

THE ROLE OF OSTEOACTIVIN IN MUSCULOSKELETAL TISSUES AS A  
REPAIR AND ANABOLIC FACTOR

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by  
Nagat Frara  
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Examining Committee Members:

Mary F. Barbe, Ph.D., Advisory Chair, Anatomy and Cell Biology, Temple University  
School of Medicine

Steven N. Popoff, Ph.D., Examining Committee Chair, Anatomy and Cell Biology,  
Temple University School of Medicine

Lynn Kirby, Ph.D., Anatomy and Cell Biology; Center for Substance Abuse Research,  
Temple University School of Medicine

Victor Rizzo, Ph.D., Anatomy and Cell Biology; Cardiovascular Research Center; Sol  
Sherry Thrombosis Research Center, Temple University School of Medicine

Fayez F. Safadi, Ph.D., Original Advisor, Anatomy and Neurobiology, Northeast Ohio  
Medical University

Michael Tytell, Ph.D., External Member, Neurobiology and Anatomy, Wake Forest  
School of Medicine

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## ABSTRACT

Osteoactivin (OA) is a novel osteogenic and repair factor. It has the ability to regulate cell proliferation, adhesion, differentiation, and synthesis of extracellular matrix proteins in various cell types under both normal and pathological conditions.

Initial identification of osteoactivin (OA)/glycoprotein non-melanoma clone B (gpnmb) was demonstrated in an osteopetrotic rat model, where OA expression was increased 3-fold in mutant bones, compared to normal. OA mRNA and protein expression increase during active bone regeneration post-fracture, and primary rat osteoblasts show increased OA expression during differentiation *ex vivo*. To further examine OA/gpnmb as an osteoinductive agent, we characterized the skeletal phenotype of a transgenic mouse overexpressing OA/gpnmb under the CMV-promoter (OA-Tg). Western blot analysis showed increased OA/gpnmb in OA-Tg osteoblasts, compared to wild-type (WT). In OA-Tg mouse femurs versus WT littermates, micro-CT analysis showed increased trabecular bone volume and thickness, and cortical bone thickness; histomorphometry showed increased osteoblast numbers, bone formation and mineral apposition rates in OA-Tg mice; biomechanical testing showed higher peak moment and stiffness. Given that OA/gpnmb is also over-expressed in osteoclasts in OA-Tg mice, we evaluated bone resorption by ELISA and histomorphometry, and observed decreased serum CTX-1 and RANK-L, and decreased osteoclast numbers in OA-Tg, compared to WT mice, indicating decreased bone remodeling in OA-Tg mice. The proliferation rate of OA-Tg osteoblasts *ex vivo* was higher, compared to WT, as was alkaline phosphatase staining and activity, the latter indicating enhanced differentiation of OA-Tg osteoprogenitors. Quantitative RT-PCR analysis showed increased TGF- $\beta$ 1 and TGF- $\beta$  receptors I and II expression in OA-Tg

osteoblasts, compared to WT. Together, these data suggest that OA overexpression has an osteoinductive effect on bone mass *in vivo* and stimulates osteoprogenitor differentiation *ex vivo*.

OA expression increases during tissue degeneration and regeneration, fracture repair, and after denervation-induced disuse atrophy, concomitant with increased matrix metalloproteinases (MMPs). However, OA's expression with repetitive overuse injuries is unknown. We sought to evaluate in an animal model of upper extremity repetitive overuse, at low force loads the following: 1) OA expression in an operant rat model of repetitive overuse; 2) expression of MMPs; 3) inflammatory cytokines; and 4) the inducible form of heat shock protein 70 (HSPA1A/HSP72), a protein known to increase during metabolic stress and be involved in cellular repair. We hypothesized that OA is functioning as a growth factor during periods of tissue repair. Young adult, female Sprague-Dawley rats performed a high repetition negligible force (HRNF) food retrieval task for up to 6 weeks, and were compared to control rats. Quantitative PCR, Western blot analyses and immunohistochemistry showed increased OA mRNA and protein expression in flexor digitorum muscles of 6-week HRNF rats, compared to controls. OA protein levels increased similarly in 6-week HRNF flexor digitorum tendons. Increased OA immunostaining was localized to the myofiber sarcolemma, macrophage-like cells and tenocytes. In muscles, Western blot analyses showed progressive increases in MMP-1, -2 and -3, whereas tendons had increased MMP-1 and -3, with HRNF task performance. ELISA and immunohistochemistry showed increased HSP72 in 6-week HRNF muscles, and co-localization with OA in the myofiber sarcolemma. HSP72 increased in 6-week HRNF tenocytes, compared to controls. Inflammatory cytokines IL-1alpha or beta showed transient increases at 3 weeks in muscles and tendons, while IL-1alpha was significantly decreased in 6-week HRNF muscles.

The simultaneous increases of MMPs and HSP72 with OA, factors involved in tissue repair, supports a role of OA in tissue regeneration after repetitive overuse.

We extended the study above to examine the expression of OA during high repetition high force loading in our animal model of upper extremity overuse, in combination with anti-inflammatory drug, to evaluate OA's link to inflammatory processes. Young adult female rats underwent an initial training period to learn the task (10 min/day, 5 days/wk, for 6 wks), before then performing a high repetition high force (HRHF) task for 11 weeks (2 hours/day, 3 days/week). Results were compared to age-matched control (C) rats. At the end of HRHF task week 3, two cohorts of HRHF rats received 5 intraperitoneal injections of saline (HRHF+Veh) or anti-rat TNF- $\alpha$  antibody (HRHF+anti-TNF) across 4-7 weeks, as did controls (C+Veh and C+anti-TNF). Two other cohorts rested during weeks 4-7 with or without treatment (HRHF+anti-TNF/Rest and HRHF+Veh/Rest), to parallel their partner groups. Motor behavior was assessed and revealed decreased grip strength in HRHF+Veh rats beginning immediately post training (HRHF task week 0), and that anti-TNF- $\alpha$  treatment prevented this grip strength decline. The 4-week anti-TNF- $\alpha$  therapy extended maintenance of grip strength near control levels through week 9, despite no further treatment after week 7. By experimental week 11, ELISA showed no significant differences in OA levels in forearm flexor digitorum muscles, and histomorphometry showed no difference in the circumference of this muscle in any HRHF group, compared to controls, matching findings of no gain in grip strength above control levels in any HRHF group. However, ELISA of distal radius and ulna homogenates showed increased OA levels in HRHF+anti-TNF rats, as well as increased IL-18 in bones of both anti-TNF treated HRHF groups (HRHF+anti-TNF and HRHF+anti-TNF/Rest rats), compared to controls. Micro-CT analysis showed that rats receiving anti-TNF- $\alpha$  treatment, with or without rest, had increased

bone mass (detected as increased trabecular bone volume, thickness, and number and reduced trabecular separation), compared to the other groups. Histomorphometry showed increased osteoblast numbers in HRHF+anti-TNF rats, compared controls, yet decreased osteoclast numbers, compared to HRHF+Veh rats, indicative of increased bone anabolism in anti-TNF- $\alpha$  treated rats. Thus, these findings suggest that TNF- $\alpha$  blocks OA expression in bones, and that its increase when combined with prolonged repetitive loading, enhances osteoblast activity and bone formation.

**DEDICATION**

To my children:

Dania, Muath, and Mohamed

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# CHAPTER 1

## INTRODUCTION

### 1.1 MUSCULOSKELETAL SYSTEM

During evolution, the emergence of the musculoskeletal system has enormously increased the structural complexity of the body as well as the range of its functions. The musculoskeletal system is a multicomponent system, comprised of bones of the skeleton, cartilage, muscles, and tendons, innervated by nerves and vascularized by blood vessels (Kardon, 2011). It evolved in mammals to perform various functions that include enabling accurate, efficient and diverse movement while maintaining the stability of the entire organism, facilitating breathing, and protecting internal organs (DiGirolamo et al., 2013; Shwartz et al., 2013). Musculoskeletal development is a complex process whereby bone, tendon, and muscle tissues develop in a spatially and temporally coordinated manner, and integrate into a cohesive functional unit by forming specific connections unique to each region of the musculoskeletal system (Pineault and Wellik, 2014). The vertebrate skeleton is composed of bone and cartilage linked to skeletal muscle through tendons (DiGirolamo et al., 2013). Once formed, the skeleton is continually remodeled throughout life, which allows for repair of microdamage and adaptive response to increased or decreased mechanical loads (DiGirolamo et al., 2013).

### 1.2 Bone Overview

Bone is a complex and dynamic tissue that continuously undergoes renewal and repair throughout life to fulfill its functions. Bone provides the framework for and bears the weight of the body and maintains its shape. It supports organs, anchors muscles and

permits mechanical movement and locomotion by providing levers for the muscles (Clarke, 2008). Bone also protects the vital organs and structures, hosts hematopoietic cells and provides the environment for hematopoiesis within marrow spaces. Bone is a major source of inorganic ions and actively maintains calcium/phosphate homeostasis and acid-base balance (Deng et al., 2008; Taichman, 2005). Bone serves as a reservoir for several minerals of the body, such as calcium, which is stored in the matrix, and iron, which is stored in the marrow (Yang, 2010).

### **1.2.1 Anatomy of the Bone**

According to their anatomical shape, bones are classified into four general categories: long, short, flat, and irregular bones. These bones form via different mechanisms during embryonic development (Gartner and Hiatt, 2007). Since long bone is investigated in this study, a detailed description of this category is given below.

Long bones have two extremities (epiphyses), a cylindrical tube in the middle (diaphysis) and a transitional zone between them (metaphyses). The epiphysis is the region between the growth plate or growth plate scar and the expanded end of bone, covered by articular cartilage. The metaphysis is the junctional region between the growth plate and the diaphysis. The diaphysis is the shaft of long bones and is located in the region between metaphyses. The epiphyseal plate, or growth plate, is the region that separates the epiphysis from the metaphysis. It is the zone of endochondral ossification that mediates growth in bone length in an actively growing bone or the epiphyseal scar in a fully grown bone (Yang, 2010).

### **1.2.2 Types of Bone Tissue**

Bone tissue is made of individual cells organized to form a porous and textured tissue. Bone tissue is called “osseous tissue,” with “osseous” referring to bone. All bone tissue is chemically identical, but the arrangement and density of cells affects the texture of each bone. Bone tissue can be classified by texture, matrix arrangement, maturity, or developmental origin (Yang, 2010). There are two types of bones categorized by texture: cortical (dense, compact) and trabecular (spongy, cancellous) bones. The bulk of our skeleton, approximately 80% is made up of cortical bone while 20% is made up of trabecular bone (Carter and Hayes, 1977). Cortical bone is ivory-like and dense in texture with few or no cavities. It is surrounded externally and internally by a periosteum and endosteum, respectively (Yang, 2010). Cortical bone is typically seen as the hard outer shell that surrounds trabecular bone or the centrally located marrow cavity. Cortical bone is organized into Haversian systems or osteons, in which the lamellae are concentrically organized around a vascular canal, the Haversian canal. The blood supply enters from the periosteum via Volkmann canals, which also connect one Haversian canal to another (Brooks, 1964). Trabecular bone is sponge-like with numerous cavities. It consists of extensively connected bony trabeculae that are oriented along the lines of stress. In contrast to cortical bone, complete osteons are usually absent from the trabecular bone due to the thinness of the trabeculae (Yang, 2010). Trabecular bone is also more metabolically active than cortical bone because of its much larger surface area for remodeling. Trabecular bones are found mainly at the ends of many long bones, and in areas like the ears and nose (Cooper et al., 1966).

Based on matrix arrangement and maturity, bone tissue can be classified into woven bone (primary bone tissue) or lamellar (secondary bone tissue) bone. Woven bone is temporary, found during embryonic development or after repair, and is eventually converted to lamellar bone except in a few places, such as areas near the sutures of the flat bones of the skull, tooth sockets, and the insertion site of some tendons. Woven bone is immature bone in which collagen fibers are arranged in irregular random arrays, contains smaller amounts of mineral substance, and a higher proportion of osteocytes than lamellar bone. Lamellar bone is mature bone in which collagen fibers are arranged in lamellae that are parallel to each other in trabecular bone or concentrically organized around a vascular canal in cortical bone. Almost all bones in adults are lamellar bones (Cooper et al., 1966).

Based on developmental origin, bones can be classified into: intramembranous (mesenchymal) bone or endochondral (intracartilaginous) bone. Intramembranous bone such as the flat bones of the skull develops from direct transformation of condensed mesenchyme. Endochondral bones such as long bones form by replacing a reformed cartilage model (Mackie et al., 2011). These processes will be discussed in greater detail later in this chapter.

### **1.2.3 Bone Cells**

Bone cells, which are found within the bone tissue, are responsible for the make-up of the skeleton of vertebrates. They all work together inside of the bones to perform different functions such as the development of new bones, the maintenance of bones, and the regulation of minerals in the body. Bone cells communicate with each other by

producing a various growth factors and cytokines which control cell division, differentiation, and survival. There are four classical bone cells which include: osteoblasts, osteocytes, osteoclasts, and bone-lining cells.

### ***1.2.3.1 Osteoblasts***

Osteoblasts, derived from osteoprogenitor cells, are responsible for the synthesis of the organic components of the bone matrix, also known as osteoid. Osteoid is composed of type I collagen, proteoglycans, and glycoproteins. Moreover, these cells synthesize the enzyme alkaline phosphatase (ALP), one of the earliest markers of the osteoblast phenotype and required locally for the mineralization of osteoid (Epstein, 1988). In addition to the production of type I collagen, osteoblasts also produce a variety of other non-collagenous proteins including osteocalcin, osteopontin, bone sialoprotein and osteonectin. These proteins are also markers of the osteoblast phenotype and each has a unique temporal pattern of expression during osteoblast differentiation (reviewed in (Khurana, 2009)). Osteoblasts are also involved in the subsequent mineralization of osteoid via the liberation of matrix vesicles and the deposition of calcium and phosphate (Anderson, 2003; Potter et al., 2002). During bone remodeling, osteoblasts deposit a layer of osteoid (approximately 10  $\mu\text{m}$  thick) on the surface of preexisting bone, which in approximately 20 days begins to mineralize. This interval is known as the 'mineralization lag time'. As a result of the normal remodeling process, up to 20% of the bone surface may be covered by osteoid. An increased amount of osteoid is seen in pathologic conditions in which the remodeling rate is accelerated or in which the mineralization lag time is increased (Melsen and Mosekilde, 1980).

*Osteoblast commitment and differentiation.* Osteoblasts originate from pluripotent mesenchymal stem cells that have the potential to proliferate and differentiate into several connective tissue-cell types depending on the stimulus within the local microenvironment. Given the appropriate stimuli, osteoprogenitor cells of mesenchymal origin will first give rise to pre-osteoblasts (Caplan, 1991; Friedenstein et al., 1987; Owen, 1988; Pittenger et al., 1999; Yamaguchi et al., 1991). The commitment of mesenchymal stem cells to the osteoblast lineage is dependent on the canonical Wnt/ $\beta$ -catenin pathway and specific transcription factors (Logan and Nusse, 2004). One of the most important transcription factors is runt-related transcription factor 2 (Runx2), also called the core-binding factor  $\alpha$ 1 (Cbfa1). Runx-2 plays an important role in the commitment of multipotent mesenchymal cells to the osteoblastic lineage and in osteoblast differentiation at early stages (Komori et al., 1997; Otto et al., 1997). Runx2 is highly expressed in osteoblast lineage cells and up-regulates the expression of various osteoblast-specific genes of bone matrix proteins, such as type I collagen, bone sialoprotein and osteocalcin (Banerjee et al., 1997; Ducy et al., 1997; Harada et al., 1999; Ji et al., 1998; Komori, 2010). In Runx2 null mice, osteoblast differentiation is completely arrested as bone formation fails to occur (Komori et al., 1997; Otto et al., 1997). The expression of Runx2 is regulated by a number of growth factors, namely, bone morphogenetic proteins (BMPs) and transforming growth factor-beta (TGF- $\beta$ ) (Ducy et al., 1997; Lee et al., 1999). Two studies showed that transgenic mice that overexpress Runx2 in osteoblasts developed osteopenia (low bone formation) with multiple fractures, indicating that the correct dose of Runx2 is crucial for normal bone physiology (Geoffroy et al., 2002; Liu et al., 2001).

The progressive development of the osteoblast phenotype from a proliferating immature cell to a mature osteoblastic cell that can synthesize specific bone proteins, is characterized by a definite sequential expression of tissue-specific genes that identifies three distinct periods of osteoblast phenotype development *in vitro*: proliferation, maturation and extracellular matrix (ECM) synthesis, and matrix mineralization (Lian and Stein, 1993).

During the active proliferation phase, osteoprogenitor cells, also called pre-osteoblasts, express genes that support proliferation and cell cycle progression together with the expression of several genes encoding for ECM proteins, such as type I collagen, osteopontin, and fibronectin, and gene encoding growth factors, such as TGF- $\beta$  and BMPs. For instance, it was established that TGF- $\beta$  stimulates the replication of osteoprogenitor cells and directly increases collagen synthesis (Antosz et al., 1989), whereas BMP-2 increases the synthesis of ALP, one of the earliest markers of the osteoblast phenotype that is required for osteoid mineralization (Yamaguchi et al., 1991). Thus, the accumulation of matrix proteins contributes, in part, to the cessation of cell proliferation (Lian et al., 1991). Osterix (Osx), the osteoblast-specific transcription factor, is also required for osteoblast differentiation (Nakashima et al., 2002). It acts downstream of Runx2 in the pathway of osteoblast differentiation because Runx2 expression is normal in Osx-null mice, while no Osx transcripts are detected in Runx2-knockout mice (Nakashima et al., 2002).

After the completion of the proliferation period, the post-proliferative phase begins, which consists of ECM synthesis. In this stage, genes of cell cycle and cell growth control are down-regulated and the expression of genes encoding proteins for

ECM maturation is initiated, which lead to the high synthesis of ALP, the characteristic marker of this stage, and other osteoblast-related proteins such as type I collagen and osteocalcin (Stein et al., 1992). The ECM synthesis phase progresses into the mineralization phase in which osteoblasts express genes related to the accumulation of hydroxyapatite in the ECM together with genes encoding several proteins, such as osteocalcin (OCN) and bone sialoprotein (BSP) associated with matrix mineralization (Franzen and Heinegard, 1985; Hauschka et al., 1989; Whitson et al., 1984). Moreover, in the late stage of osteoblast differentiation, when mature osteoblasts form mature bone, the expression of Runx2 is down-regulated (Komori, 2010). Interestingly, *Osx*-null mice die at birth due to their lack of mineralized skeleton, indicating the regulatory role of *Osx* in this stage (Nakashima et al., 2002). At the end of the synthesis and mineralization of ECM, mature osteoblasts have one of the three fates: they undergo apoptosis, differentiate further into osteocytes, or become quiescent lining cells (Lynch et al., 1998; Yang, 2010).

*Osteoblast morphology.* Active osteoblasts are generally cuboidal or columnar in shape, and are located side by side in a sheet-like arrangement (resembles simple epithelium) at the surfaces of bone or osteoid at sites of active bone formation such as bone development or fracture repair. When actively secreting matrix, osteoblasts exhibit basophilic cytoplasm. However, inactive osteoblasts have a flattened shape with less basophilic cytoplasm and low ALP (Gartner and Hiatt, 2007; Junqueira and Carneiro, 2005). Ultrastructurally, active osteoblasts are typical protein producing cells with polarized organelles so that the large nucleus is eccentrically located away from the region of secretory activity that houses enlarged Golgi structures and an extensive

endoplasmic reticulum (Clarke, 2008). Osteoblasts extend short processes that make contact with those of neighboring osteoblasts as well as long processes that make contact with processes of osteocytes (Gartner and Hiatt, 2007).

### ***1.2.3.2 Osteocytes***

Once osteoblasts become trapped and encased within the mineralized matrix they are called osteocytes. These cells are mature bone cells that live within the substance of bone and comprise 90%-95% of all bone cells (Bonewald, 2007). Osteocytes are embedded in bone matrix-occupying spaces (lacunae) in the interior of bone and are connected to adjacent cells by long cytoplasmic processes radiating from the cell body and lie within channels (canaliculi) through the mineralized matrix (Hirose et al., 2007; Lian and Stein, 2008). Thus, an osteocyte lies in its own lacuna and contacts its neighboring osteocytes within mineralized bone and osteoblasts on the bone surface through canaliculi (Bonewald, 2007; Kamioka et al., 2001). Osteocytes form a network extending throughout mineralized bone. The processes of adjacent cells make contact via gap junctions, maintaining the vitality of osteocytes by passing nutrients and metabolites between blood vessels and distant osteocytes and regulating ion homeostasis in bone (Jiang et al., 2007).

The osteocyte is considered the most mature or terminally differentiated cell of the osteoblast lineage, not capable of cell division *in vivo* (Lian and Stein, 2008). Although osteocytes have reduced synthetic activity and are not capable of mitotic division, they are actively involved with the maintenance of the bony matrix, and their death is followed by resorption of this matrix (Noble et al., 1997).

### ***1.2.3.3 Osteoclasts***

Osteoclasts are bone cells that are known to be capable of resorbing mineralized bone matrix. They lie within enzymatically etched depressions in bone matrix known as Howship's lacunae that found on bone surfaces (Roodman, 1996). The surface-facing bone matrix is folded into irregular fingerlike projections forming a ruffled border, the most characteristic feature of osteoclasts. This region is rich in lysosomes that contain the lytic enzyme tartrate-resistant acid phosphatase (TRAP) (Boyle et al., 2003; Vaananen and Zhao, 2002). The ruffled border is surrounded by an osteoclastic specific structure called clear zone or sealing zone which separates the acidic resorptive environment from the rest of the extracellular space, forming an organelle free area (Katagiri and Takahashi, 2002; Vaananen and Horton, 1995). Osteoclasts active in bone resorption are very large, multinucleated cells that possess many lysosomes and mitochondria and extensive Golgi system (Baron, 1989).

Osteoclasts are differentiated from the fusion of bone marrow-derived mononuclear precursor cells of the monocyte-macrophage lineage in a process termed osteoclastogenesis that requires two essential factors, macrophage colony stimulating factor (M-CSF) and receptor activator nuclear factor  $\kappa$ B ligand (RANKL) (Teitelbaum, 2000). M-CSF, produced by osteoblasts and stromal cells, binds to its receptor, c-fms, on early osteoclast precursors thereby promoting their survival and proliferation (Felix et al., 1994; Hodge et al., 2007). RANKL, expressed by osteoblasts, binds to RANK, a receptor that is present on osteoclasts and their precursors (Lacey et al., 1998; Xu et al., 2000). RANKL stimulates the M-CSF-expanded precursors to commit to the osteoclast phenotype. These cells then express key osteoclast markers including tartrate-resistance

acid phosphatase (TRAP). Upon further stimulation with M-CSF and RANKL the pre-osteoclasts fuse to form multinucleated cells which begin to express more specific osteoclast markers such as calcitonin receptor and Cathepsin K (Lam et al., 2001). RANKL activity can be antagonized by the presence of the soluble decoy receptor osteoprotegerin (OPG) that is produced by osteoblasts. OPG sequesters RANKL from binding to RANK thus inhibits osteoclast differentiation (Kostenuik and Shalhoub, 2001; Simonet et al., 1997).

Osteoclast activity is regulated by a range of hormones such as parathyroid hormone (PTH), estrogen and calcitonin. When PTH binds its receptor on mature osteoblasts, it increases RANKL expression and decrease OPG expression (Lee et al., 1999; Thomas et al., 2001). Estrogen has a dual effect; it increases bone formation and reduces bone resorption by enhancing osteoblast proliferation and function (Ernst et al., 1989; Majeska et al., 1994) and further reduce osteoclast activity by increasing OPG production in osteoblasts (Hofbauer et al., 1999). Calcitonin, a known inhibitor of bone resorption, acts directly on osteoblasts by increasing proliferation, enhancing OPG expression and inhibiting RANKL expression (Tian et al., 2007).

