActRIIB-Fc treatment resulted in 2.5-fold increase in *Osterix* ($p<0.05$) and 5-fold increase in *Runx2* ($p<0.01$) expression compared to controls. As both of these transcription factors are required for normal osteoblast differentiation, these data suggests that ActRIIB-Fc treatment enhances osteoblastogenesis and bone formation. This is further supported by the increased expression of Alkaline phosphatase (*ALP1*) expression also increased by 76% ($p<0.05$) compared to PBS

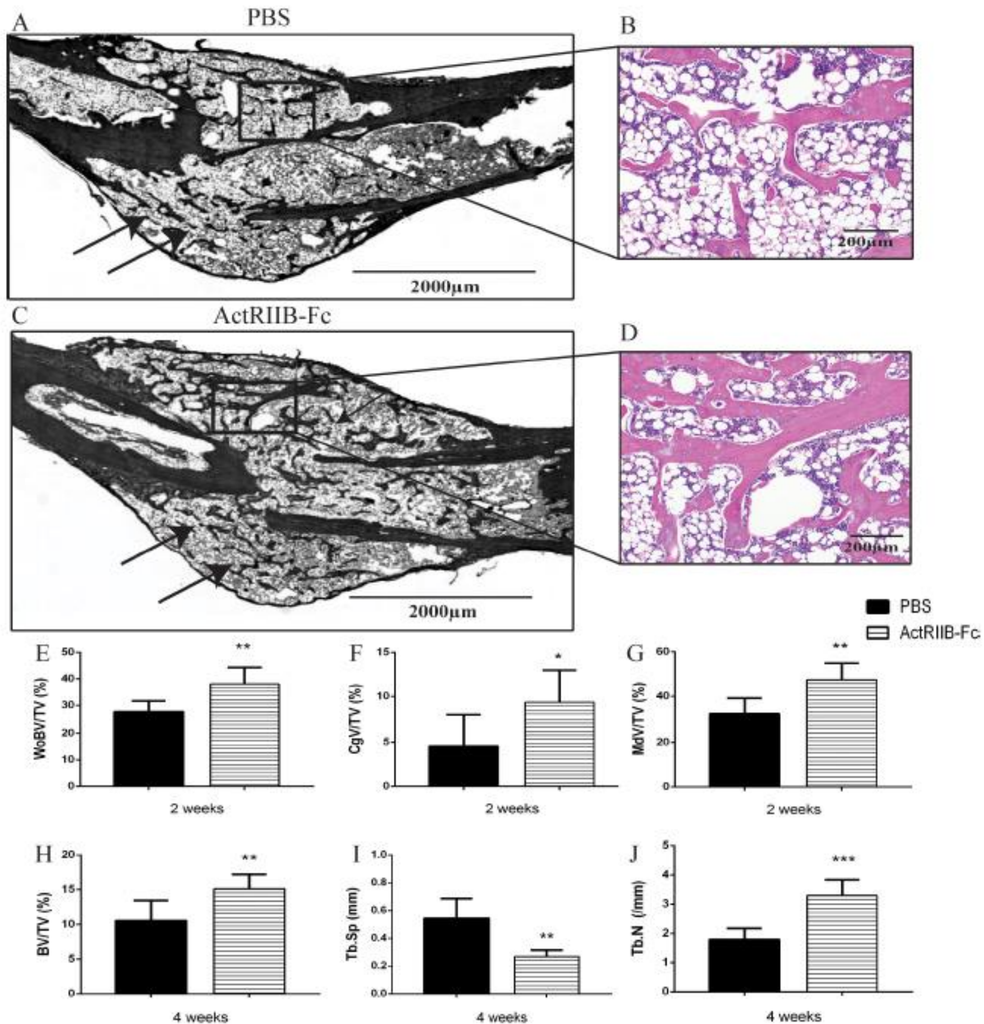

Fig 3. Histological analysis of the calluses. Representative hematoxylin and eosin stained histological images of fracture calluses at four weeks. (A and C) Overview image of the callus (in black and white in order to distinguish the newly formed trabecular bone more easily) and (B and D) larger magnification of the trabecular bone within the callus at four weeks. Black arrows pinpoint newly formed trabeculae. At the two week time point, ActRIIB-Fc treatment caused increased (E) woven bone volume and (F) cartilage volume compared to PBS controls resulting in increased (G) mineralized tissue per tissue volume. At the four-week time point ActRIIB-Fc treatment greatly enhanced (H) bone volume/tissue volume and (J) trabecular numbers and decreased their (I) separation * = $p<0.05$ ** = $p<0.01$, *** = $p<0.001$. n = 6 for PBS 2 weeks, 6 for ActRIIB-Fc 2 weeks, 7 for PBS 4 weeks and 7 for ActRIIB-Fc 4 weeks.

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controls. Conversely, the expression levels of markers of osteoclast activity, cathepsin K (*Ctsk*) and Tartrate resistant acid phosphatase (*ACP5*), decreased by 94% and 85%, respectively ($p<0.01$ in both) indicating decreased bone resorption in ActRIIB-Fc-treated calluses. The

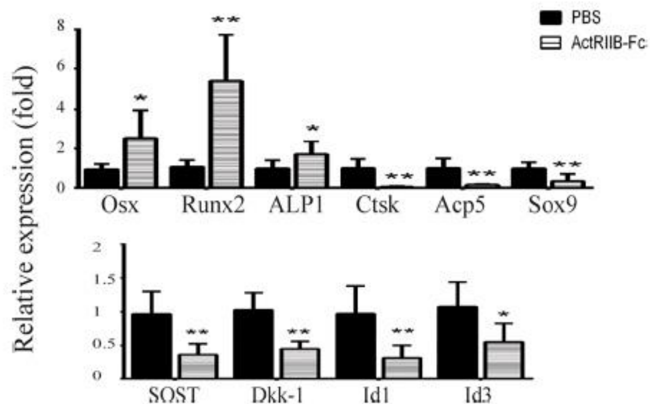