*Regulation of osteoclastogenesis.* Besides their role in bone formation, osteoblasts play an important role in the regulation of osteoclast differentiation and resorption activity by the secretion of cytokines or by direct cell contact (Kular et al., 2012; Neve et al., 2011). Osteoblasts secrete RANKL, a protein that plays an essential role in the recruitment, differentiation, activation and survival of osteoclasts (Burgess et al., 1999). In addition, osteoblasts secrete M-CSF that is also required for osteoclast formation (Tsurukai et al., 2000). Osteoprotegrin (OPG) is the other essential molecule that is

synthesized by osteoblasts and plays a regulatory role in the inhibition of osteoclast differentiation (Lacey et al., 1998).

#### ***1.2.3.4 Bone-lining Cells***

Bone-lining cells are flattened, squamous cells found lining surfaces in areas where there is no active bone formation. They can be thought of as surface osteoblasts that ceased to form matrix and revert to a quiescent state (Miller et al., 1989). Bone-lining cells are found in close proximity and joined together with tight junctions and desmosomes. Although these cells appear to be similar to osteoprogenitor cells, they are most likely incapable of dividing but can be reactivated to the secreting form with the proper stimulus and under conditions that warrant active bone formation such as during remodeling and fracture repair (Miller et al., 1989). Bone lining cells therefore represent a pool of cells capable of rapid response to an osteoinductive signal, such as PTH or mechanical stimulation (Chow et al., 1998).

#### **1.2.5 Bone Development and Growth**

Bones of the axial and appendicular skeleton are formed by one of two independent processes, intramembranous or endochondral bone formation, also called ossification. The primary difference between the two processes is the absence or presence of a cartilaginous intermediary. However, in both processes osteoblasts play a central role in the production of a characteristic ECM and subsequent mineralization of the bone matrix that gives strength to the skeleton and provides a reservoir of minerals and growth factors (Yamaguchi et al., 2000).

### ***1.2.5.1 Intramembranous Bone Formation***

Most flat bones, including bones of the skull, the mandible and the clavicle, are formed by intramembranous ossification. This process begins when osteoprogenitor cells of mesenchymal origin converge within a richly vascularized milieu to form ossification centers. The differentiating osteoblasts in the primary ossification centers, the region of initial osteogenesis within developing bone, actively synthesize new mineralized bone matrix (osteoid). Calcification quickly follows osteoid formation leads to the formation of a network of spicules and trabeculae. Osteoblasts that become trapped in the matrix are called osteocytes. The collagen fibers of the developing spicules and trabeculae are randomly oriented in primary bone. When the primary ossification centers fuse, a loose trabecular structure known as the primary spongiosa is formed. Subsequently, blood vessels grow into the connective tissue between the trabeculae. Bone marrow stem cells from the circulating blood then give rise to hematopoietic cells. Growth and fusion of several ossification centers eventually replace the original mesenchymal tissue. These secondary ossification centers enhance the rates of mineralization. As the growth rate slows down, the bones take on a lamellar character (Gartner and Hiatt, 2007; Khurana, 2009; Yang, 2010).

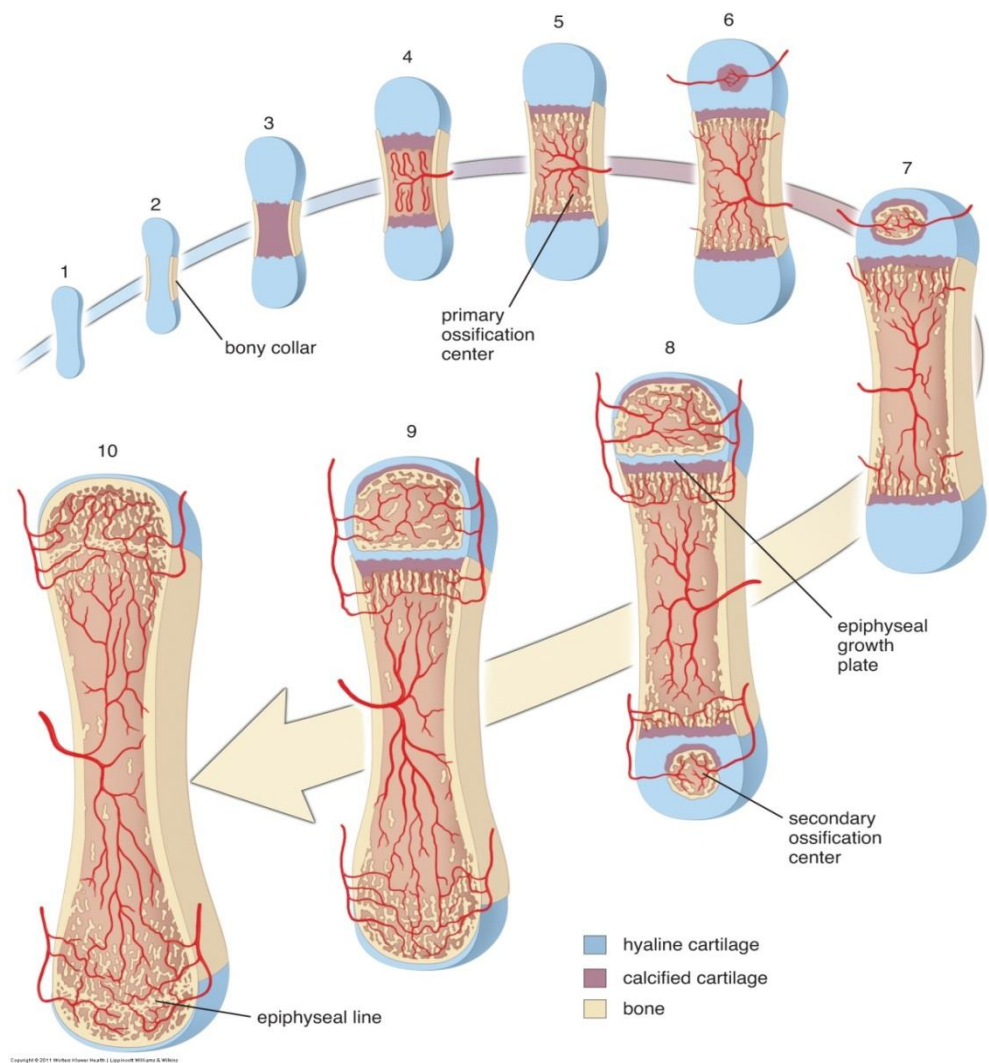
### ***1.2.5.2 Endochondral Bone Formation***

Endochondral ossification is responsible for the formation of most of the short and long bones. This process involves generation of a hyaline cartilage template in the shape of the bone to be formed, followed by ossification of this cartilage precursor (Gartner and Hiatt, 2007; Ross and Pawlina, 2011). This process starts with commitment

of the mesenchymal stem cells into compact nodules that differentiate into chondrocytes. Subsequently, chondrocytes proliferate rapidly to form the cartilage template that will eventually be replaced by bone (Figure 1-1.1). As they divide, chondrocytes secrete a cartilage-specific ECM. Later on, the chondrocytes stop dividing and become hypertrophic. Hypertrophic chondrocytes have increased production of collagen type X and fibronectin, thereby altering the remaining cartilage matrix allowing the cartilage to become mineralized. A bony collar is thus formed (Figure 1-1.2), blocking nutrient diffusion and as a result, the internal hypertrophic chondrocytes degenerate and the intervening chondroid matrix calcify (Figure 1-1.3) (Mackie et al., 2011; Ross and Pawlina, 2011). The perichondrium then becomes the periosteum, from which the osteogenic bud arises and penetrates the calcified cartilage matrix through passages that are created in the bony collar by osteoclasts (Gartner and Hiatt, 2007; Ross and Pawlina, 2011).

Osteogenic buds, composed of osteoprogenitor cells and blood capillaries, invade the spaces left by the degenerating chondrocytes (Figure 1-1.4). Osteoblasts arise from osteoprogenitor cells and lay down a layer of rapidly mineralized osteoid on the surface of the partially-degraded calcified cartilage remnants (Bruder and Caplan, 1989; Hatori et al., 1995). The complex structure of calcified cartilage overlaid with newly formed bone is known as the primary spongiosa or the primary center of ossification (Figure 1-1.5), which is later remodeled to become lamellar bone (secondary spongiosa). Calcified cartilage remnants are resorbed by osteoclasts. Thus, the cartilage model is gradually replaced by bone and marrow cavities (Yang, 2010).

In the long bones of mammals such as femur, tibia, ulna and radius, the process of endochondral ossification proceeds in both directions from the primary ossification center, which becomes the diaphysis. Long bones formed from a cartilage template grow both appositionally and interstitially. As these bones grow in length, at later stage of bone development, secondary centers of ossification form at the ends of each bone and later become the epiphyses (Figure 1-1.6 and -1.7). The area of cartilage that remains between the primary and secondary ossification centers is the epiphyseal growth plate (Ross and Pawlina, 2011). Thus, the epiphysis of the chondroid model is replaced by bone tissue, except the articular cartilage and the cartilage of epiphyseal plate (Figure 1-1.8). The epiphyseal cartilage can be divided into 5 zones, starting from the epiphyseal side of cartilage: the resting zone which consists of small chondrocytes, the proliferative zone that consists of rapidly dividing chondrocytes arranged in columns that are parallel to the long axis of the bone, hypertrophic zone which consists of large chondrocytes that contain abundant cytoplasmic glycogen, calcified cartilage zone where chondrocytes die and, and ossification zone where the primary spongiosa forms and endochondral bone tissue appears (Junqueira and Carneiro, 2005; Mackie et al., 2011). As the bone reaches maximal growth, proliferation of new cartilage within the epiphyseal plate terminates. The cartilage that has already been produced in the epiphyseal plate continues to undergo changes that lead to the deposition of new bone until there is no remaining cartilage (Figure 1-1.9). At this point, the epiphyseal and diaphyseal marrow cavities become confluent. Growth is now complete, and the only remaining cartilage is found on the articular surfaces of the bone. Vestigial evidence of the site of the epiphyseal plate is reflected by an epiphyseal line (Figure 1-1.10) (Ross and Pawlina, 2011).



**Figure 1-1. Stages of Endochondral Ossification.** The process begins with the cartilage model (1); next, bone collar forms around the diaphysis of the cartilage model (2); then, the cartilaginous matrix in the shaft begins to calcify (3). Blood vessels then invade the calcified cartilage (4), creating a primitive marrow cavity in which remnant spicules of calcified cartilage remain at the two ends of the cavity. As a primary ossification center develops, the endochondral bone is formed on spicules of calcified cartilage. Periosteal bone continues to form (5); the periosteal bone is formed as a result of intramembranous ossification. Blood vessels invade the proximal epiphyseal cartilage (6), and secondary center of ossification is established (7). A similar secondary ossification center forms at the distal end of the bone (8), and an epiphyseal cartilage is thus formed between each epiphysis and diaphysis. With continued growth of the long bone, the distal epiphyseal cartilage disappears (9), and finally with cessation of growth, the proximal epiphyseal cartilage disappears (10). The metaphysis then becomes continuous with the epiphysis. Epiphyseal lines remain where the epiphyseal plate last existed. *Used by permission from Ross and Pawlina, Histology A Text and Atlas, 6<sup>th</sup> Edition, 2011.*

### ***1.2.5.3 Bone Growth in Mature Animals***

Once bones of the skeleton are fully formed in young adults, almost all of their metabolic activity concerns a process called remodeling (Eriksen, 1986). Remodeling results from the action of osteoblasts and osteoclasts. In a homeostatic equilibrium resorption and formation are balanced so that old bone is continuously replaced by new tissue so that it adapts to mechanical load and strain (Frost, 1990). The size and shape of the bones can be greatly affected by the loading or impact that occurs with physical activity. Ultimately, bones achieve a shape and size that fits best to their function. The responses to changes in mechanical force, repair of microfractures, and the maintenance of the remodeling cycle, are determined by cytokines and growth factors, as well as hormones (Raisz, 1999). The response of bone to the application of load, whether internal, external, static or dynamic, may be anabolic or catabolic depending on many factors, including load magnitude and duration (Bentley et al., 2007; Bourrin et al., 1994; Rubin et al., 2002). Muscle force may place greater loads on bones than gravitational forces, such as body weight and bone quality can be enhanced by exercise and other forms of repeated muscle loading (Burr, 1997; Frost, 1999). Since there is a biomechanical link between muscles and bone, bones should adapt to increased loads imparted by muscles (Daly et al., 2004; Gross et al., 2010; Turner, 2000). Different studies indicate that the response of bone to excessive applied loads, including that from muscle loading, can be maladaptive, while low loading is more likely to induce bone formation (Barbe et al., 2013; Sun et al., 2011; Umemura et al., 1997).

### 1.2.6 Bone Extracellular Matrix

Extracellular matrix (ECM) plays a critical role as a scaffold on which and within which tissues can organize; it also contains an immense amount of information that regulates cell shape, cytoskeletal organization, cell motility and polarity, gene expression, proliferation, and survival (Damsky and Werb, 1992). Bone ECM consists of inorganic and organic components. The association of organic and inorganic substances gives bone its hardness and resistance (Boskey, 1989). Osteoblasts are responsible for the synthesis of the organic components of bone matrix, which subsequently calcifies, whereas osteoclasts degrade the calcified matrix and dissolve mineral components. It is thought that the bone ECM influences the behavior of postmitotic osteoblasts and also serves as a reservoir of growth factors needed for bone remodeling (Delany and Canalis, 2001; Ruoslahti and Yamaguchi, 1991).

The inorganic component of bone represents about 60% of the dry weight of bone matrix and composed mainly of abundant calcium and phosphorus, as well as smaller amounts of bicarbonate, citrate, magnesium, potassium, and sodium. Calcium forms hydroxyapatite crystals  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$  with phosphorus but is also present in an amorphous form (Junqueira and Carneiro, 2005; Khurana, 2009). Calcium hydroxyapatite crystals are arranged parallel to collagen fibers. This orientation maximizes the collagen's resistance to tensile (stretch) forces and calcium hydroxyapatites resistance to compressive forces (Gartner and Hiatt, 2007; Sela et al., 1987).

The organic component of bone makes up 40% of the dry weight of bone matrix and is composed of numerous bone-specific or bone-related ECM proteins that play a role in the regulation of mineralization and attachment of osteoblasts and osteoclasts to

the bone matrix (Lynch et al., 1995; Malaval et al., 1994; Sommer et al., 1996). Organic components of bone ECM include collagen fibers and a number of non-collagenous proteins, such as osteocalcin (OCN) and bone sialoprotein (BSP), which may orchestrate mineralization as well as various growth factors, including BMPs and TGF- $\beta$  (Hu et al., 2009; Robey et al., 1993).

Type I collagen is the most abundant protein in the bone matrix, comprising nearly 90% of the organic matrix and 97% of the collagenous proteins. It is composed of three polypeptide chains two  $\alpha 1$  and one  $\alpha 2$ , which form a triple helical structure (Bornstein and Traub, 1979). Collagen, synthesized by active osteoblasts, is deposited into an osteoid layer which subsequently mineralizes. Hydroxylation and glycosylation are post-translational modifications of collagen and are specific to bone which explains why mineralization only occurs in bone and not in other sites (Ressoret and De Crombrughe, 2002). Recently, data have accumulated that collagen cross-linking, a major post-translational modification of collagen, affects not only the mineralization process, but also micro-damage formation and consequently affects the mechanical properties of bone (Saito and Marumo, 2010).

Osteocalcin (OCN), also called bone Gla protein, is a calcium-binding protein, which constitutes approximately 15% of the non-collagenous protein fraction in bones. It is produced by mature osteoblasts during matrix mineralization (Price et al., 1983). OCN is found in the more mature and fully developed mineralized matrix as an important marker of osteoblast differentiation (Kaartinen et al., 1997). Osteocalcin was previously thought to function as a promoter or initiator of calcium deposition at the nodes between the ends of collagen fibrils and therefore regarded as a marker of bone formation.

However, mice deficient in osteocalcin develop a phenotype marked by higher bone mass and improved bone quality suggesting that osteocalcin functions normally to limit or inhibit bone formation without compromising mineralization (Ducy et al., 1996).

The interaction between osteoblasts and the ECM is essential for both bone formation and maintenance by anchoring cells and triggering signals that direct osteoblast proliferation and differentiation. These interactions are mediated by growth-promoting factors, made by osteoblasts themselves or neighboring cells, their specific interactions with ECM proteins of fundamental importance in understanding the role of osteoblast in skeletal turnover (Chen et al., 2007; Garcia et al., 2002; Gronowicz and Derome, 1994). However, the precise role of these interactions with respect to the regulation of osteoblast differentiation and mineralization is not yet fully understood. Also, contribution of the surrounding ECM to regulating growth factor signaling to neighboring cells has been largely unexplored (Bhat et al., 2011).

### **1.3 Skeletal Muscle Overview**

Skeletal muscle is the most abundant tissue in the mammalian body accounting for approximately 40% of body weight, and is involved in many different important functions (Fanzani et al., 2012; Zammit and Beauchamp, 2001). The primary function of adult skeletal muscle is to generate force in a rapid and directed manner. In addition, skeletal muscle is the primary target of glucose uptake, possesses a remarkable ability to regenerate through rapid repair following severe injury caused by exercise, toxins or diseases, and is subjected to size remodeling (Fanzani et al., 2012; Zammit and Beauchamp, 2001).

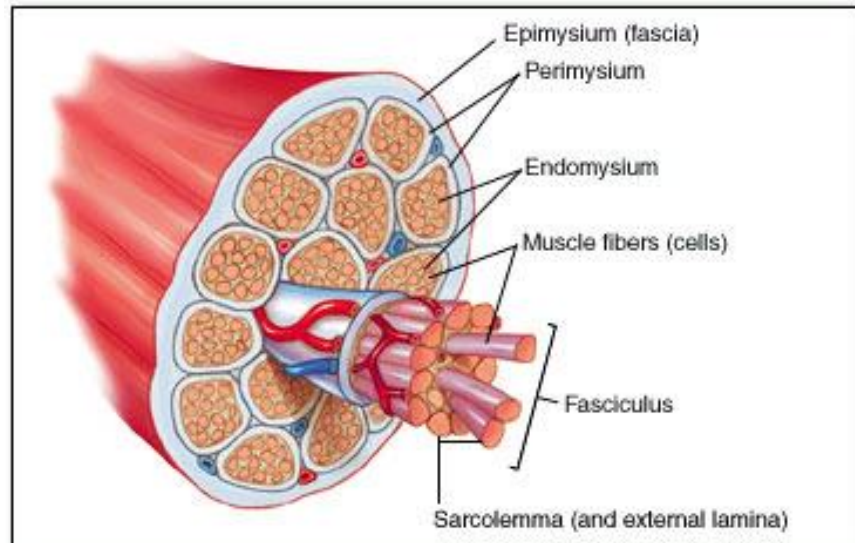
### **1.3.1 Anatomy of the Skeletal Muscle**

Skeletal muscle tissue composed primarily of contractile material, as well as connective tissue, blood vessels and nerves (Gillies and Lieber, 2011). Mammalian skeletal muscles are heterogeneous in nature composed of muscle fibers and an ECM (Gao et al., 2008; Schiaffino and Reggiani, 2011). This heterogeneity is the basis of the flexibility, which allows the same muscle to be used for various tasks from continuous low-intensity activity (e.g., posture), repeated submaximal contractions (e.g., locomotion), and to fast and strong maximal contractions (e.g., exercise) (Schiaffino and Reggiani, 2011). Although, skeletal muscle consists predominantly of contractile elements, the structural and functional properties of the fibers, as well as intimate association between muscle fibers and the surrounding connective tissue is important for maintenance of the integrity and proper function of the entire muscle, this property is defined muscle plasticity (Cohn and Campbell, 2000; Takala and Virtanen, 2000).

Muscle fibers, or myocytes (Figure 1-2), the basic cellular unit of skeletal muscle, are formed from the fusion of multiple embryonic cells i.e. myoblasts and are therefore multinucleated with a very long cylindrical appearance therefore called myotubes (Exeter and Connell, 2010). The differentiation of skeletal muscle cells is responsible for their specialized structure and is achieved by multiple steps that consist of (1) the proliferation of mononuclear myoblasts, (2) the cell–cell fusion of myoblasts to form multinuclear myotubes, and (3) the maturation of myotubes into myofibers (Tajika et al., 2015). As mentioned previously, muscle fibers are surrounded by intramuscular connective tissue that normally has a three-level organization, namely: endomysium, perimysium and

epimysium, throughout the muscle, except at the myotendinous junction (Jarvinen et al., 2002). Each muscle fiber is surrounded by a network of connective tissue known as the endomysium (Figure 1-2) (Rowe, 1981; Swatland, 1975). Adjacent fibers are also grouped together into bigger unit called bundle, or fasciculus, that surrounded by a more robust connective tissue known as the perimysium (Figure 1-2) (Borg and Caulfield, 1980; Rowe, 1981). Bundles of muscle fibers, also known as fasciculi are in turn bound together to form the complete muscle, and the entire skeletal muscle itself is wrapped in a stronger connective tissue known as the epimysium or fascia (Figure 1-2) (Exeter and Connell, 2010). These connective tissue sheaths provide pathways for neurovascular structures, and through their collagen fibers they provide continuity with the tendons and aponeuroses that form at the ends of the muscle (White and Esser, 1989). Muscle fibers themselves are surrounded by a plasma membrane, known as a plasmalemma (Exeter and Connell, 2010). Each muscle fiber in vertebrate skeletal muscles is ensheathed by a basement membrane (Sanes, 1982). The basement membrane, in turn, is composed of two layers: an internal basal lamina directly linked to the cell membrane, and an external lamina (Figure 1-2) (Timpl, 1996). The fibrils of the reticular lamina are collagenous, and they are embedded in an amorphous proteoglycan-rich ground substance. The basal lamina contains non-fibrillar collagen, non-collagenous glycoproteins, and proteoglycans (Timpl, 1996). The combination of the plasmalemma and various layers of the basement membrane is known as the sarcolemma (Figure 1-2) (White and Esser, 1989). A small fraction of the basement membrane, ~ 0.1% of the total, occupies the synaptic cleft between nerve and muscle at the neuromuscular junction (Sanes, 1982). Although the basement membrane is considered to be distinct from endomysium, the two are

intimately connected and probably involved in the transmission of force from the myofiber to the tendon (Grounds et al., 2005; Purslow and Trotter, 1994).



**Figure 1-2 Schematic Representation of Skeletal Muscle Structure.** Each muscle fiber (myocyte) is surrounded by a delicate external lamina composed primarily of reticular fibers. When observed through the light microscope, the external lamina cannot be distinguished from the cell membrane of the muscle fiber, the sarcolemma. The endomysium, a delicate network of loose connective tissue with numerous reticular fibers, surrounds each muscle fiber outside the external lamina. A bundle of muscle fibers with their endomysium is surrounded by a more robust connective tissue layer called the perimysium. Each bundle ensheathed by perimysium is a muscle fasciculus, and the entire skeletal muscle is wrapped in a stronger connective tissue known as the epimysium or fascia.

([http://www.mhhe.com/biosci/esp/2001\\_saladin/folder\\_structure/su/m4/s1/assets/images/sum4s1\\_1.jpg](http://www.mhhe.com/biosci/esp/2001_saladin/folder_structure/su/m4/s1/assets/images/sum4s1_1.jpg); *in the public domain*)

Although most descriptions of “cells” within muscle tissue refer to the multinucleated, post-mitotic, highly differentiated muscle fibers, muscle tissue, in general, and ECM, in particular, is resident to a wide variety of mononuclear cell types, that are involved in the maintenance of ECM and the regeneration of muscle (Gillies and Lieber, 2011).

As well as myocytes, muscle also contains satellite cells that were identified in the early 1960s in adult muscular tissue as resident mitotically quiescent myoblasts (Mauro, 1961). In contrast, the satellite cells of young animals are more active and are referred to as myoblasts that initiate the muscle differentiation process (Ishikawa, 1966; Snow, 1977). Upon muscle injury, satellite cells are activated, driven out of their quiescent states and start to proliferate (Charge and Rudnicki, 2004). Proliferating satellite cells, termed myogenic precursor cells or myoblasts, then stop their proliferation, undergo differentiation into myocytes, and fuse either with each other or existing myofibers in order to repair injured muscle (Charge and Rudnicki, 2004). As they differentiate into myoblasts, these cells play a key role in muscle growth and repair as well as being instrumental in the adaptation of muscle to various stimuli (Exeter and Connell, 2010). While forming myotubes, a minor fraction of satellite cells regenerate themselves or self-renew, and eventually return to a quiescent state as satellite cells under normal physical conditions (Collins et al., 2005). This capacity, that is the ability to maintain the number of satellite cells ready to participate in repetitive muscle regeneration, is an essential characteristic of these cells (Motohashi and Asakura, 2014). Satellite cells are normally located beneath the basal lamina and adjacent to the plasmalemma of muscle fibers (Mauro, 1961; White and Esser, 1989). This close association of satellite cells with muscle fibers is considered to be an important niche for maintaining the satellite cell pool (Bischoff, 1990).

### ***1.3.1.1 Skeletal Muscle Extracellular Matrix***

The skeletal muscle extracellular matrix (ECM) plays an important role in muscle fiber force transmission, maintenance, and repair. ECM strongly affects muscle's normal function, its ability to adapt, and the biological reservoir of muscle stem cells that it provides. In both injured and diseased states, ECM adapts dramatically, a property that has clinical manifestations and alters muscle function. As previously mentioned, muscle ECM is often subdivided into endomysial, perimysial, and epimysial connective tissues (Gillies and Lieber, 2011).

Collagen is the major structural protein in skeletal muscle ECM; it accounts for 1–10% of muscle mass dry weight (Dransfield, 1977; Schiaffino and Reggiani, 2011). Different types of collagen are expressed during skeletal muscle development, yet fibrillar types I and III predominate in adult endo-, peri-, and epimysium (Bailey et al., 1979; Light and Champion, 1984; Listrat et al., 2000; Marvulli et al., 1996; Nishimura et al., 1997). Type V collagen, another fibril-forming collagen, associates with types I and III and may form a core for type I collagen fibrils in peri- and endomysium (Fitch et al., 1984), whereas, muscle basement membrane consists primarily of a type IV collagen network (Sanes, 1982).

Many glycoproteins function as linker molecules between type IV collagen in the basement membrane and sarcolemma (Ervasti and Campbell, 1993). Interactions between these glycoproteins provide potential mechanisms for lateral force transmission from the myofiber (Grounds et al., 2005). Together with the branched network structure of type IV collagen; these glycoproteins form the basis for basement membrane architecture (Sanes,

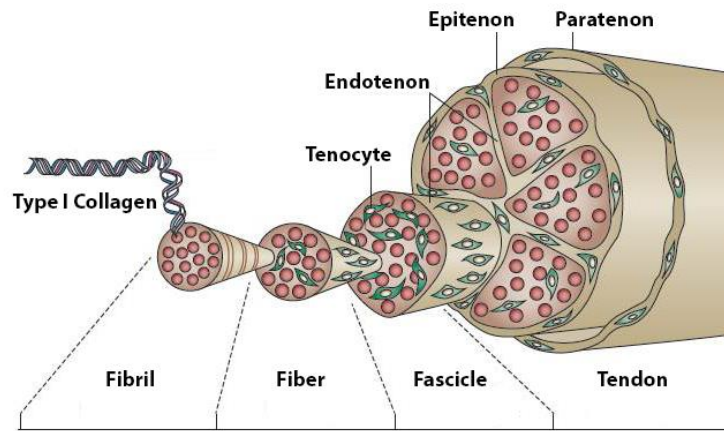
2003; Timpl et al., 1981). Although matricellular proteins do not provide structural support, they are vital in ECM signaling and maintaining ECM organization (Gillies and Lieber, 2011).

#### **1.4 Tendon Overview**

Tendons are anatomic structures of connective tissue that anchor muscles to bones. Basically, each muscle has two tendons, proximal and distal, at the origin and insertion of muscle. The point of union with a muscle is called a myotendinous junction (Kannus, 2000). Tendons transfer tensile force created in muscles to bone and absorb sudden shocks to limit muscular damage (Selvanetti et al., 1997). Although tendons are characterized by their great tensile strength (Benjamin and Ralphs, 1998), they have dynamic characteristics that belie their appearance. They are capable of repair after injury and respond to exercise or immobilization by altering their tensile strength (Woo and Buckwalter, 1988). Tendons are dominated by collagen fibers; consist of cells that are generally arranged in longitudinal rows separated by collagen fibers.

At the microscopic level, and as shown in Figure 1-3 (Nourissat et al., 2015), a bunch of collagen fibrils forms a collagen fiber, which is the basic unit of a tendon and is aligned from end to end in a tendon (Curwin, 1997). At the macroscopic level, a bunch of collagen fibers forms a bundle, also called fascicle (Figure 1-3). A fascicle is the basic unit of structure of many tendons that make up the entire tendon and represents the highest unit of subdivision in the organizational hierarchy of tendons (Kastelic et al., 1978). A fine sheath of loose connective tissue called endotenon (Figure 1-3) invests each collagen fiber and binds fibers together. The whole tendon is surrounded by a fine layer

of connective tissue sheath called epitenon (Figure 1-3) (Kannus, 2000). A condensation of the surrounding connective tissue forms a false sheath called paratenon (Figure 1-3) surrounds a tendon but is completely separate from it (Benjamin and Ralphs, 2000).



**Figure 1-3 Schematic Representation of Tendon Architecture.** Type I collagen assembles into fibrils that combine to form fibers. Tenocytes reside between collagen fibers. Fibers are surrounded by a connective tissue, the endotenon. Fibers combine to form a fascicle. Tendons are ensheathed by an outer layer of connective tissue, the epitenon, which is surrounded by another layer of connective tissue, the paratenon. Together, the epitenon and paratenon external sheaths compose the peritenon. (Figure used with permission from copyright clearance center, (Nourissat et al., 2015)).

#### 1.4.1 Tendon Cells

A variety of distinctive cell types occur within tendons. Tenocytes, a common synonym for tendon fibroblasts, and their immature form tenoblasts comprise about 90–95% of the cellular elements of the tendon and can be found in all regions within the fascicles themselves or in the endotenon, epitenon, or paratenon. There are different subpopulations of fibroblasts at these sites with different roles in matrix synthesis and cell migration during wound healing (Jozsa and Kannus, 1997). The other 5–10%

includes the chondrocytes, synovial cells, endothelial cells, nerve fibers, and Schwann cells. In pathological conditions and during wound healing many other types of cells, such as inflammatory cells (neutrophils, macrophages, and lymphocytes) and myofibroblasts, can be observed in the tendon tissue (Jozsa and Kannus, 1997).

The tendon cells, tenocytes and tenoblasts are flat, tapered cells, spindle-shaped longitudinally and stellate in cross section, and they lie sparingly in rows between collagen fibrils (Butler et al., 1978). Tenoblasts are motile and highly proliferative, and have well-developed capacity of repair (Borynsenko and Beringer, 1989). Tenocytes synthesize collagen and all other compounds of the ECM, which enable the tendon to sustain mechanical load (Evans and Barbenel, 1975; Kjaer, 2004).

#### **1.4.2 Tendon Extracellular Matrix**

The extracellular matrix (ECM) of tendon is composed of collagen, ground substance, elastic fibers and inorganic components (Kannus, 2000). Collagen makes up 60–85% of the tendon dry weight (Kjaer, 2004). Approximately 95% of this is type I collagen, with small amounts of other collagen types (Banos et al., 2008; Riley, 2004). The collagen-rich, hierarchically arranged structure results in a tissue with high tensile strength. Each level of the hierarchy is interspersed with a small amount of non-collagenous ground substance which primarily made up of proteoglycans, glycoproteins and the fibrous protein elastin that forms the elastic fibers (Kastelic et al., 1978; Thorpe et al., 2013; Yoon and Halper, 2005). The inorganic components of tendon ECM, including calcium and magnesium, are known to be involved in growth, development, and normal metabolism of tendon tissue (Kannus, 2000).

## **1.5 Work-Related Musculoskeletal Disorders**

Work-related musculoskeletal disorders (WMSDs), also known as repetitive overuse injuries are the most reported types of work-related ailments, the leading cause of work disability and productivity losses in the USA and other developed nations, and now considered a leading cause of long-term pain and physical disability worldwide (Baldwin, 2004; Bureau of Labor Statistics, 2012). WMSDs are disorders of the muscles, nerves, tendons, ligaments, joints, cartilage, or spinal disks in several body regions including the neck, back, lower extremities, shoulder, elbow, wrist and hands in response to identified risk factors in the workplace (Bureau of Labor Statistics, 2012). Forceful and/or repetitive motions are considered the leading cause of these disorders (Gallagher and Heberger, 2013; Punnett and Wegman, 2004). Ergonomic injuries involving upper extremities account for 34% of all WMSDs, require an average 11 days away from work and are estimated to cost over \$60 billion annually (Bureau of Labor Statistics, 2012). Despite widespread awareness, this type of injury remains prevalent in the workplace, partly due to a lack of clarity regarding the inducing risk factors or an inability to avoid contributing risk factors.

### **1.5.1 Musculoskeletal Injury**

Musculoskeletal injuries and disorders are a common cause of pain and disability, and result in enormous health care costs (Bureau of Labor Statistics, 2014). In general, they are the second greatest cause of disability globally and have increased 45% worldwide; according to the 2010 Global Burden of Disease Study (Horton, 2012). Musculoskeletal injuries caused by repetitive and/or forceful tasks are due to repeated

overstretching, overloading, deformation, compression, friction, or ischemia (Armstrong et al., 1991; Byl et al., 2002; Stauber, 2004; Willems and Stauber, 2000). As forces and consequent stress on affected tissues increase, tissues may be deformed enough to reach their “elastic” limit, where the material may start to exhibit an inability to return to its original configuration. This would, at some point, be likely to produce muscle or tendon microtears or disruption (Fung et al., 2010; Neviasser et al., 2012; Perry et al., 2005), and/or bone microscopic damage (e.g. increased resorption spaces and microcracks, which are small linear or elliptical cracks between osteons (Conlon et al., 2009; Kummari et al., 2009; O'Brien et al., 2005). Epidemiological studies have linked upper extremity overuse musculoskeletal injuries with occupational physical activities involving repetitive hand and arm movements, especially when those tasks included other risk factors for WMSDs, such as high force, long duration and female gender (Bernard, 1997; Silverstein et al., 1986). Increased incidence of hand/wrist osteoarthritis and reduced bone mass has been identified in female dentists and teachers with heavy or one-sided hand workloads (Ding et al., 2010; Solovieva et al., 2005; Vehmas et al., 2005). Bone scan studies of patients with upper extremity musculoskeletal disorders show increased blood flow and pooling (suggestive of inflammation) in affected forearm bones, which is important since presence of chronic inflammatory processes in bones is known to reduce bone quality by increased osteoclastic activity (al-Nahhas et al., 1997; Amorim et al., 2006; Kulkarni et al., 2012; Nanes, 2003). Related to forelimb bone, it was reported that prolonged performance of either a high repetition low force task or a low repetition high force task lead to trabecular bone adaptation, such as increased trabecular bone volume density (Barbe et al., 2013).

## **1.6 Factors Involved in Growth, Remodeling and Repair**

The growth, remodeling and repair processes of mammalian tissues are influenced by a number of variables, including extracellular matrix components. A key role takes place within a milieu of a various group of regulatory molecules known as the growth factors and cytokines that affect cell survival, proliferation and differentiation (Cross and Dexter, 1991). Those molecules may be secreted endogenously by local cells or absorbed from the serum and can act in autocrine, paracrine, or endocrine fashion. Through a variety of mechanisms, growth factors and cytokines may have stimulatory, inhibitory, or mixed activity that is either dose- or time-dependent (Allori et al., 2008). The effect of any growth factor is determined by the target cell which responds in different ways, the concentration of factor, and the stimuli of other growth factors under different circumstances. Growth factor stimuli are transmitted into the cell via activation of specific transmembrane receptors that modify key regulatory proteins in the cytoplasm. These in turn affect the decisions controlling proliferation and differentiation, including changes in gene expression and reactivity to other factors, which explains why some factors may function both extra- and intra-cellularly (Cross and Dexter, 1991). Because cytokines are structurally related to growth factors, they may also affect cellular growth and function; for example, in morphogenesis, and tissue repair, they play a pivotal role (Allori et al., 2008).

### **1.6.1 Osteoactivin**

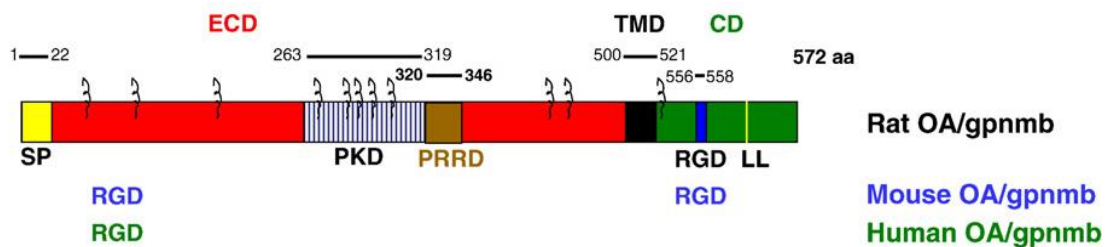
Osteoactivin (OA) was initially identified in studies using an osteopetrotic rat model (Marks and Popoff, 1989). mRNA differential display showed a novel cDNA, that

is highly up-regulated in osteopetrotic bones, compared to normal bones, and subsequently named osteoactivin. Northern blot analysis demonstrated that OA expression was increased 3-to 4-fold in long bones and calvaria of osteopetrotic versus normal (Safadi et al., 2001). Subsequent cloning and sequencing of OA cDNA demonstrated that it has 77% identity on the DNA level and 65% identity of the protein level to human glycoprotein non-melanoma protein B (*gnmb*) in melanoma cell lines and melanocytes (Anderson et al., 2002; Weterman et al., 1995). OA also shares sequence homology with dendritic cell heparan sulfate proteoglycan integrin dependent ligand (DC-HIL) in dendritic and T cells (Shikano et al., 2001) and human hematopoietic growth factor inducible neurokinin (HGFIN) in tumor cells (Bandari et al., 2003). Furthermore, mouse *gpnmb* protein was identified to be homologous to human and mouse OA sequences (Bachner et al., 2002). A comparison of rat and mouse OA revealed 88% identity (Safadi et al., 2001). The OA gene was mapped on different chromosomes in different species: chromosome 4 for rat, chromosome 6 for mouse, and chromosome 7 for human OA respectively. Due to its homology to *gpnmb* and *Pmel17*, OA was suggested to belong to the *Pmel17* gene family (Owen et al., 2003).

#### ***1.6.1.1 Osteoactivin Structure***

The protein coding region of the OA cDNA is composed of 1716 base pairs and it codes for a protein of 572 amino acid residues. OA exists as: a 65-kDa type I transmembrane protein, and a highly glycosylated 115-kDa secreted glycoprotein (Abdelmagid et al., 2008; Safadi et al., 2001). Based on the predicted protein sequence, OA consists of three main domains (Figure 1-4): an N-terminal extracellular domain

(ECD) or luminal domain (amino acids 23-500), a middle short transmembrane domain (TMD) rich in hydrophobic residues (amino acids 501-521), and a C-terminal cytoplasmic domain (CD) (amino acids 522-572). The first 22 amino acids at the N-terminal domain constitute a signal peptide (SP) that aids the entry of OA into its secretory pathway (Abdelmagid et al., 2008).



**Figure 1-4 Structure of Osteoactivin Protein.** OA consists of three main parts, the extracellular domain (ECD), the transmembrane domain (TMD) and the cytoplasmic domain (CD). Numbers correspond to amino acid position. SP, signal peptide; PKD, polycystic kidney disease domain; PRRD, proline rich repeat domain; LL, dileucine sorting sequence; RGD, integrin binding domain. (Figure used with permission from copyright clearance center, (Abdelmagid et al., 2008)).

The ECD can be further divided into three subdomains, including an Arg-Gly-Asp (RGD) domain, a polycystic Kidney disease-like domain (PKD), and a proline-rich repeat domain (PRRD). Each of these domains has specific functions. The RGD domain, which is present in mouse and human OA in its N-terminal domain but absent in the N-terminal domain of rat OA, is suggested to function as an attachment site for integrins and contributes to integrin-mediated cell attachment and spreading (Abdelmagid et al., 2008; Safadi et al., 2001; Shikano et al., 2001). The PKD domain has an immunoglobulin-like folding structure that plays a role in the protein-protein and protein-carbohydrate interactions (Scheffers et al., 2002). The PRRD domain function is not clear in OA but

has been linked to O-linked glycan of Pmel-17 (Hoashi et al., 2006). The transmembrane domain has an alpha helical structure and is suggested to play a role in anchoring OA protein to the cell membrane. OA also contains a di-leucine amino acid (LL) sorting signal sequence in close proximity to the C-terminal domain, with a potential role in sorting the protein through the rough endoplasmic reticulum (RER) (Piccirillo et al., 2006; Setaluri, 2000; Theos et al., 2006; Zerivitz and Akusjarvi, 1989). The roles of the different domains of OA have not yet been determined experimentally. However, one study showed that the PKD and, to a lesser extent, the PRRD domains negatively regulate T-cell proliferation (Chung et al., 2007a). Further studies sought to examine OA processing in osteoblasts. Cell fractionation experiments revealed two distinct isoforms of OA both present in the cytosol; however a more mature form was only present in membrane fractions. Further studies on osteoactivin will be described in the next section.

#### ***1.6.1.2 Osteoactivin Expression and Function in Musculoskeletal Tissues***

OA has the ability to regulate cell proliferation, adhesion, differentiation, and synthesis of extracellular matrix proteins in different tissues and in various cell types in both normal and pathological processes.

In bone, the high level of OA expression was initially described in a model of osteopetrosis in rat and its expression was increased 3- to 4-fold in mutant bones compared to normal ones (Safadi et al., 2001). OA expression was further studied *in vivo* during active bone regeneration using a model of fracture repair in rat. Temporal and spatial expression patterns of OA mRNA and protein in a femur fracture model in rat were reported (Abdelmagid et al., 2010). OA mRNA and protein were found to be highly

expressed in osteoblasts localized in the metaphysis of the intact long bone (tibia), and in the hypertrophic chondrocytes in the growth plate, as determined by *in situ* hybridization and immunohistochemistry, respectively. In addition, Northern blot analysis showed a high expression of OA mRNA in day-3 and day-10 post-fracture (PF) calluses in femurs of fracture model compared with intact rat femurs (Abdelmagid et al., 2010). *In situ* hybridization technique showed that positive OA mRNA signals were seen as early as day 3 PF and reached a maximal intensity at day 5 PF, followed by decreasing levels of OA mRNA at day 21 PF. Also, a higher level of OA mRNA expression was observed in the soft callus compared to intact femurs. Similarly, high levels of OA protein was detected throughout the reparative phase of the hard callus compared to intact femurs. Furthermore, a secreted isoform of OA protein was also detected within the newly made cartilage matrix and unmineralized osteoid tissue (Abdelmagid et al., 2010).

OA was also emerged as a vital glycoprotein for the differentiation and function of both osteoblasts and osteoclasts *ex vivo*. Primary rat osteoblast cultures, showed OA expression to increase during osteoblast differentiation and function with maximal expression during the final stages of differentiation (Abdelmagid et al., 2008; Safadi et al., 2001). However, expression of OA in bone is not restricted to osteoblasts. Different studies demonstrated that mature osteoclasts also express high levels of OA. Sheng et al. reported the temporal expression of OA during osteoclastogenesis (Sheng et al., 2008), and our group showed that OA acts as a negative regulator of osteoclast differentiation and survival but not function (Abdelmagid et al., 2015).

To examine the roles of osteoactivin in bone, the effect of exogenous OA on bone formation *in vivo* was studied. Recombinant OA (rOA) was administered into the marrow

cavity of rat femur through a local delivery system via a single injection. Histological evaluation revealed that rOA-injected femurs had islands of newly formed woven bone within the marrow cavity. Many blood vessels adjacent to those islands were observed. Also, multiple rows of osteoblasts and/or osteoprogenitors near the periphery were detected. However, no evidence of an osteogenic response was observed in the control-injected group. These results show that the rOA is an osteoinductive agent *in vivo* (Singh et al., 2010).

To identify the specific role of OA during osteoblast development, osteoblast cell cultures at different stages of development were treated with either anti-OA functional blocking antibody or OA antisense oligonucleotides to neutralize secreted OA protein or to down-regulate OA expression, respectively. Both treatments blocked osteoblast differentiation associated with inhibition of matrix maturation and calcium deposition (Abdelmagid et al., 2008; Owen et al., 2003). Subsequent studies examined the effects of overexpression of OA using CMV promoter to drive OA cDNA expression in osteoblasts. These experiments showed that overexpression of OA induced osteoblast differentiation (Abdelmagid et al., 2008). To further elucidate a mechanism whereby OA regulates osteoblast differentiation, investigators first identified whether the N- or C-terminus of OA protein is involved in osteoblast differentiation. Using blocking antibodies directed against either the N- or C-terminus revealed an important role for C-terminus of OA in osteoblast differentiation (Selim et al., 2007). Similarly, osteoblast progenitor cells, obtained from OA-mutant mice that lacked the C-terminus domain of OA, showed a significant decrease in differentiation (Abdelmagid et al., 2008).

Similar roles of OA were observed in osteoclasts, where treatment of osteoclast progenitor cells with an OA antibody decreased osteoclasts' fusion and migration and resulted in smaller osteoclasts with decreased resorption activities (Sheng et al., 2008), whereas, targeted overexpression of OA under control of the TRAP promoter in an osteoclastic lineage promoted their bone resorptive activity (Sheng et al., 2012).

In muscle, OA has been found to have a protective function in skeletal muscle against long-term denervation. Previous study showed that OA is upregulated under muscle unloading conditions including denervation and space flight (Nikawa et al., 2004). Rat gastrocnemius muscle undergoing atrophy following denervation showed an eight-fold up-regulation of OA expression which has been reported in muscle fibers and especially in the sarcolemma of myofibers (Ogawa et al., 2005). Studies using a mouse model of muscle denervation showed an increase in OA expression associated with increased expression of MMP-3 and -9 mainly in fibroblast-like cells infiltrated into denervated muscle as well as in the sarcolemma adjacent to these fibroblast-like cells (Furochi et al., 2007a). Further studies to confirm the functional role of OA in muscle were performed using OA transgenic mice under the control of cytomegalovirus (CMV) promoter. Muscle denervation in these mice further enhanced the expression of MMP-3 and MMP-9 in fibroblasts infiltrated into gastrocnemius muscle when compared with the muscles of WT mice (Furochi et al., 2007a). These results suggested that the OA-mediated increase in MMPs in skeletal muscle might be useful for protecting injured muscle from fibrosis, leading to full regeneration after denervation. A more recent study identified that OA attenuates skeletal muscle fibrosis after distraction osteogenesis by

regulating ECM degradation/remodeling through expressions of MMPs and improved the function of the distracted muscle (Tonogai et al., 2015).

### ***1.6.1.3 Regulation of Osteoactivin***

Studies have shown that regulation of osteoactivin is cell type specific. In osteoblasts it occurs via the BMP/Smad signaling pathway. BMP-2 was identified as a stimulating factor for OA expression via Smad1/4 signaling in osteoblasts. Furthermore, the OA promoter has multiple Smad1 binding sites that could regulate OA transcription following BMP-2 stimulation. Treatment of osteoblasts with OA antisense oligonucleotides inhibited BMP-2-stimulated osteoblast differentiation and function (Abdelmagid et al., 2007). In addition to Smad1/4, deletion and mutation studies from our laboratory suggested that Runx2 also regulates OA transcriptional activity (Singh et al., 2010). These studies suggest that OA acts as a downstream mediator of BMP-2.

In breast cancer cells OA is regulated via the ERK pathway. Some insights on OA regulation have emerged from therapeutic targets in malignancy. For instance, treatment with inhibitors of the ERK pathway enhanced cell-surface expression of OA in melanoma cells (Qian et al., 2008). It was also showed that utilizing CDX-011, an antibody-drug conjugate (OA-auristatin E) to treat breast cancer, in combination with ERK pathway inhibitors, increased the sensitivity of breast cancer cells to CDX-011 treatment (Rose et al., 2010).