Fig 4. Increased expression of osteogenic marker genes in ActRIIB-Fc-treated calluses at two weeks. Quantitative real-time PCR analyses of the two-week time point revealed higher expression of essential osteoblast markers osterix, runt-related transcription factor 2 and alkaline phosphatase. Expression of cathepsin K and tartrate resistant acid phosphatase decreased compared to PBS controls which could demonstrate impaired cartilage and bone resorption. Furthermore expression of Sox9 was also lower compared to controls. Expression levels of Sclerostin and Dkk-1, negative regulators of Wnt-signaling, were also lower in ActRIIB-Fc-treated mice. Expression of Smad1/5/8 target genes Id1 and Id3 were decreased as well. * = $p<0.05$, ** = $p<0.01$. n = 7 for PBS groups and 8 for ActRIIB-Fc groups.

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expression of Sox9, an essential transcription factor for chondrogenesis, was significantly decreased and therefore Runx2:Sox9 expression rate was higher due to ActRIIB-Fc treatment compared to PBS controls. We also noticed significant decreases in the expression of negative regulators of Wnt-signaling including sclerostin and Dkk-1 ($p<0.01$ in both). Additionally we measured the expression levels of known Smad-targeted transcription factors Id1 and Id3 which were also significantly down-regulated compared to PBS controls ($p<0.01$ in both).

Immunohistochemical analyses of calluses

To provide further evidence for increased bone formation and decreased bone resorption, we performed immunohistochemical staining on the calluses (Fig 5). Trends of decreased number of cathepsin K positive cells at both two and four weeks as well as increased number of Runx2 positive cells at four weeks were noticed and, despite being non-significant, support our results of ActRIIB-Fc enhancing callus formation. Serum levels of marker of bone resorption, C-terminal telopeptide (CTX), and marker of bone formation, N-terminal type I procollagen (P1NP), were not significantly changed at two or four weeks (S1 Fig). To elucidate the mechanism for these changes, we quantified number of phospho(p)-Smad1 and p-Smad2 positive cells in the fracture calluses. We noted no differences in the number of p-Smad1+ cells between the groups at either time point but we observed trends of increased number of p-Smad2+ cells at two and four week time points in the ActRIIB-Fc-treated mice.