Additional studies showed that RANKL, via microphthalmia-induced transcription factor (MITF), enhances OA expression during osteoclast differentiation (Ripoll et al., 2008; Sheng et al., 2008). Based on the current literature, it is clear that OA

is an important molecule in osteoblast and osteoclast differentiation and is a key target for bone-specific transcription factors that integrate multiple osteogenic regulatory signals in bone. However, the transcriptional regulation of OA in osteoblasts is still not fully understood.

#### ***1.6.1.4 Animal Models examining the function of Osteoactivin***

Few animal models have been generated to demonstrate the novel function of OA in various tissues. Transgenic mice that overexpress osteoactivin (OA) under the control of the tartrate-resistant acid phosphase (TRAP) exon 1B/C promoter showed significant bone loss due to an increase in bone resorption. Sheng et al. reported that histomorphometric bone formation parameters and plasma levels of bone formation biomarkers were not different between transgenic mice and WT littermates, supporting the idea that bone loss in OA-Tg mice is due to an increase in bone resorption and not to reduction in bone formation (Sheng et al., 2012). In targeted muscle expression, transgenic mice that overexpress OA under the CMV promoter showed increased muscle mass and enhanced expression of MMP-3 and MMP-9 in fibroblasts in a model of denervated skeletal muscle (Ogawa et al., 2005). Finally, in transgenic rats that overexpress OA two-fold in the liver showed that OA attenuates the development of hepatic fibrosis by suppressing platelet-derived growth factor receptor- $\alpha$  (PDGFR- $\alpha$ ) and tissue inhibitor of metalloproteinase-1 (TIMP-1), the key genes required for disease pathogenesis (Abe et al., 2007).

OA mutation data is equally interesting. A mouse line exists with a natural mutation in the OA gene, causing a premature stop codon, resulting in the generation of a

truncated OA protein of only 150 amino acids (Anderson et al., 2006; Anderson et al., 2002; Anderson et al., 2001; Ott et al., 2003). A study using this animal model revealed that the presence of truncated OA causes both iris pigmentary dispersion and iris stromal atrophy. Both phenotypes are strongly associated with the development of pigmentary glaucoma (Anderson et al., 2002). These mice also have increased macrophage function (Ripoll et al., 2007). To characterize the function of truncated OA, our group used bone marrow-derived stromal cells that are osteoblast progenitor cells from the OA mutant mice. The results showed that truncated OA had reduced osteoblast differentiation in comparison with their normal littermates (Abdelmagid et al., 2008). Moreover, bone mass in OA mutant mice was significantly reduced and remained relatively constant with age, which may indicate a decrease in bone remodeling capability (Abdelmagid et al., 2014). Since this truncated OA decreased osteoblast differentiation and reduced bone mass, osteoactivin transgenic mice were generated (under the control of the CMV promoter) in order to examine the effects of OA overexpression on skeletogenesis. This study forms the basis for part of this project to characterizing the skeletal phenotype of this transgenic model, which will be discussed in greater detail in Chapter 2.

### **1.6.2 Heat Shock Proteins**

Heat shock proteins (HSPs), or stress proteins, are a superfamily of a number of proteins that are synthesized by cells in response to different stimuli in order to protect the cell from damaging influences (Wong and Wispe, 1997). They are present in all organisms and in all cells of all organisms and are highly conserved across species (Lindquist, 1986). Selected HSPs, also known as chaperones, play crucial roles in

folding/unfolding of proteins, stabilization and assembly of multiprotein complexes, reactivation of denatured proteins, transport/sorting of proteins into correct subcellular compartments, cell-cycle control and signaling, and protection of cells against stress/apoptosis (Craig et al., 1993; Lindquist, 1986; Wong and Wispe, 1997). In addition, HSPs are immune-active compounds that induce the maturation and activation of inflammatory cells (Cohen-Sfady et al., 2005; Haug et al., 2005; Kuppner et al., 2001; Srivastava, 2002), and regulate the expression of pro-inflammatory factors (Asea et al., 2000). HSPs are classified according to their weight into five major groups: the HSP100s, HSP90s, HSP70s, HSP60s, and the HSP20s or the small HSPs; however, most investigations were conducted using HSP70 (Hartl, 1996; Schlesinger, 1986). Of particular interest is the inducible form of HSP70 (HSP72, commonly known as HSPA1A) that has widely been used as a marker for cell stimulation experiments because it can be induced dramatically by stress (Beck et al., 1995; Schlesinger, 1986).

In skeletal muscle, HSPs are suggested to be important factors in tissue repair or regeneration and adaptation to exercise and stress (Banfi et al., 2004; Koh, 2002; McArdle et al., 2004; Paulsen et al., 2007). Specifically, HSP72 is known to play a significant role in protecting injured muscle against damage and dysfunction and to mediate matrix remodeling and repair (Marber et al., 1995; McArdle et al., 2004; Miyabara et al., 2006). Following skeletal muscle injury, induced HSP72 regulates both the early inflammatory and regenerative phases, protects from atrophy and aids regeneration (Gehrig et al., 2012; Miyabara et al., 2012; Senf et al., 2008; Senf et al., 2013). In the event of skeletal muscle injury HSP72 activates the immune response by binding to both neutrophils and macrophages and recruiting them to the site of injury,

which subsequently regulates muscle regeneration (Chen and Cao, 2010; Kovalchin et al., 2006; Ortega et al., 2009; Tsan and Gao, 2009).

In early tendon damage, stressed tenocytes release various inflammatory mediators and associated HSPs that play a role in tissue healing response and drive the tendon matrix toward a reparative process (Millar and Murrell, 2012). The increased expression of HSP72 in response to repetitive mechanical stretching decreases the apoptosis rate of tenocytes (Barkhausen et al., 2003). Furthermore, tendon fibroblasts subjected to mechanical stress induced by cyclic longitudinal stretching showed upregulated HSP72 expression, suggestive of a tissue repair mechanism (Jagodzinski et al., 2006). In both rat and human models of tendinopathy increased levels of inducible HSP70 protects supraspinatus tendons from apoptosis (Millar et al., 2008).

### **1.6.3 Matrix Metalloproteinases**

The interaction of cells with extracellular matrix (ECM) is critical for their normal functions. Modulation of cell-matrix interactions occurs through the action of unique enzyme systems responsible for the ECM turnover and remodeling (Chakraborti et al., 2003). Matrix metalloproteinases (MMPs) are a large family of zinc-dependent endopeptidases known for their ability to degrade one or several ECM constituents to regulate cell-matrix composition and modulate ECM turnover (Mandal et al., 2002; Mandal et al., 2003; Massova et al., 1998; Visse and Nagase, 2003).

In muscle, the turnover of ECM is required for cell migration, myotube formation, and reorganization of the matrix during muscle adaptation. Remodeling of the ECM by MMPs is a major feature of skeletal muscle repair, which is required to remove damaged

tissue, restore the ECM scaffold, and release reservoirs of stored growth factors, to eventually result in the renewal of functioning muscle fibers (Lei et al., 2013). MMP levels in uninjured muscle are generally low, but after muscle injury, satellite cells known to express MMP become activated; an elevated level of MMP production is thought to result in cell migration into injured areas with subsequent differentiation and fusion of satellite cells into myofibers (El Fahime et al., 2000; Kherif et al., 1999; Lewis et al., 2000). MMPs expressed in skeletal muscle include the gelatinases MMP-2 and MMP-9 (Kherif et al., 1999) that degrade type IV collagen, fibronectin, proteoglycans, and laminin, as well as the collagenases MMP-1 (Singh et al., 2000) and MMP-13 (Wu et al., 2003) that degrade types I and III collagen. MMP-3 is part of the stromelysin family, broad-spectrum proteinases that have important regulatory functions such as the activation of other MMPs (Pasternak and Aspenberg, 2009). Inhibitors of MMPs, tissue inhibitors of matrix metalloproteinases (TIMPs) 1–3, either bind to active MMPs or stabilize inactive forms, thereby inhibiting their enzymatic activity (Chin and Werb, 1997; Singh et al., 2000).

Tenocytes of injured tendons show increased expression of MMPs (Tajana et al., 2009). The role of MMPs in tendon injury is well established and it was shown that injury causes alterations in the levels of several of the matrix MMPs within tendon which degrade specific components of the ECM (de Mos et al., 2007; Jones et al., 2006; Riley, 2008). Studies demonstrated that the alterations in the expression levels of several MMPs including MMP-1 and -3 occurring immediately post-loading (Asundi and Rempel, 2008a, b; Sun et al., 2010). MMP-1 was suggested to play a role in the early stages of degradation and remodeling of damaged collagen fibers within the matrix, while, MMP-

3, the ubiquitously expressed MMP in tendon tissue, was suggested in the degradation of many minor proteins within the tendon matrix, including the different types of collagen, proteoglycans, and elastin (Birch et al., 2008).

#### **1.6.4 Transforming Growth Factor Beta**

Transforming growth factor beta (TGF- $\beta$ ); a member of a superfamily of growth and differentiation factors was originally identified in neoplastic tissues and then found in a wide variety of normal cells and tissues (Assoian et al., 1983; Childs et al., 1982; Frolik et al., 1983; Roberts et al., 1981; Roberts et al., 1983). In mammals, TGF- $\beta$  exists in 3 isoforms TGF- $\beta$ 1, - $\beta$ 2 and - $\beta$ 3, among them, TGF- $\beta$ 1 is the most abundant isoform in bone (Seyedin et al., 1985). The biological effects of the TGF- $\beta$  are mediated through specific cell surface receptors, designated TGF- $\beta$  type I and II receptors which are high-affinity serine/threonine kinases that couple to various secondary messenger pathways (Allori et al., 2008). Upon ligand binding, the constitutively active type II receptor phosphorylates type I receptor forming a heterodimer which then phosphorylates Smad2 and 3. Activated Smad2/3 then forms a complex with Smad4 and translocate to the nucleus where they regulate transcription of target genes (Derynck and Zhang, 2003; Massague et al., 1994; Shi and Massague, 2003). The biological function of TGF- $\beta$  in the induction of cell chemotaxis, proliferation, and synthesis of collagen at wound sites suggests its role in inflammation, wound healing and tissue repair (Derynck et al., 1985; Pierce et al., 1989a; Pierce et al., 1989b; Postlethwaite et al., 1987; Sporn et al., 1983). Studies showed TGF- $\beta$  to either stimulate fibroblast-colony formation or inhibit the growth of variety of human cancer cell lines. Whether TGF- $\beta$  acts to stimulate or inhibit

the growth of a particular cell type depends on many variables, such as the physiological condition of the cell and the presence of additional growth factors (Roberts et al., 1985; Tucker et al., 1983). *In vivo* and *in vitro* studies that showed TGF- $\beta$  stimulates the biologic activities of osteoblasts, suggest its role in osteogenesis, especially during active matrix secretion (Centrella et al., 1986; Joyce et al., 1990a; Joyce et al., 1989; Joyce et al., 1990b; Linkhart et al., 1996; Noda and Camilliere, 1989; Sandberg et al., 1993). The role of TGF- $\beta$  in bone will be discussed in greater detail in Chapter 2.

### **1.6.5 Tumor Necrosis Factor Alpha**

Tumor necrosis factor alpha (TNF- $\alpha$ ) is a pro-inflammatory cytokine was initially identified to be produced by the immune system during acute inflammation with an optional capacity to induce apoptosis (Wajant et al., 2003). Although, TNF- $\alpha$  is produced mainly by activated macrophages and other immune cells, it can be also expressed by other cell types including endothelial cells and fibroblasts (Carswell et al., 1975; Granger and Williams, 1968). TNF- $\alpha$  exists in two forms, a membrane bound form, and a soluble form; each form carries out a distinct physiological role (Granger and Williams, 1968). TNF- $\alpha$  is first produced as a type II membrane protein of 26 kDa. This pro-TNF- $\alpha$  can be cleaved in the extracellular domain and released as a soluble mature protein of 17 kDa. This soluble cytokine exists in solution as a homotrimer of 51 kDa and this is the form that binds and cross-links receptors (Black et al., 1997; Kriegler et al., 1988; Tang et al., 1996). The biologic activities of TNF- $\alpha$  are initiated by high-affinity binding to specific cell surface receptors. The extracellular portion of these membrane receptors can be proteolytically cleaved to form the soluble receptors. Both soluble receptors are able to

bind ligand and compete with cell-associated receptors for free TNF- $\alpha$  (Kohno et al., 1990; Loetscher et al., 1990). The biological activities of TNF- $\alpha$  are dependent on its interaction with other cytokines as TNF has a pivotal role in regulating both pro-and anti-inflammatory mediators (Feldmann et al., 1994; Le and Vilcek, 1987).

Although TNF- $\alpha$  was originally shown to exert significant cytotoxicity on many tumor cell lines leading to necrosis or apoptosis, it can exert an extreme spectrum of bioactivities and most cells show at least some TNF responsiveness (Carswell et al., 1975; Idriss and Naismith, 2000; Kriegler et al., 1988; Wajant et al., 2003). In pathological or physiological situations, TNF- $\alpha$  shows a remarkable functional duality, being strongly engaged both in tissue regeneration/expansion and destruction (Carswell et al., 1975; Idriss and Naismith, 2000; Kriegler et al., 1988; Wajant et al., 2003). Even though TNF- $\alpha$  action in various tissues is complex, various studies have examined its effect in musculoskeletal tissue, namely muscle and bone. TNF- $\alpha$  was shown to play a physiological role in muscle repair and myogenesis. While the high circulating levels of TNF- $\alpha$  mediate muscle wasting and inflammatory myopathies, the low concentrations of it induce proliferation of satellite cells in muscles *in vitro* (Chen et al., 2005; Li and Schwartz, 2001; Warren et al., 2002), indicating that a therapeutic potential of TNF- $\alpha$  is hampered by its systemic toxicity at effective concentrations (Idriss and Naismith, 2000; Kontoyiannis et al., 1999). TNF- $\alpha$  was identified as a skeletal catabolic agent that stimulates osteoclastogenesis and inhibits osteoblast function *in vitro* (Bertolini et al., 1986; Canalis, 1987; Nanes, 2003; Stashenko et al., 1987). An *in vivo* study reported that mice null for TNF- $\alpha$  had significantly increased peak bone mass, resulting exclusively

from elevated bone formation, suggesting that TNF- $\alpha$  also has catabolic action on *in vivo* bone metabolism (Li et al., 2007).

### **1.7 Significance of Dissertation**

Osteoporosis has emerged as a global health concern that affects vast populations, consequently placing immense pressure on global health care systems. In the United States, osteoporosis is the most common bone disease. Approximately 10 million people have been diagnosed with osteoporosis, while an additional 34 million people have been diagnosed as osteopenic (low bone mass) and are on the cusp of developing osteoporosis. (Boonen and Singer, 2008; Orsini et al., 2005). In 2005, costs associated with osteoporosis-related fractures in the U.S. were estimated at \$19 billion, and they are projected to reach \$25.3 billion in 2025 (Burge et al., 2007). Both osteopenia and osteoporosis are a result of an abnormal decrease in overall bone mass.

In normal bone remodeling, osteoclast-mediated bone resorption is coupled with an equivalent amount of osteoblast-mediated bone formation. However, the low bone density that is observed in both osteopenia and osteoporosis is the result of an imbalance in the bone remodeling process in which the rate of bone resorption exceeds the rate of bone formation. There are two basic approaches that can potentially reverse this: the inhibition of the bone resorbing cells, or the stimulation of the bone forming cells, and if each alone is not sufficient, the synergistic effect of the combination of both approaches may be useful.

Recent studies have shown promising results of bone loss reversal through the stimulation of the bone-forming osteoblasts. Increased knowledge of the mechanisms involved in the regulation of osteoblastic bone development and bone mass maintenance have enhanced the understanding as to why a variation in normal osteoblastogenesis regulation can result in various bone diseases. Further research on the regulation signals may promote the discovery of therapeutic targets to stimulate bone formation, and combined with other therapies, it may provide the ultimate treatment for osteoporosis.

Osteoactivin has been shown to be a prominent growth factor in bone and has a novel role in bone formation. To date, studies have identified OA as an osteoinductive agent that can enhance bone formation and stimulate osteoblast differentiation. Once we understand the full effect of osteoactivin on bone and how it works to promote bone formation, this information in combination with other recent advances in the field, will be helpful in developing new therapeutic strategies to selectively enhance bone formation in patients with clinically significant bone loss.

## 1.8 Specific Aims

Osteoactivin (OA) has emerged as a vital glycoprotein required for the differentiation and function of both osteoblasts and osteoclasts. The high level of OA expression in bone was initially described in a model of osteopetrosis in rat and its expression was shown to increase during osteoblast differentiation. In primary rat osteoblast cultures, OA expression increases over time, with maximal expression during the final stage of differentiation, matrix mineralization (days 17-21). Our group showed that treating osteoblast cultures at different stages of osteoblast differentiation, with either an anti-OA blocking antibody or OA antisense oligonucleotides inhibited matrix maturation and calcium deposition. Moreover, our laboratory reported in a rat femur fracture model that the expression of OA mRNA was higher in day-3 and day-10 post-fracture callus when compared with intact rat femur. Also, high levels of OA protein were detected by immunohistochemistry throughout the reparative phase of the hard callus compared to intact femurs. Furthermore, using a model of local delivery system in which recombinant OA (rOA) was administered via a single injection into the marrow cavity of rat femur, our group showed that rOA-injected femurs had islands of newly formed woven bone within the marrow cavity. Many blood vessels adjacent to those islands were observed. Also, multiple rows of osteoblasts and/or osteoprogenitors near the periphery were detected. However, no evidence of an osteogenic response was observed in the control-injected group. Collectively, these data indicate that OA enhances bone formation and serves as an osteoinductive agent. Based on these findings, we hypothesize that over-expression of OA can positively regulate bone mass *in vivo*. Therefore, the specific aims of this study are:

**Aim 1a.** To characterize skeletal phenotypes in OA transgenic mice. Effects on long bone from OA transgenic (OA-Tg) mice were studied using micro-CT, biomechanical, histological and histomorphometric analyses at different ages and gender. Together with data on biochemical markers for bone formation and resorption, this work provided insights on the role of OA on bone formation *in vivo*.

We hypothesized that over-expression of OA would positively regulate bone mass *in vivo* in a transgenic mouse model, and enhance osteoblast differentiation *ex vivo*, using cells from these transgenic mice.

**Aim 1b.** To study the function of OA over-expression *ex vivo*. Primary osteoblasts were isolated from the calvaria and bone marrow of wild type (WT) and OA-Tg mice. These osteoblasts were differentiated and compared for the expression for the levels of differentiation markers during various stages of differentiation. From these *ex vivo* data, we evaluated the effect of OA over-expression on osteoblast differentiation.

We hypothesized that over-expression of OA would enhance osteoblast differentiation *ex vivo*, using cells from OA transgenic mice.

**Aim 2.** To characterize the temporal and spatial expression of OA as well as MMPs and HSP72 in musculotendinous tissue in rats performing an upper extremity high repetitive negligible force (HRNF) task for 3-6 weeks compared to age-matched control rats. Inflammatory cytokines were also examined in order to determine if injury/inflammatory processes were present in the tissues at the same time points.

We hypothesized that OA would increase in the overloaded skeletal muscles and tendons, as would two known mediators of repair, MMPs and HSP72.

**Aim 3.** To evaluate OA expression in a rat model of repetitive overuse in musculoskeletal tissues in young adult rats performing a high repetition high force (HRHF) task treated with anti-inflammatory drugs (specifically, a rat anti-TNF-alpha ( $\alpha$ ) drug) or task cessation (i.e. rest), compared to untreated HRHF rats.

We hypothesized that adaptive remodeling in musculoskeletal tissues undergoing prolonged repetitive loading at high force loads are modulated by a superimposed inflammatory response, specifically an increased TNF- $\alpha$  response known to enhance catabolic tissue changes. We further hypothesized that anti-TNF- $\alpha$  treatment or task cessation provided in weeks 4-7 of an 11 week HRHF task paradigm would alter the balance towards anabolic responses, such as increased OA production and tissue growth.

## CHAPTER 2

### TRANSGENIC EXPRESSION OF OSTEOACTIVIN/GPNMB ENHANCES BONE FORMATION *IN VIVO* AND OSTEOPROGENITOR DIFFERENTIATION *EX VIVO*

\*Extracted from previously published work: Frara, N., Abdelmagid, S.M., Sondag, G.R., Moussa, F.M., Yingling, V.R., Thomas A. Owen, T.A., Popoff, S.N., Barbe, M.F., & Safadi, F.F. (2015) *Journal of Cellular Physiology*.

#### 2.1 Introduction

In normal bone remodeling, osteoclast-mediated bone resorption is coupled with an equivalent amount of osteoblast-mediated bone formation. However, low bone density with both osteopenia and osteoporosis is the result of an imbalance in the bone remodeling process in which the rate of bone resorption exceeds the rate of bone formation. There are two basic approaches to potentially reverse this: the inhibition of bone resorbing cells or the stimulation of bone forming cells, and if each alone is not sufficient, the synergistic effect of the combining both approaches may be useful.

Osteoactivin (OA)/gpnmb has been shown to be a prominent factor in bone and plays a novel role in bone formation. To date, studies have identified OA/gpnmb as an osteoinductive agent that enhances bone formation and stimulates osteoblast differentiation (Abdelmagid et al., 2008; Abdelmagid et al., 2014; Safadi et al., 2001; Selim et al., 2003). High levels of OA/gpnmb expression in bone were initially described in a rat model of osteopetrosis in which its expression was increased during osteoblast differentiation (Safadi et al., 2001). In primary rat osteoblast cultures, OA/gpnmb

expression increases over time, with maximal expression during matrix mineralization, the final stage of differentiation (Abdelmagid et al., 2008; Owen et al., 2003; Safadi et al., 2001). In a rat femur fracture repair model, OA/gpnmB mRNA expression was higher in the callus at 3- and 10-days post-fracture, and OA/gpnmB protein immunoeexpression was elevated throughout the reparative phase of the hard callus, compared to intact femurs suggesting that the OA/gpnmB plays a role in osteogenesis (Abdelmagid et al., 2010). Furthermore, using a model of local delivery system in which recombinant OA/gpnmB (rOA/rgpnmB) was administered via a single injection into the marrow cavity of rat femur, our laboratory showed that rOA/rgpnmB-injected femurs had islands of newly formed woven bone within the marrow cavity (Singh et al., 2010). Angiogenesis was observed adjacent to those islands, and multiple rows of osteoblasts and/or osteoprogenitors were detected near the periphery of the new bone. In contrast, no evidence of an osteogenic response was observed in the control-injected group (Singh et al., 2010).

Few animal models have been generated to demonstrate the function of OA/gpnmB in various tissues. The importance of OA/gpnmB in osteogenesis was confirmed in mice with a natural mutation in the OA/gpnmB gene in which a premature stop codon resulted in the generation of a truncated OA/gpnmB protein. These mice also exhibited decreased bone mass (Abdelmagid et al., 2014). Transgenic mice that overexpress OA/gpnmB under the control of the tartrate-resistant acid phosphatase (TRAP) exon 1B/C promoter show significant bone loss due to increased bone resorption (Sheng et al., 2012). Histomorphometric bone formation parameters and plasma levels of bone formation biomarkers were not different between transgenic mice and WT

littermates, supporting the idea that bone loss in OA-Tg mice is due to an increase in bone resorption and not to a reduction in bone formation (Sheng et al., 2012).

In the current study, given that OA/gpmb is a vital glycoprotein required for the differentiation and function of both osteoblasts and osteoclasts, osteoactivin transgenic mice were generated (under the control of the CMV promoter) in order to examine the effects of OA/gpmb overexpression on skeletogenesis. Specifically, our aim was to characterize the skeletal phenotype of this transgenic model. We hypothesized that overexpression of OA/gpmb positively regulates bone mass *in vivo* in a transgenic mouse model, and enhances osteoblast differentiation *ex vivo*, using cells from these transgenic mice.

## **2.2 Material and Methods**

### *2.2.1 Animals*

The colony of OA-transgenic (OA-Tg) mice was generated by over-expression of OA/gpmb under control of the CMV promoter. The targeting vector was confirmed for the presence of the OA transgene using MC3T3-E1 osteoblast-like cells; we found that OA/gpmb is over-expressed over 3-fold, compared to empty-vector control cells (data not shown). This targeting vector was used to generate the OA/gpmb transgenic (OA-Tg) mice using the University of Pennsylvania Transgenic Core Facility. Six lines were established by breeding the founders with CD-1 wild type mice. Breeding of the hemizygous OA-transgenic mice with CD-1 mice showed Mendelian inheritance of the transgene with an equal sex distribution among the litters. Mice were backcrossed for 8-10 generations using C57BL/6 mice. OA/gpmb-expression in the transgene was

examined and was markedly up-regulated. A second line of OA-transgenic mice was then established and used for further analysis of the skeletal phenotype. Mice from this line survive and breed well. OA/gpnm expression was increased over 3-fold in transgenic mice, compared to age-matched wild type mice (data not shown). A C57BL/6 mouse strain was used as wild-type (WT) controls. All animals were maintained at Temple University School of Medicine Central Animal Barrier Facility, and used according to the principles in the NIH Guide for the Care and Use of Laboratory Animals, and with the respect to the guidelines established by the Institutional Animal Care and Use Committee.

#### *Micro-Computed Tomography (Micro-CT) Analysis*

Femurs from WT and OA-Tg mice that were 4- and 12-weeks of age, and 12 months of age, were scanned ( $n \geq 4/\text{group}$ ), utilizing a Skyscan 1172 system (Bruker, Billerica, MA), at a pixel size of 7.5  $\mu\text{m}$ , with a source voltage and current of 70 kV and 114  $\mu\text{A}$ , respectively. A 0.5 mm Al Filter was used to minimize the beam hardening from the polychromatic nature of the sealed X-ray source. Following scanning, three-dimensional (3D) microstructural images were reconstructed using SkyscanNRecon software, and structural indices of bone were computed using a marching-cubes algorithm in 3D. Volumes of interest were defined and structural indices calculated using Skyscan CT Analyzer software. The region of interest for trabecular micro-architectural variables started 0.375 mm below the growth plate of the femoral distal epiphysis and then extended 2.5 mm proximally towards the mid-diaphysis to measure trabecular bone volume (BV) per total volume (BV/TV), mean trabecular thickness (Tb.Th), mean trabecular number (Tb.N), and mean trabecular separation (Tb.Sp) indices. The volume

of interest for cortical micro-architectural variables started 3 mm below the growth plate of the femoral distal epiphysis and then extended 1.5 mm proximally towards the central diaphysis to measure mid-diaphyseal cortical thickness (Ct.Th), bone perimeter (B.Pm), and percent cortical porosity (Ct.Po) indices. 3D reconstructed images of the sagittal and axial planes of the femoral metaphysis and diaphysis were generated using SkyScan CTvox software.

### *2.2.2 Three-Point Bending*

Breaking strength of the right femur was measured under 3-point bending using a material testing machine (Bose, Eden Prairie, MN) fitted with a 1,000 N load cell. Wild-type and OA-Tg mice were sacrificed at 12 weeks of age and femurs were collected ( $n \geq 4/\text{group}$ ). Superficial muscles were dissected off and the femurs were placed in 1X PBS (Cellgro-Mediatech, Manassas, VA). Samples were preserved at  $-20^{\circ}\text{C}$  until testing. Prior to testing, the bones were thawed in saline at room temperature to ensure hydration. At the time of testing, femurs were individually positioned on the loading fixture anterior side down and loaded first in the anterior–posterior plane, and then loaded to failure at a rate of 0.05 mm/s, during which displacement and force were collected (100 Hz). Bending moments were calculated from the force (F) data ( $M=FL/4$ ; N mm), as previously described (Joshi et al., 2011). Displacement data were divided by  $L^2/12$  ( $\text{mm}/\text{mm}^2$ ), where L was the distance between the lower supports. Whole-bone mechanical properties were then determined from the moment versus normalized displacement curves, including peak moment (N mm, ultimate load the specimen

sustained) and stiffness ( $\text{N mm}^2$ , the slope of the initial linear portion of the moment–displacement curve).

### *2.2.3 Histological and Histomorphometric Analyses*

After sacrifice, femurs of 12-weeks-old WT and OA-Tg mice were dissected out ( $n \geq 4/\text{group}$ ), fixed in buffered 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), dehydrated, and embedded undecalcified in methacrylate. To measure dynamic bone formation parameters, calcein (i.p., 10 mg/kg body weight) had been previously injected at 7 and 2 days before euthanasia. Bones were sectioned into 3  $\mu\text{m}$  longitudinal sections, placed onto slides, and dried at 60°C overnight. Unstained plasticized longitudinal sections were used for the measurement of calcein fluorochrome label, using a Nikon microscope, digital camera, and image analysis system (Bioquant Osteo 2012, v12.1, Nashville, TN). Adjacent sections were deplasticized before staining with von Kossa and toluidine blue, or Masson's Trichrome staining. Static and dynamic histomorphometric analyses were then performed, as described previously (Abdelmagid et al., 2014).

### *2.2.4 Biochemical Analysis*

Sera were collected from 4-, 8-, and 12-week old WT and OA-Tg mice ( $n \geq 5/\text{group}$ ). ELISA was conducted using the manufacturer's protocol to determine serum levels of RANKL (receptor activator of nuclear factor kappa-B ligand) and OPG (osteoprotegerin) using Quantikine<sup>®</sup> ELISA (R&D Systems, Minneapolis, MN), and for levels of CTX (C-terminal telopeptide) using BioTang (Waltham, MA). The optical absorbance of the solution was measured at 450 nm using a micro-plate reader (iMark,

Bio-Rad). The concentration of proteins present in the sera was quantified from the slope standard curve estimation and normalized to  $\mu\text{g}$  protein per ml of serum assayed.

### *2.2.5 Primary Osteoblast and Bone Marrow-Derived Mesenchymal Stem Cell Isolation and Culture*

For calvarial primary osteoprogenitors, parietal calvaria were dissected out from WT and OA-Tg neonatal pups (approximately 3-6 days of age). Following dissection, calvaria pieces were digested using 0.1% Collagenase (Sigma) in 2.5% trypsin (Invitrogen) at 37°C. Cells released from subsequent digestions were plated in 100 mm dishes (Corning Life Sciences, Lowell, MA) at a density of  $5 \times 10^5$  cells/plate in Alpha Minimal Essential Medium ( $\alpha$ -MEM; Cellgro-Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; HyClone), hence called growth medium, and 1% penicillin/streptomycin solution (Invitrogen). The cells were incubated at 37°C with 5% CO<sub>2</sub> and culture media were changed every three days until they reached ~80% confluence. For mesenchymal stem cell (MSC) isolation, bone marrow was flushed out of the long bones of 8-week-old WT and OA-Tg mice under aseptic conditions, centrifuged and resuspended into growth media and plated in 100 mm dishes in an incubator at 37°C with 5% CO<sub>2</sub>. The next day, non-adherent cells were washed off the plates with 1X PBS. The adherent cells were maintained and the culture media were changed every three days. In order to differentiate osteoblasts from osteoprogenitors, cells were cultured in the growth medium containing 50  $\mu\text{g}/\text{ml}$  ascorbic acid (Sigma Aldrich) and 10 mM  $\beta$ -glycerophosphate (Sigma Aldrich), hence called osteogenic medium. In addition to

osteogenic media, stromal cell cultures were also supplemented with 0.1  $\mu\text{M}$  dexamethasone (Sigma; Australia Register Number: 16375; Melbourne, Victoria, Australia). Osteoblast cultures were maintained by changing the osteogenic medium every third day and stopped at specific time points according to the desired assay.

#### *2.2.6 Cell Proliferation*

To determine cell numbers, a CyQUANT<sup>®</sup> NF Cell Proliferation Assay Kit (Molecular Probes) was used according to the manufacturer's protocol. Briefly, WT and OA-Tg primary osteoblasts were plated at a density of  $4 \times 10^3$  cells/well in a 96-well plate (Falcon), with a minimum of six replicates per condition per independent experiment, in growth media and incubated at 37°C in the presence of 5% CO<sub>2</sub>. On day 3, the culture media was aspirated from all wells to be tested and replaced with DNA binding dye solution. Cells were incubated at 37°C for 1 hour and samples were measured using a Wallac 1420 fluorometer. Cell numbers were calculated based on a standard curve generated for primary osteoblasts.

#### *2.2.7 Alkaline Phosphatase Staining and Activity*

For alkaline phosphatase (ALP) staining, WT and OA-Tg osteoprogenitors or MSCs were plated at a density of  $5 \times 10^4$  cells/well in a 24-well plate (Corning) with osteogenic media ( $n \geq 4$  replicates/condition/experiment). On day 14, osteoblast cultures were stained for ALP using the Leukocyte Alkaline Phosphatase Kit (Sigma) according to the manufacturer's protocol. Briefly, wells were washed with 1X Hank's Balanced Salt Solution (HBSS) (Cellgro-Mediatech), fixed, rinsed twice with ddH<sub>2</sub>O, and incubated for

15 minutes at RT in a staining solution (sodium nitrate, FRV-Alkaline, and Naphthol AS-B Alkaline solutions). Following incubation, wells were rinsed with ddH<sub>2</sub>O and allowed to air dry. To examine the staining pattern and to capture images, a Nikon Eclipse TE300 inverted microscope interfaced with a Nikon Digital Sight DS Camera and NIS Elements F software was used. For ALP activity, osteoblast cultures at day 14 were evaluated using a LabAssay<sup>TM</sup> ALP kit (Wako, Richmond, VA). Briefly, wells were washed with 1X HBSS and lysed using assay buffer with 0.2% Triton X-100, and the ALP assay was carried out according to the manufacturer's protocol. Prior to beginning the assay, the following reagents were prepared: working assay solution and a dilution series of the standard buffer solution. Briefly, samples were incubated with the working solution (*p*-Nitrophenylphosphate Disodium 6.7 mmol/L) for 15 minutes at 37°C. After incubation, Stop Solution was added and the absorbance was measured at 405 nm using a microplate reader (Bio-Rad). The absorbance readings were used to generate the standard curve. ALP activity was calculated and normalized to the total µg protein/sample. Protein concentrations were quantified by BCA protein assay kit (Pierce).

#### *2.2.8 RNA Isolation and Quantitative Real-Time PCR*

Total RNA was isolated from cell cultures of WT and OA-Tg osteoblasts at days 7, 14, and 21 using TRIzol reagent (Invitrogen). RNA was purified using the RNeasy Mini Kit (Qiagen) and treated with RNase-Free DNase (Qiagen). RNA concentrations were measured using DU<sup>®</sup>640 spectrophotometry (Coulter, Jersey, NJ). RNA integrity of all samples was confirmed using 1% formaldehyde-agarose gels. For cDNA synthesis, 1 µg of cDNA was transcribed from total RNA using SuperScript<sup>®</sup> First-Strand Synthesis

kit (Invitrogen). Gene expression profile for alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx-2), collagen type I alpha 1 (Col1a1), transforming growth factor beta 1 (TGF- $\beta$ 1), transforming growth factor beta receptors I and II (TGF- $\beta$  RI, TGF- $\beta$  RII) was determined by quantitative real time PCR (qPCR) using SYBER Green Master Mix (Applied Biosystems, Foster City, CA). qPCR was performed using an ABI Model 7500 Real-Time PCR system (Applied Biosystems) in triplicate. Specific PCR primers were synthesized and optimized for amplification of each cDNA. Primers are listed in Table 2. The cycling parameters were as follows: an initial step of 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Gene expression analysis was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using delta delta C<sub>T</sub>. At least three independent experiments were performed for each gene expression.

#### *2.2.9 Osteogenesis Array*

Total RNA from WT and OA-Tg osteoblast cultures was isolated, purified, and quantified, as described above. cDNA was synthesized using RT<sup>2</sup> First Strand Kit (SABiosciences; Qiagen). Gene expression profiling was performed using RT<sup>2</sup> Profiler PCR Array; mouse osteogenesis (SABiosciences; Qiagen) and qRT-PCR was performed, as described (Abdelmagid et al., 2014).

#### *2.2.10 Protein Isolation and Western Blotting*

WT and OA-Tg cells were washed with 1X PBS and lysed in 1X RIPA (Millipore). Protein concentrations in the cell lysates were determined using a BCA Protein Assay Reagent Kit (Pierce, IL). Protein Samples (30 $\mu$ g) were mixed with

denaturing buffer, heated to 100°C, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Total proteins were transferred to nitrocellulose membrane (Bio-Rad) using semi-dry transfer apparatus (Bio-Rad). The membranes were blocked with 5% milk in Tris-buffered saline (TBS)-0.1% Tween-20 (TBST) for 1 h and then incubated with the following primary antibodies: custom made anti-chicken OA (concentration of 1:250), produced as previously described (Abdelmagid et al., 2007), and anti-rabbit actin (concentration of 1:1000; Sigma), overnight. Blots were washed with 1X TBS-0.1% Tween-20 (TBST) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies as appropriate (donkey anti-chicken or anti-rabbit IgG, Jackson Immunoresearch), in a blocking buffer. Blots were washed with TBST, incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and exposed to X-ray film, which was developed using Konica SRX-101A Medical Film Processor (Taiwan). Quantification of the immunoblots was performed using Image J software (version 1.49n, NIH Image), and results of OA were normalized to  $\beta$ -actin.

### *2.2.11 Immunocytochemistry*

Primary osteoblasts from WT and OA-Tg mice were plated in 2-well chamber slides (Lab-Tek, Naperville, IL) at a density of 3,000 cells per well. The next day, cells were washed with 1X PBS, fixed with 4% PFA, washed with (1X PBS-0.05% Tween). Cells were permeabilized by 1X PBS-0.1% Triton-X100, rinsed twice with wash buffer, blocked with (1X PBS-2.5% BSA), and incubated with anti-chicken OA primary antibody (concentration of 1:250) at 4°C overnight. The next day, chambers were washed

with wash buffer and incubated with the appropriate secondary antibody (concentration of 1:500; Jackson ImmunoResearch) for 1hr. Cells were then rinsed with wash buffer, mounted with DAPI-Vectashield (Vector Laboratories, CA) and coverslipped. Slides were examined using confocal microscope. Captured images were merged using Image J software (version 1.49n, NIH Image).

#### 2.2.12 Statistical Analysis

The statistical differences between WT and OA transgenic groups for the *in vivo* and *in vitro* data were determined using unpaired, two-tailed Student's t-tests. One-way analysis of variance (ANOVA) with Bonferroni post-hoc tests were used for multiple comparisons and adjusted *p*-values are reported. All statistical analyses were generated using GraphPad Prism Software version 4 (San Diego, CA). The differences were considered statistically significant if the *p*-value < 0.05. All data are represented as mean plus standard error of the mean (SEM). All *in vitro* data are representative of at least three independent experiments.

Table 2-1. qPCR primers

Gene Symbol	Primer Sequence (5'-3') Forward, Reverse	Product size: bp	Accession number
GAPDH	ATCTTGGGCTACACTGAGGA CAGGAAATGAGCTTGACAAAGT	122	NM_008084
ALP	TCCTGACCAAAAACCTCAAAGG TGCTTCATGCAGAGCCTGC	101	NM_007431
Runx-2	CCGTGGCCTTCAAGGTTGT TTCATAACAGCGGAGGCATTT	118	NM_001146038
Col1a1	TGGCAAAGACGGACTCAAC GGCAGGAAGCTGAAGTCATAA	156	NM_007742
TGF- $\beta$ 1	GCTAATGGTGGACCGCAACAACG CTTGCTGTACTGTGTGTCCAGGC	682	NM_011577
TGF $\beta$ RI	AGTGGTCTTGCCCATCTTC GGCAATAGCTGGTTTTCTT	60	NM_009370
TGF $\beta$ RII	AGATGGCTCGCTGAACACTACCAA AGAATCCTGCTGCCTCTGGTCTTT	100	NM_009371

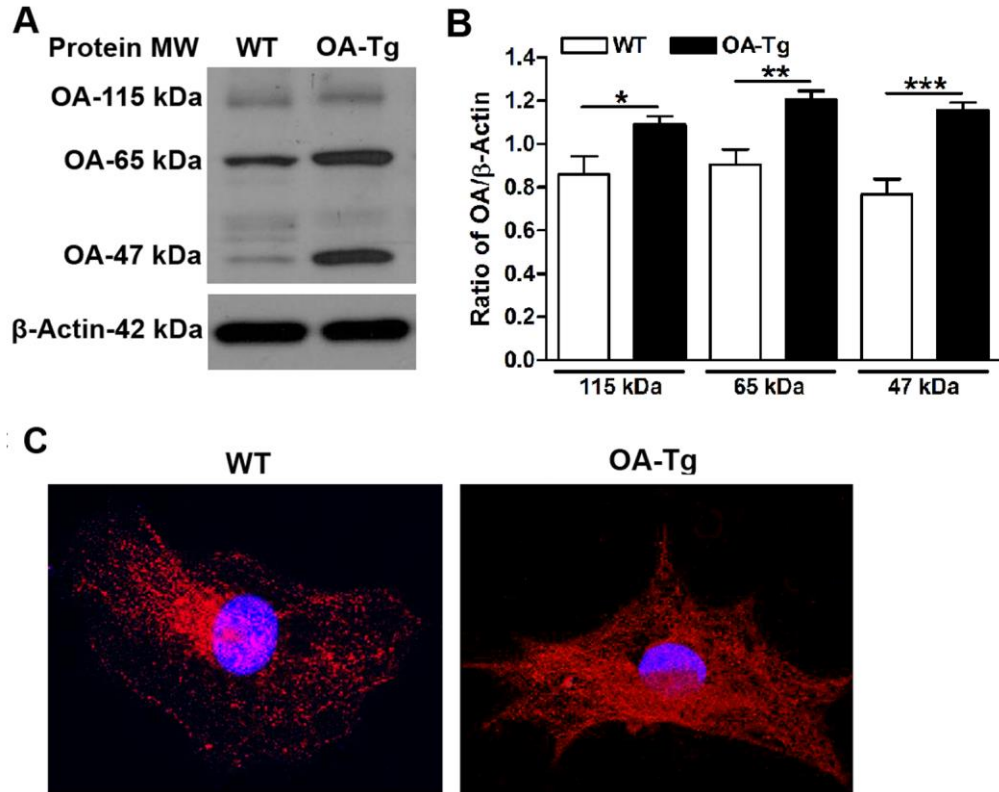
GAPDH: glyceraldehyde-3-phosphate dehydrogenase; ALP: alkaline phosphatase; Runx-2: runt-related transcription factor 2; Col1a1: collagen type 1 alpha 1; TGF- $\beta$ 1: transforming growth factor beta 1; TGF $\beta$  RI: transforming growth factor beta receptor I; TGF $\beta$  RII: transforming growth factor beta receptor II.

## 2.3 Results

### 2.3.1 Generation of osteoactivin/gpnmb transgenic (OA-Tg) mice

Our group previously showed that OA/gpnmb is a principal bone anabolic factor (Abdelmagid et al., 2008; Abdelmagid et al., 2014; Safadi et al., 2001). To confirm the importance of OA in osteogenesis, osteoactivin/gpnmb transgenic mice (OA-Tg) were generated (under the control of CMV promoter). The targeted over-expression vector was confirmed for the presence of OA in MC3T3-E1 osteoblast-like cells (data not shown). Our results demonstrating that OA is over-expressed > 3 fold when compared to control untransfected control cells (data not shown). The targeted over-expression vector was used to generate the OA-Tg transgenic mice. To examine expression of the OA-transgene, multiple tissues were used to examine OA-transgene mRNA using PCR. The OA-transgene expression was confirmed using Northern blot analysis. Transgene expression was markedly significant in all tissues examined (data not shown).

To confirm that OA-Tg mice over-expressed OA/gpnmb in bone, the cellular OA/gpnmb protein levels were measured via Western blot in osteoblast cultures derived either from neonatal calvaria of OA-Tg and WT or from bone marrow of 8-week-old OA-Tg mice and WT littermates. Fig. 2-1A and B show that the different molecular weights (115, 65, and 47 kDa) of the OA protein were increased in OA-Tg osteoblasts, compared to WT. OA/gpnmb overexpression in OA-Tg versus WT mice was also confirmed via immunofluorescent staining in primary osteoblasts cultured for 24 hours, then fixed and stained with the anti-OA/gpnmb antibody (red signal) (Fig. 2-1C).



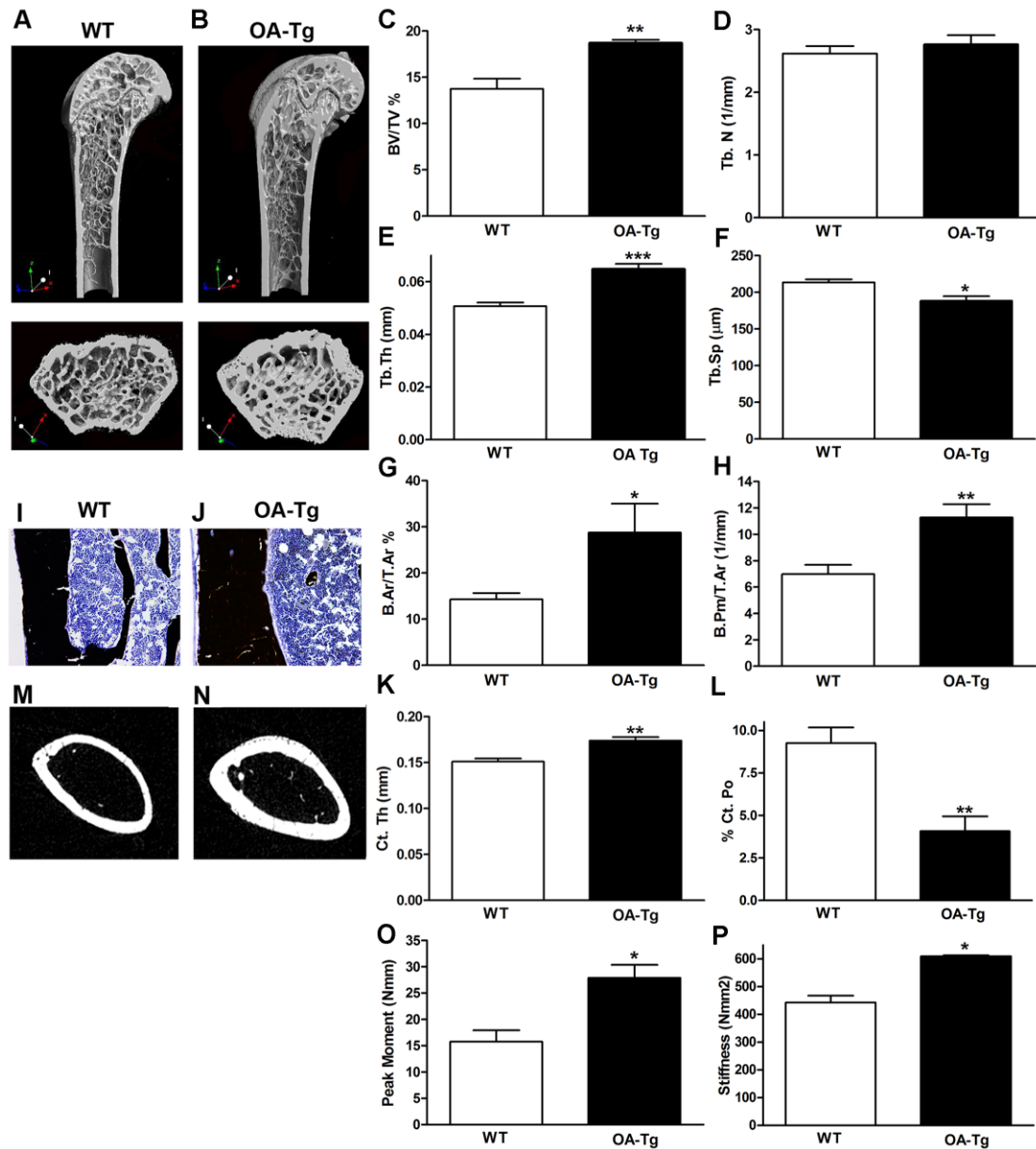
**Figure 2-1. Over-expression of OA/gpnmb in osteoblasts from transgenic mice.** (A) Representative Western blot of OA/gpnmb protein level in primary osteoblasts from OA/gpnmb transgenic (OA-Tg) mice and wild-type (WT) littermates. (B) Densitometric analysis of the relative density of a three OA protein bands (115, 65 and 47 kDa). OA/gpnmb expression levels were normalized to  $\beta$ -actin. (C) Immunofluorescent staining of OA/gpnmb (red) and nuclei (DAPI stained blue) in WT and OA-Tg primary osteoblasts using anti-OA/gpnmb antibody. Images were obtained using a confocal microscope with a 60 x objective. Experiments were repeated three times with similar results. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , compared to WT mice.