Discussion

The physiological changes associated with aging predispose older patients to musculoskeletal pathologies such as postmenopausal osteoporosis and frailty. Both of these conditions are risk factors for bone fractures. The optimal treatment approach would thus maintain or improve

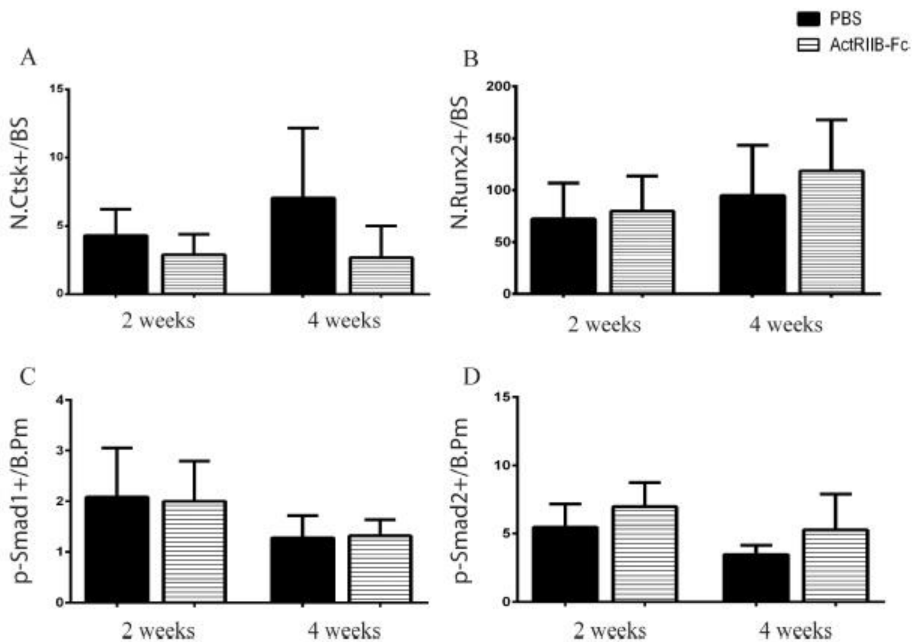

Fig 5. Immunohistochemical analyses of the calluses. Trends towards decreased number of cathepsin K+ cells in ActRIIB-Fc-treated mice were seen at both two and four weeks (A). The number of Runx2 positive cells was slightly increased at four weeks compared to PBS controls ($p = 0.346$) (B). There were no changes in the number of p-Smad1+ cells at either two or four weeks (C) but trends of increased number of p-Smad2+ cells were observed at two ($p = 0.056$) and four weeks ($p = 0.209$) due to ActRIIB-Fc-treatment (D). n = 8–9 for PBS 2 weeks, n = 10 for ActRIIB-Fc 2 weeks, n = 5–8 for PBS 4 weeks and n = 5–8 for ActRIIB-Fc 4 weeks.

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bone mass while enhancing/maintaining muscle mass and strength. The use of activin type II receptors as recombinant fusion proteins have already been shown to increase bone and muscle mass in different animal models but their effects on bone fracture healing are less well known. In this study we evaluated the effects of ActRIIB-Fc on fracture healing in a closed, diaphyseal tibial fracture mouse model. As we hypothesized, ActRIIB-Fc treatment improved fracture healing after four weeks of treatment when compared to PBS-treated controls. Both μCT and histological analyses at the four-week time point demonstrated increased amount trabecular bone in the calluses of ActRIIB-Fc-treated mice compared to control groups. This translated into improved biomechanical properties, as callus stiffness and strength were also increased by ActRIIB-Fc treatment. Our data indicate that these positive effects of ActRIIB-Fc were likely due to enhanced osteoblast differentiation and function and suppressed bone resorption in the fracture callus. Therefore, our results suggest that ActRIIB-Fc could be used as a novel approach to augment fracture healing.