### 2.3.2 Over-expression of osteoactivin enhances bone mass *in vivo*

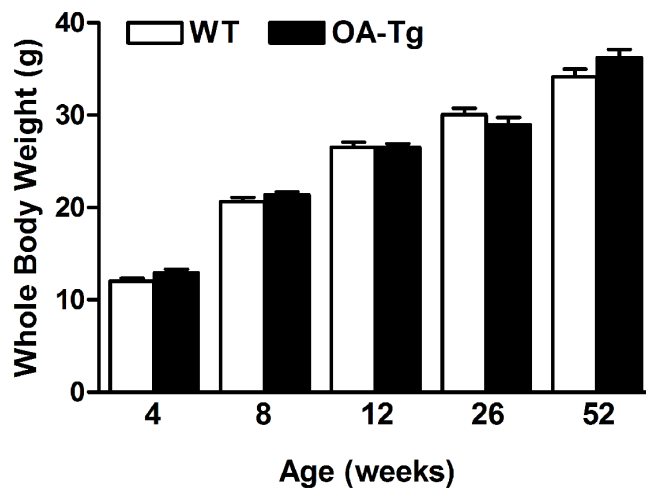
Given that OA/gpnb expression is increased in bones in a rat model of osteopetrosis (Safadi et al., 2001), we sought to evaluate the effect of systemic OA over-expression in bones *in vivo*. Femurs harvested from WT and OA-Tg mice that were 4- and 12-weeks of age as well as 12-months of age, of both genders, were scanned using micro-CT, and the distal metaphyseal region was analyzed for trabecular bone parameters. All data are listed in Table 1, while data from 12-week old males is shown in Figure 2-2. Cross-sectional and longitudinal sections from distal metaphyseal regions of OA-Tg femurs showed increased bone, compared to WT mice (Fig. 2-2A and B). Specifically, we observed increased trabecular bone volume (BV/TV) and trabecular thickness (Tb. Th) in OA-Tg femurs, compared to WT femurs (Fig. 2-2C and E). In contrast, there was a reciprocal reduction in trabecular separation (Tb. Sp) in OA-Tg mice, compared to WT littermates (Fig. 2-2F). The increase in trabecular bone in OA-Tg mice was also demonstrated using histomorphometry, which showed increased trabecular bone area as a percentage of total bone area (B.Ar/T.Ar %), and increased trabecular bone perimeter (B.Pm/T.Ar) (Fig. 2-2G and H).

To examine the effect of OA/gpnb over-expression on cortical bone, histological sections stained with von Kossa were examined, and showed an increase in cortical bone thickness in 12 week OA-Tg mice, compared to WT (Fig. 2-2I and J). This was confirmed using micro-CT in which the central diaphyses were scanned and analyzed for cortical bone parameters (See Table 1 for all data and Figure 2-2 for 12-week old male mice data). We observed an increase in cortical thickness (Ct.Th) and a

significant decrease in total % cortical bone porosity in OA-Tg femurs, compared to WT (Fig. 2-2K and L). The 2D cross-section images of the mid diaphyseal region of OA-Tg femurs showed thicker cortical bone, compared to WT (Fig. 2-2M and N). Biomechanical testing showed a significant increase in the three-point bending strength of femurs from OA-Tg mice, compared to WT mice (Fig. 2-2O and P).



**Figure 2-2. OA Over-expression increases trabecular and cortical bone mass *in vivo*.** Femurs harvested from 12 week-old WT and OA-Tg mice (n = 6/group) were scanned using a Skyscan 1172 system. For trabecular and cortical micro-CT analyses, the metaphysis of distal femur and mid-diaphysis regions were analyzed, respectively. (A, B) 3D micro-CT-reconstructed images, in the sagittal and transaxial planes, of metaphyseal regions of WT and OA-Tg distal femurs. (C-F) Micro-CT analysis results of trabecular metaphysis: BV/TV, Tb.N, Tb.Th and Tb.Sp. (G, H) Micro-CT analysis results of cortical diaphyseal areas and perimeter: B.Ar/T.Ar and B.Pm/T.Ar. (I, J) Von Kossa stained sections showing mineralized bone in WT and OA-Tg femurs, images taken using a 20X objective. (K, L) Micro-CT analysis results of diaphyseal Ct.Th and Ct.Po. (M, N) Micro-CT reconstructed images, in the axial plane, of mid-diaphyseal region of WT and OA-Tg femurs. (O, P) Biomechanical testing results of failure moment and stiffness of 12 week-old WT and OA-Tg femurs (n = 3/group). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , compared to WT mice.



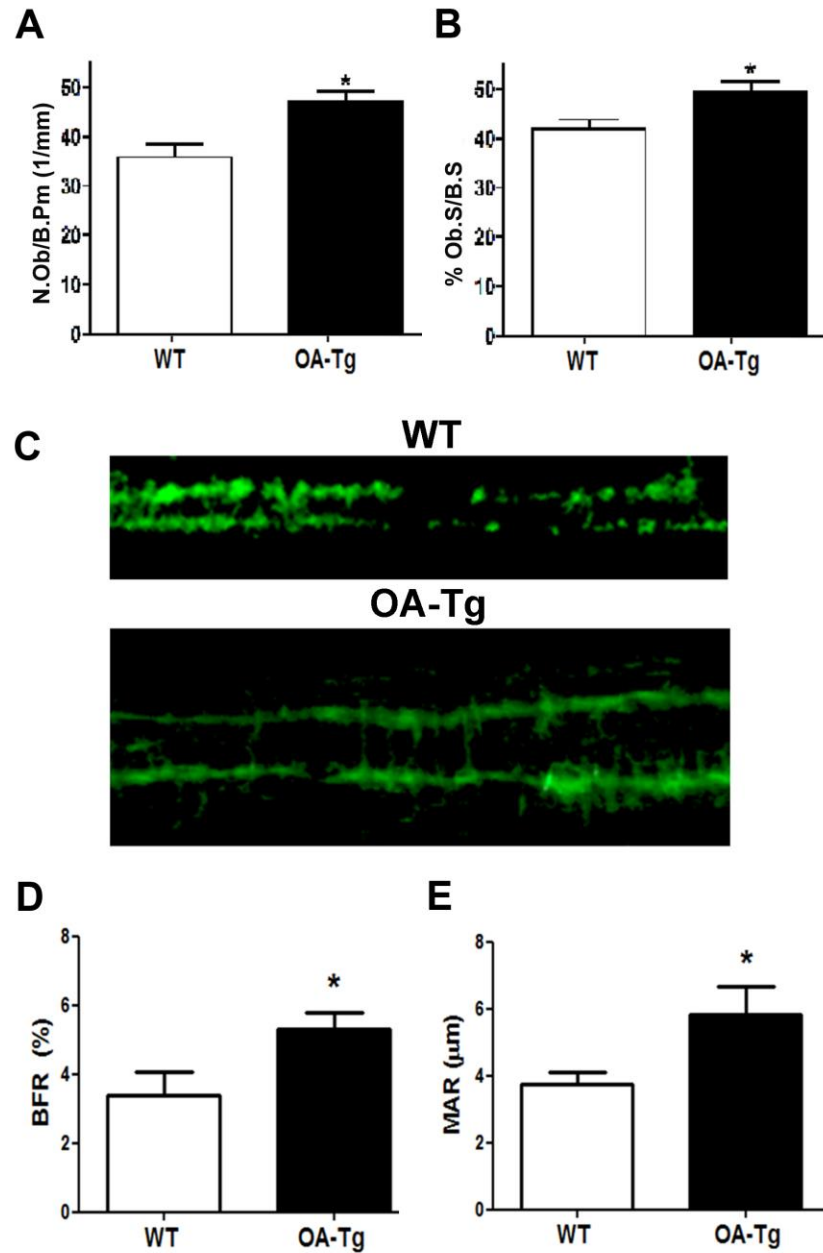
**Figure 2-3. Whole body weights across weeks.** The body weight of OA-Tg mice and corresponding WT littermates in grams (g) across 4-52 weeks of age. n  $\geq$  5.

<b><math>\mu</math>-CT Parameters</b>	<b>Genotype</b>	<b>4-week</b>		<b>12-week</b>		<b>12-month</b>	
		<b>Male</b>	<b>Female</b>	<b>Male</b>	<b>Female</b>	<b>Male</b>	<b>Female</b>
<b>BV/TV (%)</b>	<b>WT</b>	5.22±0.26	4.11±0.27	13.76±1.08	6.23±0.64	4.14±0.31	3.78±0.25
	<b>OA-Tg</b>	8.57±0.68***	9.02±0.83***	18.70±0.36**	6.30±0.53	5.90±0.40*	4.22±0.30**
<b>Tb.Th (mm)</b>	<b>WT</b>	0.04±0.00	0.04±0.00	0.05±0.00	0.04±0.001	0.04±0.002	0.05±0.005
	<b>OA-Tg</b>	0.04±0.00	0.04±0.00	0.06±0.00***	0.05±0.001	0.06±0.002*	0.06±0.003
<b>Tb.N (1/mm)</b>	<b>WT</b>	1.31±0.05	1.07±0.08	2.62±0.12	1.10±0.14	0.82±0.05	0.13±0.03
	<b>OA-Tg</b>	2.07±0.24**	2.25±0.17***	3.00±0.17	1.54±0.07*	0.96±0.07	0.70±0.05***
<b>Tb.Sp (mm)</b>	<b>WT</b>	0.50±0.01	0.53±0.01	0.21±0.00	0.25±0.01	0.30±0.005	0.62±0.04
	<b>OA-Tg</b>	0.36±0.03***	0.28±0.03***	0.18±0.00*	0.26±0.006	0.32±0.01*	0.36±0.03***
<b>Co.Pm (mm)</b>	<b>WT</b>	3.45±0.09	3.40±0.05	4.52±0.15	4.32±0.08	4.51±0.17	4.35±0.10
	<b>OA-Tg</b>	4.17±0.05***	9.65±1.04***	5.40±0.17*	4.33±0.14	5.76±0.16**	5.20±0.17**
<b>Ct.Th (mm)</b>	<b>WT</b>	0.08±0.00	0.08±0.00	0.15±0.00	0.15±0.00	0.14±0.00	0.16±0.00
	<b>OA-Tg</b>	0.09±0.00*	0.10±0.01	0.17±0.00**	0.15±0.00	0.17±0.00*	0.18±0.00*
<b>Po.N (1/mm)</b>	<b>WT</b>	1.81±0.16	1.75±0.11	9.25±0.91	2.85±0.32	1.57±0.24	1.46±0.15
	<b>OA-Tg</b>	3.02±0.53	2.94±0.49*	3.21±0.09**	1.18±0.04**	1.41±0.17	1.41±0.17

Table 2-2. Micro-CT analysis of long bone microstructure in wild type (WT) and osteoactivin transgenic (OA-Tg) femora. Values are shown as mean ± SEM; \* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001.

### **2.3.3 Over-expression of osteoactivin/gpnmnb enhances bone formation in vivo**

To determine if the higher bone mass phenotype in OA-Tg mice was due to an increase in bone formation, plastic embedded bone sections underwent Masson's Trichrome staining and histomorphometric analysis and showed an increase in osteoblast numbers per bone perimeter (N.Ob/B.Pm) and percent osteoblast surface per bone surface (% Ob.S/B.S) (Fig. 2-4A and B). Examination of calcein double-labeling in unstained plastic embedded bone sections showed increased width between labeled surfaces in OA-Tg mice, compared to WT mice (Fig. 2-4C). Dynamic histomorphometry of the calcein labeling in distal femoral trabeculae showed increased bone formation and mineral apposition rates (BFR and MAR) in femurs of OA-Tg mice, compared to WT (Fig. 2-4D and E), indicative of increased bone mineralization in OA-Tg mice.

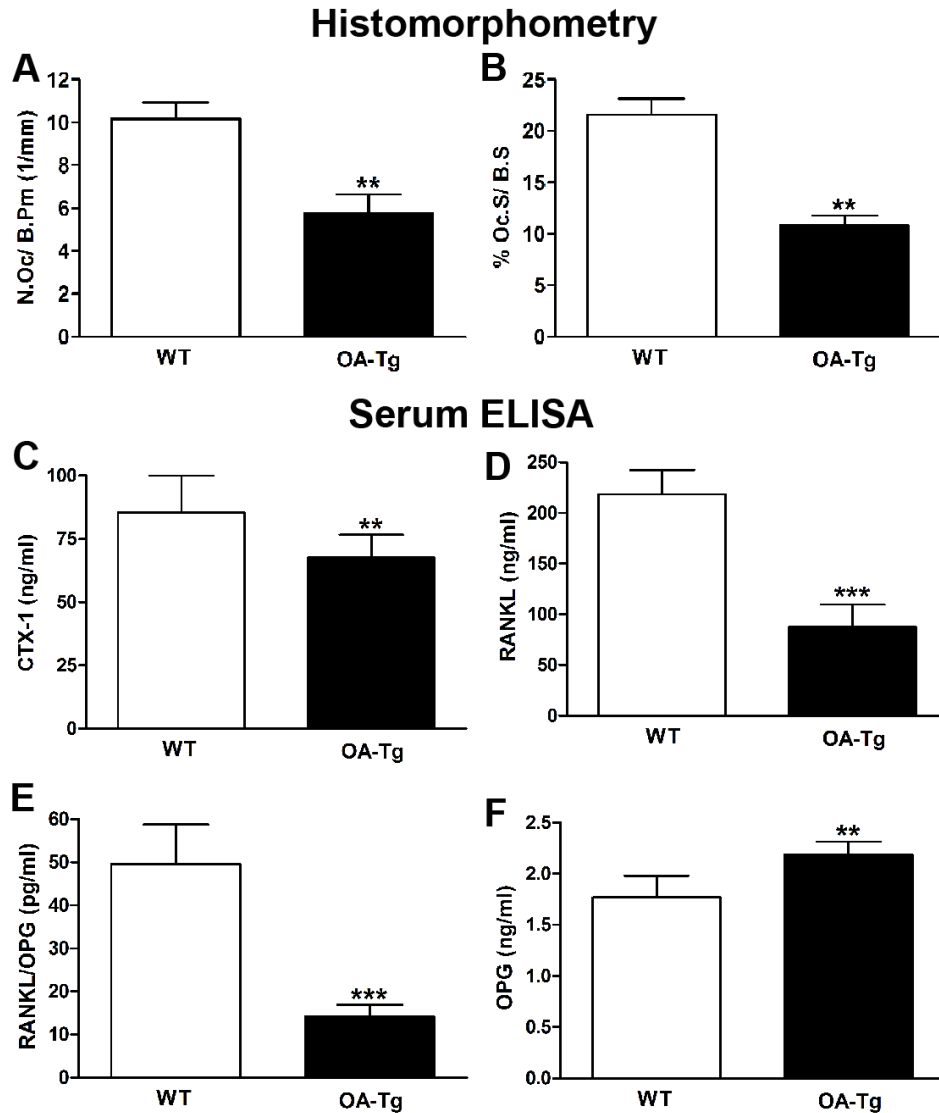


**Figure 2-4. Bone formation is enhanced in OA-Tg mice.** (A, B) Histomorphometric results of osteoblast number lining bone surfaces: N.Ob/ B.Pm and % Ob.S/ B.S ( $n \geq 4$ /group). (C) Calcein labeling showing periosteal mineralized bone width. (D, E) Dynamic histomorphometric results for BFR and MAR. \* $p < 0.05$ , compared to WT mice.

### **2.3.4 Defective bone remodeling in OA-Tg mice**

To determine if the higher bone mass phenotype in OA-Tg mice was due to a decrease of bone resorption, histomorphometric analysis for numbers of osteoclasts lining the trabecular bone surface was performed. We observed a significant decrease in osteoclast numbers per bone perimeter (N.Oc/B.Pm) and the percent of osteoclast surface per bone surface (% Oc.S/B.S) (Fig. 2-5A and B). To confirm that the high bone mass phenotype in OA-Tg mice was due to, at least in part, a generalized decrease in bone resorption, serum was analyzed for levels of C-terminal telopeptide (CTX-1), receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegrin (OPG) using ELISA (Fig. 2-5C-F). Serum levels of CTX-1, a biomarker of osteoclast activity, were reduced in OA-Tg mice, compared to WT (Fig. 2-5C), as were serum levels of RANKL, a key factor for osteoclast differentiation and activation, (Fig. 2-5D). We also evaluated serum levels of OPG, another essential molecule that is synthesized by osteoblasts and plays a regulatory role in the inhibition of osteoclast differentiation, and found that the ratio of RANKL/OPG was significantly reduced in sera of OA-Tg mice, compared to WT (Fig. 2-5E). However, OPG levels were relatively higher in serum samples from OA-Tg mice, compared to WT littermates, likely due to the increased number of osteoblasts, the source of OPG (Fig. 2-5F).

Together, overexpression of OA/gpnmB lead to increased osteoblast numbers and function (Fig. 2-4), yet decreased osteoclastic activity (Fig. 2-5).



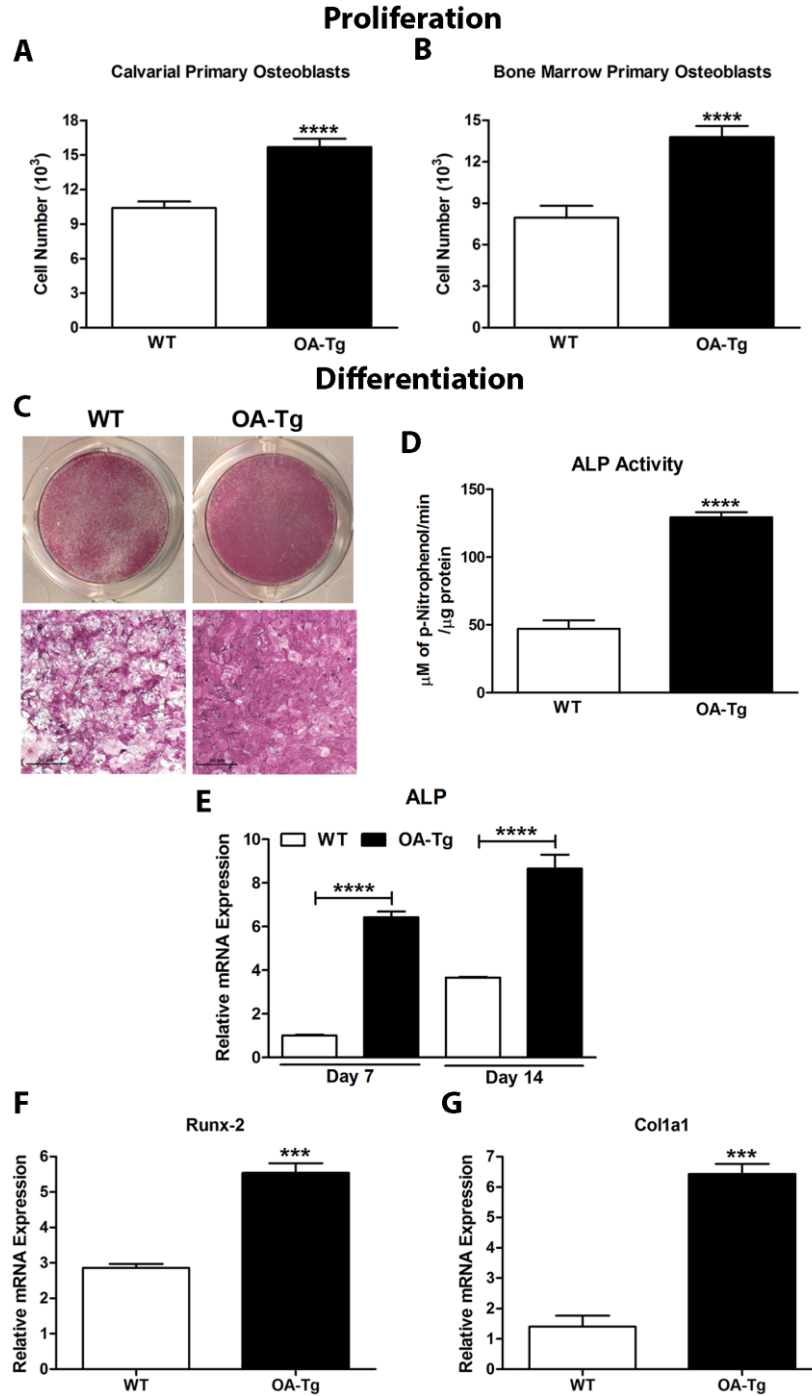
**Figure 2-5. Defective osteoclast-mediated bone resorption in OA-Tg mice.** (A, B) Histomorphometry results for osteoclast numbers lining bone surfaces: N.Oc/ B.Pm and % Oc.S/ B.S. (C-F) Serum analysis of bone resorption markers using ELISA: CTX-1, RANKL, RANKL/OPG ratio, and OPG in 8 week WT and OA-Tg mice ( $n \geq 5$ /group). \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , compared to WT mice.

### **2.3.5 Over-expression of osteoactivin stimulates osteoprogenitor proliferation and differentiation in vitro**

Previous studies have demonstrated the temporal expression of OA/gpnmB in osteoblast development *in vitro* (Abdelmagid et al., 2008; Abdelmagid et al., 2014; Safadi et al., 2001). To determine if OA/gpnmB over-expression affects osteoblast number *ex vivo*, WT and OA-Tg primary osteoblast cultures were terminated at day 3 after plating to evaluate their proliferation ability. The number of osteoblasts in 3-day OA-Tg calvarial and bone marrow-derived MSC cultures was significantly higher than in 3-day WT osteoblast cultures (Fig. 2-6A and B).

It has been reported that OA/gpnmB over-expression in osteoprogenitors *ex vivo* stimulates their differentiation and function (Abdelmagid et al., 2008). To investigate the effect of OA/gpnmB over-expression on osteoprogenitor differentiation in our transgenic mouse model, primary osteoblasts from WT and OA-Tg mice were cultured for 7 or 14 days. A subset of cultures was terminated on day 14 and evaluated for the staining and activity of alkaline phosphatase (ALP), a marker of early osteoblast differentiation. We found that both ALP staining (Fig. 2-6C) and activity (Fig. 2-6D) were significantly increased in OA-Tg primary osteoblasts, compared to WT. In addition, an increase in ALP mRNA expression was detected in OA-Tg osteoblasts at days 7 and 14, compared to WT osteoblasts (Fig. 2-6E). In culture terminated on day 7, we further evaluated mRNA levels of runt-related transcription factor 2 (Runx-2), a crucial factor for early osteoblast differentiation, and collagen type 1 alpha 1 (Col1a1), an important protein for osteoblast

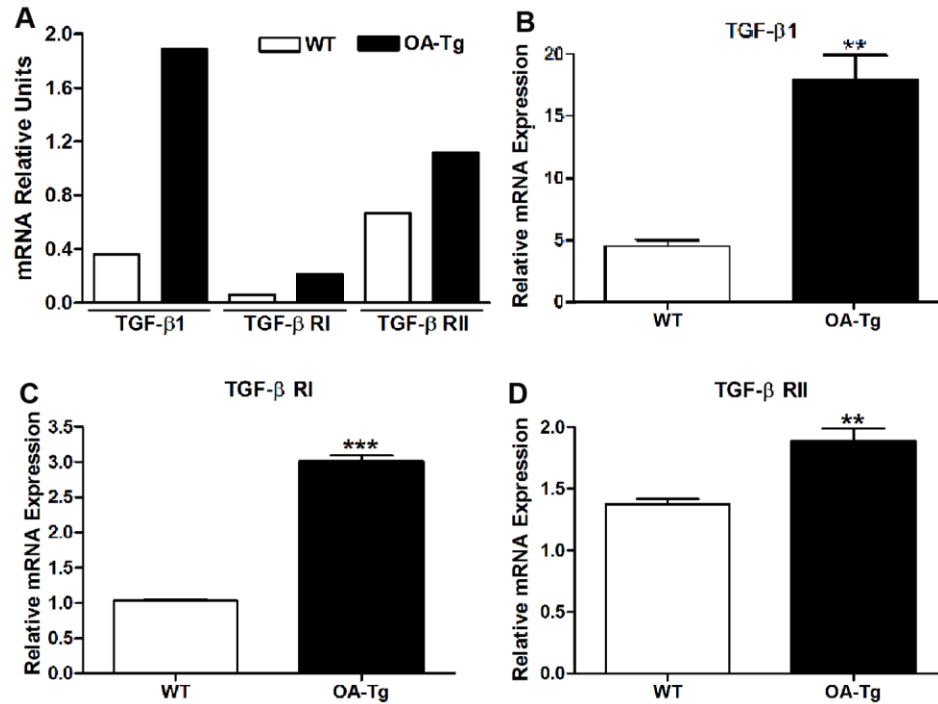
maturation phase. We found that Runx-2 and Col1a1 mRNA expressions were also increased in 7-day OA-Tg osteoblast cultures, compared to WT (Fig. 2-6F and G).



**Figure 2-6. Over-expression of OA stimulates osteoprogenitor proliferation and differentiation *in vitro*.** WT and OA-Tg primary osteoblast cultures were terminated at day three and assayed for proliferation. (A, B) Cell proliferation was measured using CyQUANT NF dye reagent. Cell numbers were based on fluorescence at 520 nm ( $n \geq 6$ /group). WT and OA-Tg primary osteoblast cultures were terminated at day 14. (C) Macro- and microscopic images of ALP staining were evaluated using ALP staining kit and an inverted microscope. (D) ALP activity quantification using ALP substrate. After the incubation period, samples were read on a plate reader at 405 nm. Activity was normalized to the total protein. (E) ALP mRNA gene expression at days 7 and 14. Values were normalized to GAPDH.  $n \geq 4$ . (F, G) Runx-2 and Col1a1 mRNA gene expression at day 7. Values were normalized to GAPDH.  $n \geq 4$ . \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ , compared to WT mice.

### 2.3.6 Over-expression of OA enhances TGF- $\beta$ 1 expression in osteoblasts

Since OA-Tg osteoblasts displayed enhanced differentiation, we utilized an osteogenesis array on osteoblast cultures terminated on day 7 to screen for differentially expressed genes. We identified that transforming growth factor beta 1 (TGF- $\beta$ 1), and transforming growth factor beta receptors I and II (TGF- $\beta$  RI and TGF- $\beta$  RII) were up-regulated in 7-day OA-Tg osteoblast cultures, compared to WT (Fig. 2-7A). Similarly, gene expression analysis, using qRT-PCR, showed increased TGF- $\beta$ 1 and TGF- $\beta$  receptors I and II in 7-day cultures of OA-Tg osteoblasts, compared to WT (Fig. 2-7B-D). Collectively, these data suggested that TGF- $\beta$ 1 and its receptors were enhanced in OA-Tg osteoblasts, which suggests, at least in part, a regulatory effect of OA on the early stages of osteoblast differentiation.



**Figure 2-7. Over-expression of OA enhances TGF-β1 expression in osteoblasts.** (A) Osteogenesis array of TGF-β1, TGF-β receptors I and II (TGF-β R.I and TGF-β R.II) in 7-day osteoblast cultures from WT and OA-Tg mice. (B-D) q-PCR analysis of (B) TGF-β1, (C) TGF-β R.I, and (D) TGF-β R.II in 7-day osteoblast cultures from WT and OA-Tg mice.  $n \geq 3$ . \*\* $p < 0.01$  and \*\*\* $p < 0.001$ , compared to WT mice.

## 2.4 Discussion

Previous studies reported on the anabolic role of osteoactivin/gpnmB in bone formation and matrix mineralization using gain-of-function and loss-of-function approaches (Abdelmagid et al., 2010; Abdelmagid et al., 2008; Safadi et al., 2001). In this study, we sought to examine the function of OA/gpnmB as an osteoinductive agent by overexpressing OA/gpnmB under the CMV-promoter in transgenic mice (OA-Tg). Wild type (WT) mice showed a physiological increase in bone mass at 4 to 12 weeks of age. Yet, overexpression of OA resulted in an exponential increase in trabecular bone mass in OA-Tg mice, compared to WT mice, evident by micro-CT and histomorphometric analyses. Additionally, dynamic bone parameters indicated a substantial increase of bone formation and mineral apposition rates in OA-Tg mice, compared to WT. Further investigations were carried out to identify the mechanism(s) of increased bone volume in the OA-Tg mice. Our data showed that osteoblast numbers were increased, while osteoclast numbers were decreased in OA-Tg mice, compared to WT. Moreover, osteoclast activity markers were significantly lower in OA-Tg than WT mice. Taken together, these results demonstrate an uncoupled bone remodeling process in OA-Tg mice, with net increases in bone formation. These findings were supported by the *in vitro* data showing a significant increase in osteoprogenitor differentiation, marked by increased ALP staining, activity and mRNA expression in OA-Tg mice, compared to WT. The increased differentiation in OA-Tg osteoblast cultures may be linked to the increased levels of TGF- $\beta$ 1 in OA-Tg osteoblasts, compared to WT.

The continued increase in bone mass of WT mice from 4 to 12 weeks of age can be described as normal bone growth. Physiological increases in trabecular bone volume is known to peak at 3 months of age, while periosteal apposition continues longer in the tibiae and femurs (Glatt et al., 2007). In contrast, overexpression of osteoactivin/gpnmB in the OA-Tg mice resulted in significant increases in bone mass in this same time period, in both distal femoral trabecular and cortical regions, based on micro-CT outcomes. The increased bone mass in OA-Tg mice was evident as increased thickness of trabeculae, increased cortical thickness and perimeter, reduced cortical bone porosity, and increased biomechanical bone strength and stiffness. These findings support those from a previous study (Safadi et al., 2001) in which high levels of OA/gpnmB expression were observed in bones of osteopetrotic mutant rats in association with increased bone mass in those animals, compared to control rats.

On the cellular level, the increased bone mass in the OA-Tg mice was due to increased osteoblast numbers and activity, as indicated by increased bone formation and mineral apposition rates. In addition, the decreased osteoclast numbers and activity likely contributed to the increased bone mass in OA-Tg mice as indicated by histomorphometry, and decreased serum levels of CTX-1 and RANKL/OPG ratio. Thus, both osteoblast and osteoclast numbers and activity were altered by OA/gpnmB overexpression in the OA-Tg mice. Our previous published data showed that intramedullary injection of recombinant OA/gpnmB (rOA/gpnmB) stimulated the formation of new woven bone within the marrow cavity with multiple tandems of osteoblasts covering the new trabeculae, compared to control-injected mice (Singh et al., 2010).

Another important observation in this study is that the *ex vivo* characterization of osteoprogenitors demonstrated increased proliferation, and earlier and enhanced differentiation into osteoblasts in OA-Tg mice, as marked by ALP expression and activity, for example. These observations confirm our previous published findings that OA/gpnmB over-expression in osteoblasts *ex vivo* stimulated their differentiation (Abdelmagid et al., 2008). We have also shown that OA increases osteoblast markers and decreases myoblast markers *in vitro* (Sondag et al., 2014). These data combined suggest that OA/gpnmB promotes osteoprogenitor differentiation primarily through up-regulation of osteoblast differentiation markers.

We also observed increased Runx-2 mRNA expression in OA-Tg osteoblast cultures, compared to WT. Our results are in accordance with a prior study reporting that Runx-2 positively regulates early osteoblast differentiation in Runx-2 transgenic mice (Geoffroy et al., 2002). Several gene transfer studies with Runx2 overexpressing cells have supported its capacity to induce osteogenesis *ex vivo* and *in vivo* (Byers and Garcia, 2004; Zhao et al., 2005; Zheng et al., 2004). Runx2 overexpression in the osteoblast-like cell line UMR-106 leads to the induction of osteoblast ECM protein expression and mineralized nodule formation (Geoffroy et al., 2002). In another study, overexpression of Runx-2 by retroviral infection, induced osteoblast-related gene expression levels, such as mRNA levels of ALP, Col I and BSP in murine fibroblast cell line (NIH3T3) cells and in dental follicle cells (DFC) (Pan et al., 2009). *In vitro* experiments demonstrate that Runx-2 transfection induce ALP activity in multipotential mesenchymal cells, C3H10T1/2 and C2C12, indicating an important role for Runx-2 in the induction of ALP activity (Harada et al., 1999; Lee et al., 2000). Forced expression of Runx-2 via transfection in

nonosteoblastic cells, such as primary fibroblasts, induced the expression of the principal osteoblast specific genes (Ducy et al., 1997).

Several studies have implicated a growing number of cytokines involved in the regulation of bone formation and turnover (Eingartner et al., 1999; Sato et al., 1999; Sato et al., 1998; Tavakoli et al., 1999). The actions of the transforming growth factor- $\beta$ s (TGF- $\beta$ 1, -  $\beta$ 2, -  $\beta$ 3) have been characterized (Linkhart et al., 1996) and studies show that TGF- $\beta$ s have an essential role in the regulation of bone formation (Bouletreau et al., 2002). Most publications report that TGF- $\beta$ s enhance bone formation *in vivo* (Beck et al., 1993; Beck et al., 1991b; Joyce et al., 1989; Marcelli et al., 1990; Noda and Camilliere, 1989). For example, TGF- $\beta$ 1 augments bone healing in endochondral critical size defect models and significantly enhances the biomechanical properties of bone (Lind et al., 1993; Peterson et al., 1997). Beck and colleagues suggested that *in vivo* injections of TGF- $\beta$  stimulate osteoblasts to proliferate and increase in number, which in turn, lead to new bone formation (Beck et al., 1991a). Biological effects of stimulating growth and cell numbers suggest that functional TGF- $\beta$  cell surface receptors are present on primary osteoblast-enriched cultures derived from adult human trabecular bone (Kells et al., 1992). These reports, combined with our observation of increased TGF- $\beta$  in primary osteoblasts overexpressing OA, might explain the increased bone formation in OA-Tg mice *in vivo*. With regard to our other findings, it has been reported that TGF- $\beta$ s increase the number of osteoprogenitor cells and promote matrix production (Centrella et al., 1987; Farhadieh et al., 1999; Hock et al., 1990). TGF- $\beta$ 1 reduces the ability of osteoblasts to secrete RANKL, thereby decreasing osteoclast formation (Chen et al., 2012). TGF- $\beta$  induces Runx2 expression in the C2C12 myogenic cell line (Lee et al., 2003), and

stimulates mesenchymal cells to increase ALP production (Long et al., 1990). Lastly, TGF- $\beta$  stimulates synthesis of type I collagen, thus, it enhances the premature osteoblast cell pool and matrix protein synthesis (Centrella et al., 1986; Centrella et al., 1987; Robey et al., 1987).

## **2.5 Conclusion**

Our findings support a role for OA/gpnmB as an osteoinductive agent, with increased bone mass and strength, as well as increased bone formation rates in OA-Tg mice, compared to WT. Several mechanisms underlying the increased bone volume in OA-Tg mice were identified, including increased numbers, differentiation and activity of osteoblasts as well as decreased numbers and activity of osteoclasts. A number of studies have shown promising results of bone loss reversal through the stimulation of bone-forming osteoblasts. A full understanding of osteoactivin's effect on bone and its promotion of bone formation will be helpful in developing new therapeutic strategies to selectively enhance bone formation in patients with clinically significant bone loss.

## CHAPTER 3

### OSTEOACTIVIN, MATRIX METALLOPROTEINASE, AND HEAT SHOCK PROTEIN 72 INCREASE WITH RESOLUTION OF INFLAMMATION IN MUSCULOTENDINOUS TISSUES IN A RAT MODEL OF REPETITIVE REACHING AND GRASPING

This Work Submitted to *BMC Musculoskeletal Disorders*, April of 2015

#### 3.1 Background

Overuse injuries are now considered a leading cause of long-term pain and physical disability world-wide (Horton, 2012), with diagnoses including tendinopathies and muscle disorders (Piligian et al., 2000; van Rijn et al., 2009). Studies have identified repetition and duration as two of the key risk factors for upper extremity overuse injuries (Gallagher and Heberger, 2013). Moreover, overuse injuries commonly occur as a result of prolonged repetitive loading of the muscle-tendon unit, even at low force levels (Barr and Barbe, 2002; Kannus et al., 1997).

Several studies have shown that repetitive movements lead to tissue injury ((Cutlip et al., 2014) and reviewed in (Barbe and Barr, 2006)). Barbe and Barr have developed an operant rat model of upper extremity overuse in which rats learn to perform repetitive tasks, such as a high repetition negligible force (HRNF) food retrieval task. In this particular task, rats reach at a rate of 4 reaches/minute into a portal to retrieve a 45-mg pellet of food for 2 hours/day (in four 30 min sessions/day), 3 days/week. Performance of this HRNF task leads to modest signs of myositis and tendinitis in forearm muscles and tendons, and increased focal sites of myotendon fray and fibroblast proliferation (Abdelmagid et al., 2012; Barbe et al., 2003). After such tissue damage, it is known that inflammatory cells infiltrate tissues, which, along with injured cells, produce

inflammatory cytokines and other mediators that either exacerbate damage or assist in tissue repair (Barr et al., 2004; Cutlip et al., 2009).

Osteoactivin (OA), a type I transmembrane protein, also known as glycoprotein nonmelanoma protein B (GPNMB), is a growth factor involved in tissue turnover during regeneration (Abdelmagid et al., 2010; Furochi et al., 2007a; Furochi et al., 2007b). OA is known to up-regulate the expression of matrix metalloproteinases (MMPs) in fibroblasts infiltrating denervated muscle, leading to increased extracellular matrix (ECM) turnover (Ogawa et al., 2005). OA increases in tissue matrices during fracture repair (Abdelmagid et al., 2010), influences adhesion and migration of select cell types, including fibroblasts, involved in tissue repair (Shikano et al., 2001), and regulates muscle regeneration in desmin-deficient cardiomyocytes (Psarras et al., 2012). In a process called ectodomain shedding, the extracellular fragments of OA is cleaved on the plasma membrane, released to the ECM where they act as cytokines or growth factors (Hoashi et al., 2010; Qian et al., 2008; Rose et al., 2010), in addition to increasing MMP production (Furochi et al., 2007a). Thus, it is clear that OA increases under pathological conditions; however, its expression in association with overuse injuries has yet to be examined.

The inducible form of HSP70 (HSPA1A; commonly known as HSP72) mediates tissue repair after injury and protects skeletal muscle from atrophy, damage and dystrophy (Gehrig et al., 2012; Miyabara et al., 2006; Miyabara et al., 2012; Parsell and Lindquist, 1993; Senf et al., 2008). HSP70/72 plays a role in skeletal muscle repair or regeneration and adaptation after high-force eccentric exercise (Koh, 2002; McArdle et

al., 2004; Paulsen et al., 2007), and increases concomitant with MMP-2 in skeletal muscle following high intensity training (Carmeli et al., 2010), apparently acting together to promote muscle matrix remodeling (Hirunsai et al., 2015). Increased expression of HSP70 following muscle injury regulates both the early inflammatory and regenerative phases of muscle regeneration (Senf et al., 2013).

Our goals here were to evaluate the temporal and spatial expression of OA, MMPs and HSP72 in forelimb flexor digitorum muscles and tendons in rats performing a HRNF task for 3 to 6 weeks, in order to determine if one or more may be a potential therapeutic target to improve muscle and tendon repair in future experiments. Inflammatory cytokines were also examined in order to determine if injury/inflammatory processes were present in the tissues at the same time points. Although OA has yet to be studied in a model of overuse, we hypothesized that it would increase in the overloaded skeletal muscles and tendons, as would the known mediators of repair, MMPs and HSP70.

## **3.2 Methods**

### *3.2.1 Animals*

All experiments were approved by the Temple University Institutional Animal Care and Use Committee in compliance with NIH guidelines for the humane care and use of laboratory animals. Rats were housed individually in the central animal facility in transparent plastic cages in a 12 hour light: 12 hour dark cycle with free access to water. Studies were conducted on a total of 34 young adult (3.5 months of age at onset of experiments), Sprague-Dawley, female rats. All rats were handled for 1 week, and then

food restricted to 80-90% of their naïve full body weights for another week before onset of the experiment to motivate interest in 45 mg food pellets used for reward and retrieval (Banana flavored 45 mg food pellets; Bio-Serve, Flemington, NJ). All rats were weighed once to twice per week, provided regular rat chow daily, and allowed to gain weight over the course of the experiments, since they were young adult rats. All rats were maintained within 5-10% of the weights of age-matched normal control rats (used only for weight matching purposes and therefore not included in the study). Rats were then randomly divided into food restricted control rats (n=12) and high repetition negligible force (HRNF) task rats (n=22) performing this task for 3 weeks (n=12), and 6 weeks (n=10).

### *3.2.2 Behavioral Task Paradigm*

The HRNF task has been described in detail previously (Barbe et al., 2003). Briefly, rats were trained for 5 minutes/day, 5 days/week, for 10-12 days, to learn to retrieve a 45-mg pellet of food from a shoulder-height portal at a reach rate of 4 reaches/min, making this a high repetition task (i.e. faster than 30 seconds/cycle) (Silverstein et al., 1986). After this initial training period, task rats then performed the HRNF task for 2 hours/day, in four 30 min sessions/day, 3 days/week, for 3 to 6 weeks, in customized operant behavioral apparatus (Med Associates, St Albans, VT), described in detail previously (Barbe et al., 2003). Retrieval of the 45 mg food pellet from the portal was estimated as <5% maximum pulling force, making this a negligible force task (Bertelli and Mira, 1995). Rats were allowed to use their preferred reach limb to reach and retrieve the food pellet, hereafter referred to as the reach limb. Tissues used in this study were from the reach limbs.

### 3.2.3 *Quantitative Real-Time PCR (qPCR)*

Twelve animals were euthanized with an overdose of sodium pentobarbital (120 mg/kg body weight) and forearm flexor muscles were collected the flexor digitorum mass from FRC, and 3- and 6-week HRNF rats (n=3/group). Each muscle belly was divided into two longitudinal parts; half was used for RNA extraction and half for protein extraction. The half for RNA extraction was put into RNAlater RNA Stabilization Reagent (QIAGEN, Valencia, CA) for 2 hours at room temperature, and then stored at -80°C. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). The concentration of each RNA sample was determined using a spectrophotometer; the integrity was monitored on 1% formaldehyde denatured gels. After confirming RNA integrity on an agarose gel, cDNA was prepared from mRNA extracts from the above tissue samples (n=3/group), using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, Foster City, CA). PCR primer sets for the following were used: OA (Cat# PPR45879B, SABiosciences, Frederick, MD) and GAPDH (Cat# PPR51520A, SABiosciences, Frederick, MD). Quantitative real time PCT (qPCR) was then performed in duplicate for 20µl reactions each containing 1µl cDNA reaction mix, 100nM of each primer and 10µl 2x SYBR® Green PCR Master Mix method (Applied Biosystems™, Foster City, CA), on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems™). PCR cycles consisted of an initial cycle of 50°C for 2 min and the second cycle of 95°C for 10 min, followed by a two-step program of 95°C for 15 sec and 60°C for 1 min for 40 cycles. Using GAPDH as the internal control, relative gene expression among samples was calculated by comparison of C<sub>t</sub> (threshold cycle) values. A dissociation curve was

checked for each qPCR run to confirm specific amplification of target RNA. The PCR primer set (OA) used was purchased from SABiosciences.

### *3.2.4 Protein Isolation and Western Blotting*

Animals were euthanized with an overdose of sodium pentobarbital (120 mg/kg body weight) before muscle and tendon collection from the flexor digitorum mass from FRC rats (n=8), and from rats that performed the HRNF task for 3 weeks (n= 4) or 6 weeks (n= 6). As described above, the muscles were divided into half longitudinally. The half for protein analysis was snap frozen and stored at -80°C until homogenization, and preparation for protein analysis, using previously described methods (Barbe et al., 2008; Rani et al., 2010). Total protein was determined using BCA-200 protein assays (Bicinchoninic Acid, Pierce, Rockford, IL). Then, 30µg of protein sample was mixed with 5X Laemmli sample buffer (Bio-Rad, Hercules, CA) mixed with denaturing buffer; 25% β-mercaptoethanol, heated to 100°C for 5 min, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred at 100 V for 1 hr at 4°C to a nitrocellulose membrane (Bio-Rad) using semi-dry transfer apparatus (Bio-Rad). The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS)-0.1% Tween-20 (TBST) for 1 h at room temperature and then incubated with the following primary antibodies: anti-rabbit beta Actin (1:1000; Sigma, St Louis, MO), anti-mouse GAPDH (1:500; Santa Cruz Biotechnology, Delaware Avenue, CA), anti-mouse Hsp72 (1:300; Stressgen, Ann Arbor, MI), anti-rabbit MMP-1 (1:500; Abbotec, San Diego, CA), anti-mouse MMP-2 (1:200; Abcam, Cambridge, MA), anti-rabbit MMP3 (1:200; Novus Biologicals, Littleton, CO), anti-mouse MMP-13 (1:200; Abcam), anti-rabbit

OA/GPNMB (1:500; Bioss Inc. Woburn, MA) and custom made anti-chicken OA (1:250), which was produced as previously described in (Abdelmagid et al., 2007), in blocking buffer (same as above) overnight at 4°C. The blot was then washed with 1X TBS-0.1% Tween-20 (TBST) and incubated with the following horseradish peroxidase-conjugated secondary antibodies: donkey anti-chicken, anti-mouse or anti-rabbit (1:5000; Jackson ImmunoResearch, West Grove, PA), in blocking buffer for 1 hour at room temperature. The blot was washed again with TBST, incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL), and exposed to film. Quantification of the bands was performed using either Image J or myImageAnalysis v2.0 software (Thermo Scientific).

### 3.2.5 ELISA

For protein analysis, tissues from above animals that had been prepared for protein analysis were used in ELISAs to determine HSP72 and inflammatory cytokine levels. All muscle and tendon samples were homogenized separately with 0.5 to 1.0 ml RIPA buffer ((NaCl, KCl, NaH<sub>2</sub>PO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, and DDH<sub>2</sub>O + NaOH) plus EDTA free complete protease inhibitor cocktail tablets (Roche Diagnostics, GMPH, Germany) using a PowerGen 125 Homogenizer. Tissue homogenates were centrifuged at 14,000 rpm for 15 min. at 4°C. Total protein was determined using BCA-200 protein assays (Bicinchoninic Acid). For ELISA, tissue lysates (50 microliter aliquots) were analyzed using a commercially available single-plex ELISA kit for HSP72 (EKS-700, Stressgen, obtained before its acquisition by Enzo Life Sciences, Inc, Farmingdale, NY), according to the manufacturers' protocol. Tissue lysates were also analyzed for IL-1alpha, IL-1beta,

TNF-alpha and IL-10 using commercially available ELISA kits according to manufacturer's protocols (each from BioSource International). Each sample was run in duplicate. Data (ng protein of HSP72 and pg protein for the cytokines) were normalized to µg total protein.