Fracture healing is initiated by an early inflammatory phase followed by chondrogenesis and subsequent endochondral fracture healing. During the first two weeks, inflammatory and chondrogenic markers are highly expressed after which, the expression of markers of chondrogenesis dwells down and in turn the expression of osteogenic markers increases rapidly. At the two-week time point, cartilage volume was increased in ActRIIB-Fc-treated mice, while no

effect was yet found in the callus mineralization despite increased expression of osteoblast marker genes. There are two possible explanations to this. Either the chondrocyte proliferation and differentiation were enhanced or the remodeling of cartilage-containing callus by endochondral healing is impaired due to ActRIIB-Fc treatment. Myostatin has been suggested to suppress chondrocyte differentiation, while some data imply that activin A could actually promote chondrogenic differentiation. Making the picture even more complex, activin A is an important regulator of inflammatory processes that are essential for proper fracture healing [26, 27]. Interestingly, at the two-week time point we already found decreased expression of chondrogenic marker mRNAs and increased expression of osteoblastic genes, despite the increased amount of cartilage in the callus. We also did find a non-significant trend towards increased number of Runx2 positive cells in ActRIIB-Fc group that likely represent pre- or mature osteoblasts at the four-week time point. This is in line with the increased osteoblastic gene expression at two weeks. Taken together our data suggest that the sum effect of the inhibition of ActRIIB-Fc ligands leads to accelerated early endochondral ossification, possibly by promoting chondrogenic and osteoblastic differentiation of mesenchymal progenitor cells at the fracture site.

As mentioned above, the increased amount of cartilage within the callus could also be due to impaired remodeling of the callus. Indeed, we did observe significantly lower expression of osteoclast marker genes *Ctsk* and *ACP5* (Fig 4) within the callus at two weeks. We also saw a non-significant trend of decreased number of cathepsin K positive cells within the calluses of ActRIIB-Fc-treated animals at both time points, that together with the decreased expression of ACP5 and Ctsk mRNAs could support slightly decreased osteoclast numbers in the ActRIIB-Fc treated animals. Activin A has been shown to have a context-dependent role as a positive regulator of osteoclast induced bone resorption [28]. Interestingly, myostatin was also recently implicated in promoting osteoclastogenesis [29]. Therefore inhibition of both myostatin and activin A with ActRIIB-Fc could lead to suppressed remodeling of the newly formed callus and in part explain the histological findings of the increased cartilage mass at the two-week time point. An interesting question is then whether inhibition of bone resorption has a negative effect on fracture healing. Bisphosphonates (BPs) are drugs that suppress osteoclastic bone resorption by inducing osteoclast apoptosis [30]. BPs are widely used in the treatment of osteoporosis, Paget's disease and in skeletal metastases [31]. Both pre-clinical and clinical studies have been performed to assess the effects of BPs on fracture healing. Based on the available data BPs do not affect osteoblasts, inflammatory cells or other factors forming the soft or hard callus but the actual remodeling of the callus could be delayed [32]. However BPs administered early after the fracture do not delay fracture healing but could even accelerate the process [33]. Further studies are warranted to elucidate the specific effects of activin receptor ligand inhibition on the different phases of fracture healing and callus remodeling.

We were able to show that ActRIIB-Fc enhances fracture healing at the four-week time point with robust increases in the trabecular bone volume and bone mineral density within the callus. These changes were also translated into enhanced biomechanical properties as ActRIIB-Fc-treated calluses were stronger and stiffer compared to their PBS-treated controls. In addition, our data provide a possible mechanism for these effects as ActRIIB-Fc greatly increased the expression of osteoblast markers genes and favors the commitment of precursor cells to the osteogenic lineage over the chondrogenic one due to the increased Runx2:Sox9 expression ratio as described before [34]. This early switch from chondrogenic to osteogenic program could lead to the robust increase in mineralized bone at four-week time point. To further explore the mechanisms by which ActRIIB-Fc enhances fracture healing, we examined the activation of the BMP and TGF-β signaling pathways by analyzing the number of p-Smad1 and p-Smad2 cells within the fracture callus using immunohistochemistry (IHC).

Unfortunately, IHC does not allow for evaluation of the level of signaling activity within the positive cells. Although non-significant, there was a trend towards increased number of p-Smad2 cells in the callus at both two and four weeks, which is somewhat surprising as with ActRIIB-Fc treatment one could expect decreased Smad2 activation. However, as TGF-β has been shown to recruit osteoblast progenitors to the sites of active bone remodeling [35], the increased number of p-Smad2 positive cells could be due to enhanced recruitment of early osteoblast progenitors at the fracture site. We also measured the expression of BMP signaling target genes Id1 and Id3 and both mRNAs were expressed at lower level in ActRIIB-Fc-treated calluses. The expression of Id1/3 are induced by acute BMP treatment but their expression normalizes within a few days [36]. In our experiment the animals have been treated for several weeks before mRNA analyses, possibly explaining the suppressed Id1/3 mRNA levels. Moreover, Id proteins induce early proliferation of osteoblast progenitors but prolonged Id expression appears to inhibit osteoblast differentiation. Two- and four-week time points represent phases of rapid bone formation and later remodeling i.e. high rate of osteoblast differentiation. We believe that actually the low Id expression in ActRIIB-Fc-treated animals reflects the enhanced osteoblast differentiation at these time points. Lastly, we found that the expression levels of Wnt1 signaling inhibitors Sclerostin and Dkk-1 were decreased upon ActRIIB-Fc-treatment, possibly leading to enhanced Wnt signaling. Thus ActRIIB-Fc treatment appears to induce multiple pathways to stimulate fracture healing.

Our findings are partly in concordance with the report from Morse et al. where the authors stated that ActRIIA-Fc treatment augmented callus formation in rats [20]. However, they did not find significant changes in callus BV/TV compared to the vehicle controls and the treatment with ActRIIA-Fc only modestly improved the biomechanical properties. Although the models used (rat vs. mouse, femur vs. tibia) are different to our study, these findings suggest a difference between the effects of ActRIIA-Fc and ActRIIB-Fc on fracture healing. Tanko et al. [21] in turn reported contradicting results stating that the use of intravenous bimagrumab, an anti-ActRII antibody, had no significant effects on rat fibula osteotomy healing as they observed no differences in mature callus size, vBMD or biomechanical properties and they suggested that ligands that signal through other receptors, such as specific bone morphogenetic proteins, have more relevant roles in fracture healing. However, Nagamine et al. previously demonstrated in an immunohistochemical study that ActRIIA/B are strongly expressed in mature and hypertrophic chondrocytes as well as in osteoblasts during the different phases of fracture healing suggesting that activin type II receptor ligands are important regulators of these events [37]. The differences between these and our studies could in part be explained by the differences in the treatment approaches as bimagrumab directly inhibits the ActRII receptors while our ActRIIB-Fc inhibits the binding of all of its ligands to ActRIIB as well as their alternative receptors. Moreover, the fracture models (closed and open fractures), the duration of healing and the animal species used varied between the studies.

Despite recent advances in the treatment of osteoporosis, osteoporotic fractures will remain as a significant disease burden to our society. Fractures lead to periods of immobilization, which result in further bone loss as well as loss of muscle mass that may further impair patient mobility. Moreover, fractures cause increased mortality [38]. We and others have previously shown that treatment with ActRIIB-Fc results in robust increases in both muscle and bone mass and could thus provide an intriguing treatment option for frail, osteoporotic patients [17, 39]. Here we demonstrate that in a closed tibial fracture mouse model treatment with ActRIIB-Fc results in enhanced fracture healing seen in improved callus bone volume and structure, which translated into biomechanically stronger calluses four weeks after the fracture. Our data suggests that this is likely due to increased osteoblastic bone formation and suppressed bone resorption. These data demonstrate that ActRIIB ligands play an important role

in regulating multiple phases of fracture healing. Moreover, ActRIIB-Fc with its effects on bone and muscle could provide a novel approach to enhance fracture healing.

Supporting information

S1 Fig. ActRIIB-Fc treatment does not affect CTX or P1NP serum levels at two or four weeks. ActRIIB-Fc treatment does not affect CTX or P1NP levels at two or four weeks compared to PBS controls. n = 7 for all groups.
(DOCX)

S1 File. Master data file. Specific values of each sample for each analysis listed as they appear in the manuscript.
(XLSX)

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