### 3.2.6 Immunohistochemical Analyses and Quantification

Animals received an overdose of sodium pentobarbital (120 mg/kg body weight) before being perfused transcardially with first sterile saline, and then 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4): FRC (n=6), 3-week HRNF (n=6), and 6-week HRNF (n=4). Tissues were collected and postfixed "en bloc" by immersion overnight. A proximal portion of the muscle mass was then removed with a scalpel for cross-sectional sectioning while the remaining flexor digitorum muscles and tendons were separated *en bloc* as a flexor mass from the bones for longitudinal sectioning, as previously depicted (Fedorczyk et al., 2010). All tissues were cryoprotected in 30% sucrose in phosphate buffered saline (PBS) before frozen-sectioning using a cryostat into 15 µm cross sectional or longitudinal slices. Sections were then placed onto charged slides (Fisher, Super Frost Plus) and allowed to dry overnight before storage at -80°C. Sections on slides were treated with 3% H<sub>2</sub>O<sub>2</sub> in methanol to block for endogenous peroxidase for 30 min (if the tissues were to be used for HRP-DAP visualization), washed in PBS, then permeabilized with 0.05% pepsin in 0.01N HCL, prior to blocking with 10% goat serum in PBS for 20 min at room temperature. Sections on slides were then probed in batches with the following primary antibodies: anti-HSP72 (1:1000, C92F3A-5, Enzo Life Sciences, Inc) or anti-osteocalcin (1:350, custom made anti-

chicken OA) produced and verified, as previously described (Abdelmagid et al., 2007). On the 2<sup>nd</sup> day, after washing, one set of sections, from all groups, that had been incubated with anti-OA antibody was incubated with goat anti-chicken secondary IgG antibody conjugated to HRP (Jackson ImmunoResearch Laboratories, West Grove, PA; diluted 1:100 in PBS, and incubated 2 hours at room temperature before washing in PBS) and visualized using DAB (Fast DAB, Sigma). These sections were counterstained lightly with eosin, then dehydrated and coverslipped with DPX mounting medium for bright field microscopy (for HRP-DAB). A second set of sections was incubated with anti-HSP72 antibody, and then with a donkey anti-mouse secondary IgG antibody conjugated to Cy3 (red fluorescent tag) diluted as above (Jackson ImmunoResearch Laboratories). A third set of slides was incubated with both anti-OA antibody and anti-HSP72 and then with appropriate secondary antibodies conjugated to Cy2 (green fluorescent tag) or Cy3 (red fluorescent tag) (Jackson ImmunoResearch Laboratories). The fluorescent tag-labeled sections were washed with PBS and coverslipped with 80% glycerol in PBS for epifluorescence microscopy.

The specificity of the OA and HSP72 antibodies are shown in western blots that are part of this study. In addition, negative control staining was performed by omitting either the primary antibody or the secondary antibody; no labeling was observed as a result of incubation of tissues with serum and then secondary antibodies alone (data not shown). Furthermore, preabsorption controls were performed to demonstrate if the antibodies bound specifically to the antigen of interest: Specifically, specificity of the OA and HSP72 antibodies was determined via the use of recombinant human Osteoactivin/GPNMB/Fc Chimera (Cat# 2550-AC, R&D Systems, Minneapolis, MN)

and recombinant human HSP70 protein (Product# SPP-755, StressGen, Farmingdale, NY; purchased from StressGen before its acquisition by Enzo Life Sciences, Inc.), respectively. A ten-fold excess of purified protein was pre-incubated with the matching antibody overnight at 4°C, the mixture centrifuged, and then the pre-absorbed antibody supernatant was incubated with the tissues (after pepsin and goat serum treatment) similarly to that described above before washing and incubation with secondary antibodies. No labeling was observed in the tissues for either pre-absorbed antibody (data not shown), matching previously published specificity of the custom made OA antibody in bone tissues (Abdelmagid et al., 2007).

The percent area with OA and HSP72 immunostaining in muscles and tendons was quantified from HRP-DAB stained and fluorescent stained slides, respectively. This quantification was performed using an image analysis program (Bioquant Osteo II, Nashville, TN) and previously described thresholded pixel count methods (Al-Shatti et al., 2005). The person performing the quantification was blinded to group assignment.

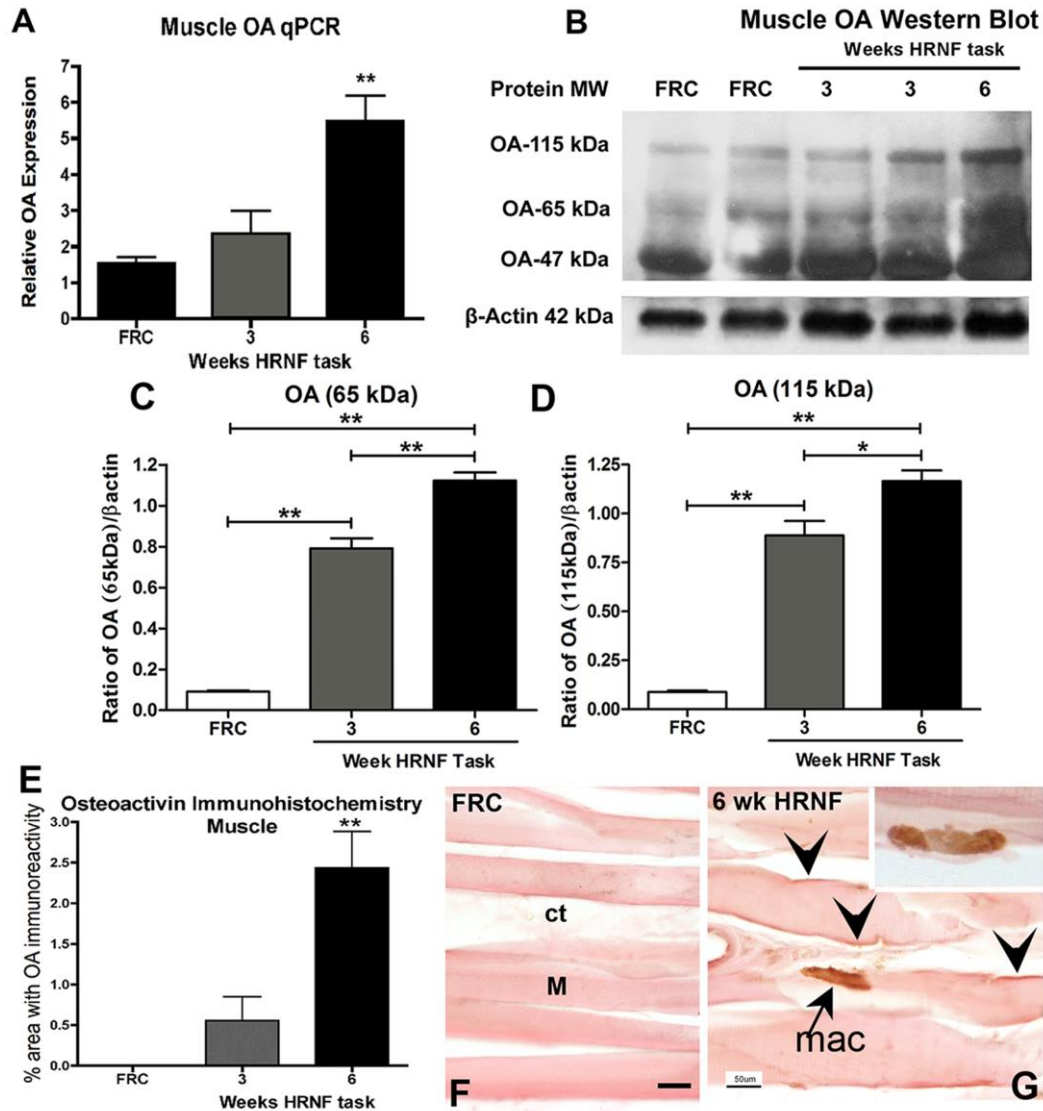
### *3.2.7 Statistical Analysis*

Statistical analyses were performed using Prism 4 and 5 (GraphPad Software, La Jolla, CA). Univariate ANOVAs were used to analyze quantitative PCR, Western blot densitometry, and ELISA results for differences in analytes between groups. Then, post hoc analyses were carried out using the Bonferroni test for multiple comparisons, with FRC used as the control group; adjusted p values are reported. An adjusted p value of < 0.05 was considered significant for all analyses. Data are presented as the mean ± standard error of the mean (SEM).

### **3.3 Results**

#### **3.3.1 Increased Osteoactivin in Flexor Digitorum Muscles with HRNF Task**

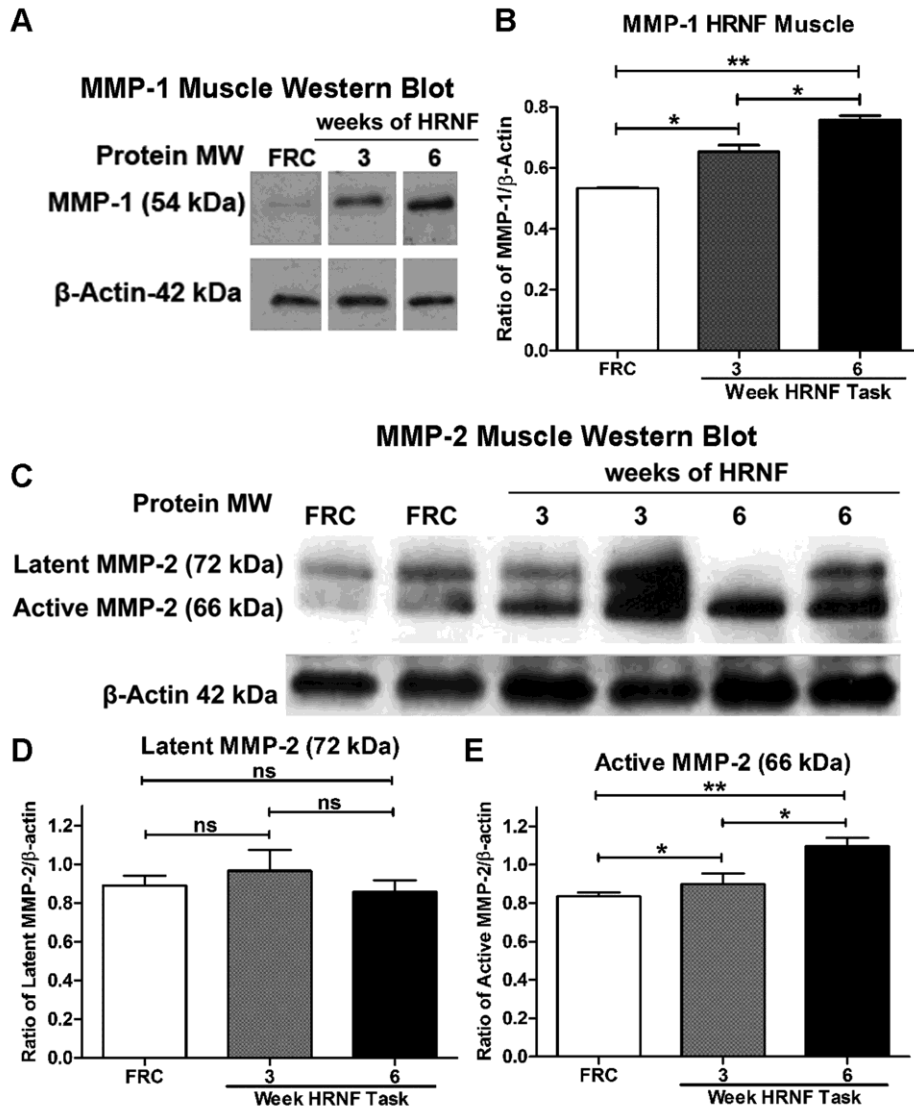
Utilizing quantitative real time PCR (qPCR) analysis, we observed a significant up-regulation of OA gene expression in muscles of 6-week HRNF animals ( $p < 0.01$ ), compared to FRC rats (Fig. 3-1A). These findings were confirmed using Western blot analysis, which showed progressive increases in OA protein levels with HRNF task performance, compared to FRC rats (Fig. 3-1B). The 65 kDa and 115 kDa molecular weight bands of OA were increased ( $p < 0.01$ ), while the 47 kDa band was not increased, with HRNF task performance (Fig. 3-1B-D). To further examine the expression of OA in the muscle tissue of our rat model, we performed immunohistochemistry and subsequent quantification of OA in the flexor digitorum muscles of HRNF rats. OA immunostaining was increased in 6-week HRNF muscles ( $p < 0.05$ ), compared to controls (Fig. 3-1E). OA was localized to the sarcolemma and to macrophage-like cells located between individual muscle fibers in 6-week HRNF muscles, but was absent in control muscles (Fig. 3-1F and G).



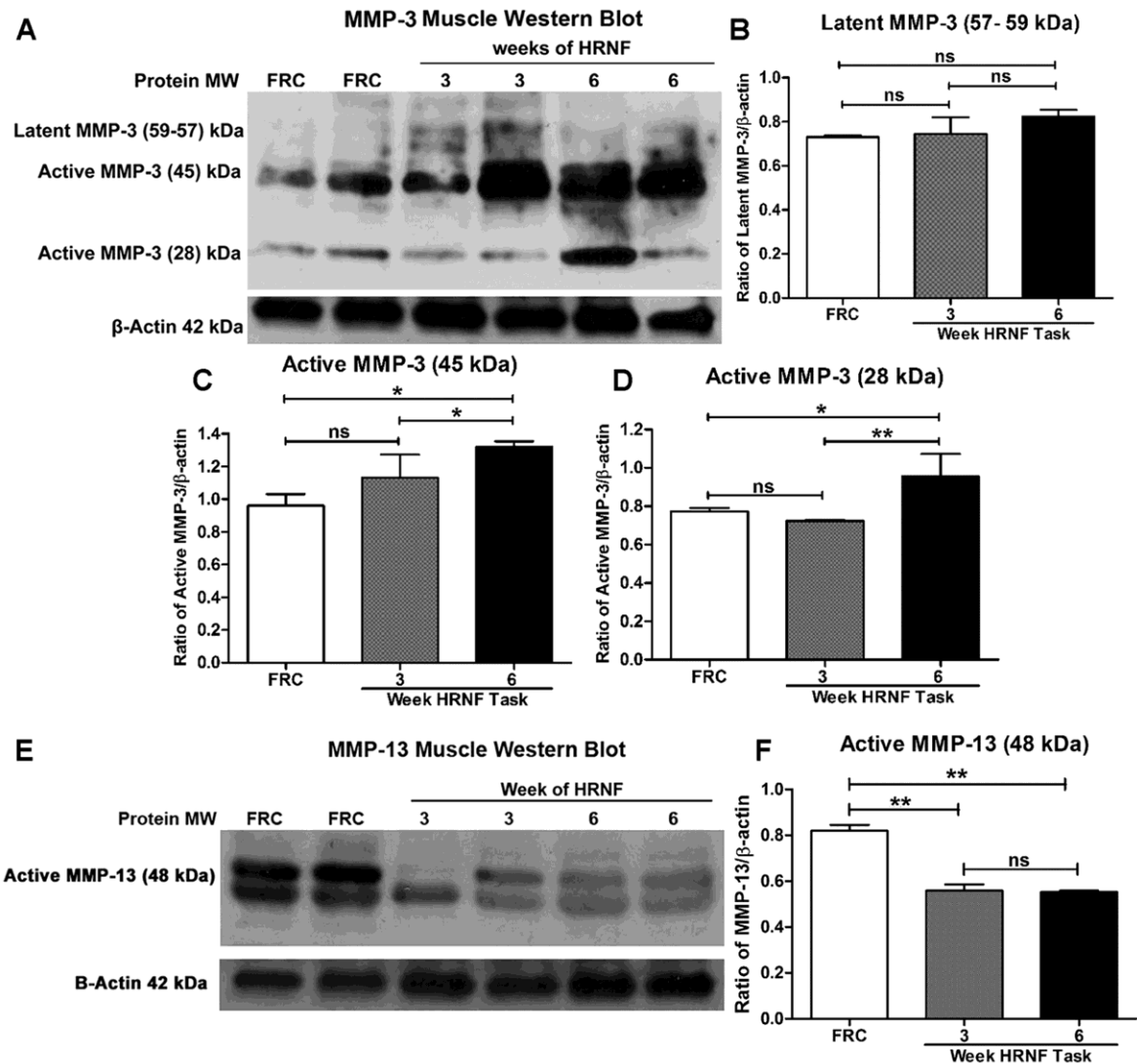
**Figure 3-1. Osteoactivin (OA) expression increases in flexor digitorum muscles with high repetition negligible force (HRNF) task.** (A) OA mRNA expression levels (determined using quantitative PCR, qPCR) in muscles of FRC rats and rats that had performed a HRNF food retrieval task for 3 or 6 weeks. Values were normalized to GAPDH mRNA levels. (B) A representative Western blot of muscle homogenates probed with anti-OA. Bands are at the expected molecular weights of OA in muscle (approximately 115, 65, and 47 kDa).  $\beta$ -actin was used as a loading control (at 42 kDa). (C, D) Densitometric analysis of two OA bands (65 and 115 kDa) from three replicate Western blots normalized to  $\beta$ -actin levels. (E) Quantification of OA-positive immunostaining as a percent of total tissue area. (F, G) Examples of OA immunostaining in longitudinal sections of FRC and HRNF muscles showing OA in the myofiber sarcolemma (arrowheads) and macrophages (mac) only in HRNF muscles. Ct = connective tissue, M = muscle. \*  $p < 0.05$ , \*\*  $p < 0.01$ , compared to FRC rats. Scale bars in F and G = 50  $\mu$ m.

### **3.3.2 MMP-1, -2, -3 and -13 are altered in Flexor Digitorum Muscles with HRNF Task**

Western blotting detection showed that MMP-1, -2 and -3 were significantly increased in 3- and 6-week HRNF rat muscles, compared to FRC rats (Fig. 3-2 and 3). Specifically, the 54 kDa molecular weight band of MMP-1 (Fig. 3-2A and B) and the 66 kDa active form of MMP-2 (Fig. 3-2C and E) was increased in HRNF rat muscles ( $p < 0.05$  each), particularly by 6 weeks of task performance. The 72 kDa latent form of MMP-2 did not change significantly across groups (Fig. 3-2C and D). Expression levels of the active forms of MMP-3 (45 kDa and 28 kDa) also increased significantly in 6-week HRNF rats ( $p < 0.05$  each), compared to 3-week HRNF and FRC rat muscles (Fig. 3-3A, C and D). In contrast, the latent form of MMP-3 (57-59 kDa) did not change (Fig. 3-3A and C), and the level of active MMP-13 (48 kDa) decreased with HRNF task performance ( $p < 0.01$ ), compared to controls (Fig. 3-3E and F).



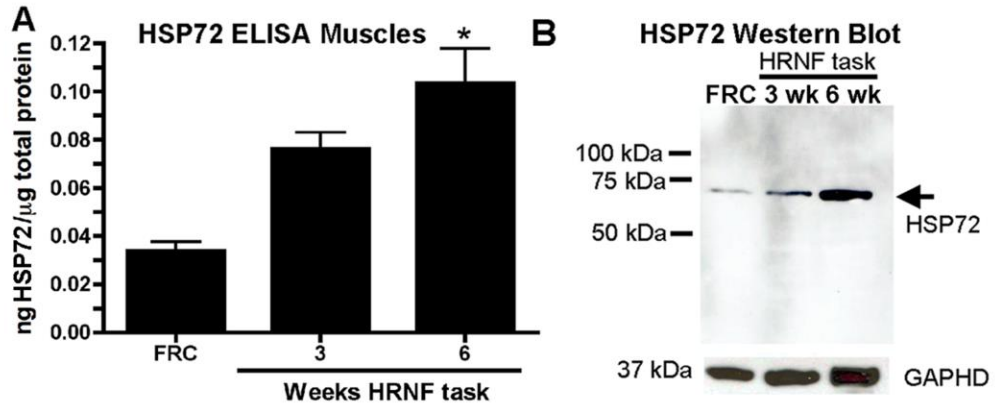
**Figure 3-2. MMP-1 and -2 protein levels increase in flexor digitorum muscles with HRNF task.** (A) Representative Western blot of muscle homogenates probed with anti-MMP-1. An immunoreactive band at the expected molecular weight of 54 kDa was detected.  $\beta$ -actin was used as a loading control (42 kDa). (B) Densitometric analysis of the MMP-1 band from three replicate Western blots, normalized to  $\beta$ -actin levels, documents significant increases relative to FRC after 3 and 6 weeks of the HRNF task and between 3 and 6 weeks of the task. (C) Representative Western blot of muscle homogenates probed with anti-MMP-2. Bands are detected at the expected molecular weights for the latent (72 kDa) and active (66 kDa) forms. (D, E) Densitometric analyses of the MMP-2 protein bands (72 and 66 kDa) from three replicate Western blots, normalized to  $\beta$ -actin levels show that only the active form is increased. \*  $p < 0.05$ , \*\*  $p < 0.01$ , compared to FRC rats; ns = not significant.



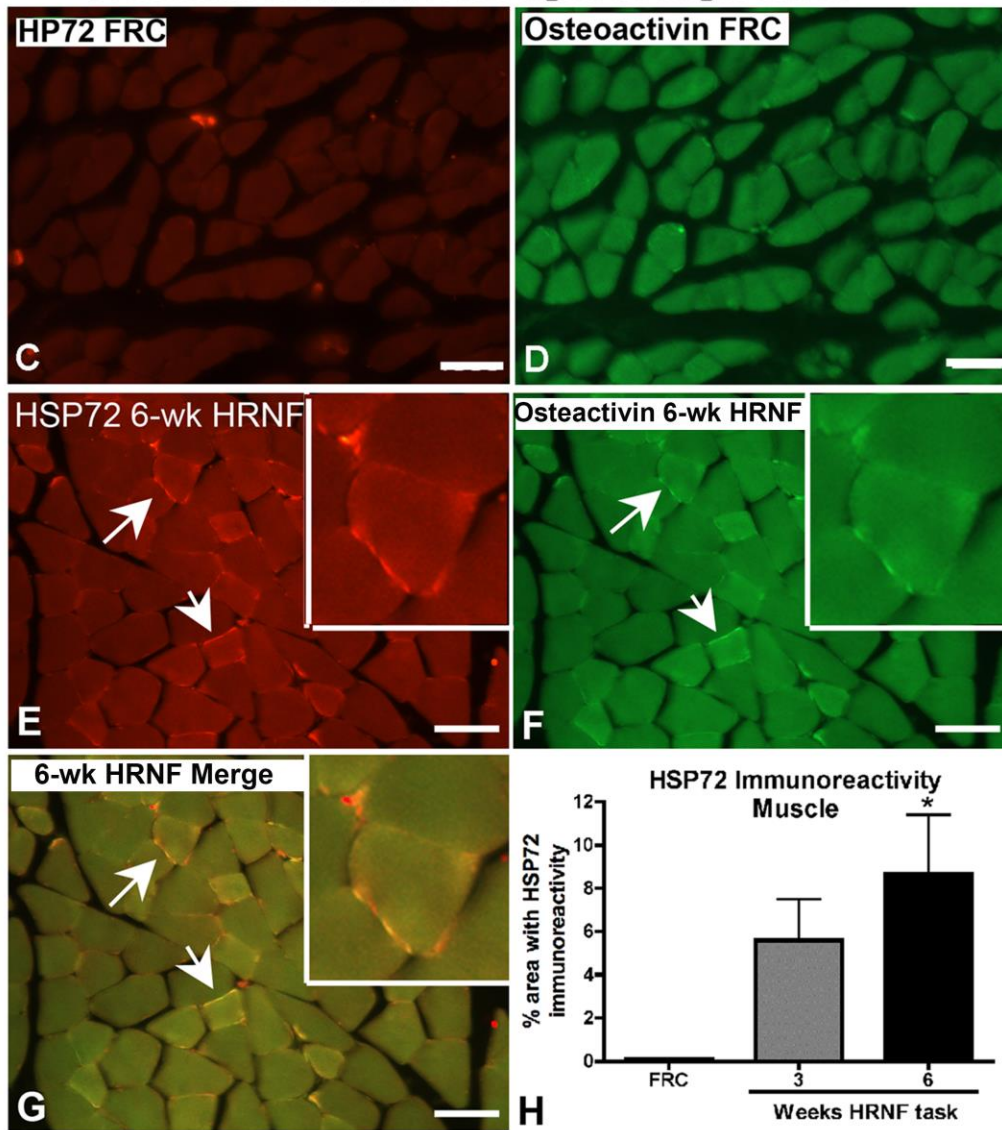
**Figure 3-3. MMP-3 levels increase whereas MMP-13 decreases in flexor digitorum muscles with HRNF task.** (A) Representative Western blot of muscle homogenates probed with MMP-3. Bands are at the expected molecular weights of latent and active forms of MMP-3 (approximately 57-59, 45, and 28 kDa).  $\beta$ -actin was used as a loading control. (B-D) Densitometric analysis of latent (57-59 kDa) and active (45 and 28 kDa) bands of MMP-3 from three replicate Western blots, normalized to  $\beta$ -actin levels. (E) A representative Western blot of muscle homogenates probed with MMP-13. Doublet bands, at the expected molecular weight of active MMP-13 (approximately 48 kDa), were analyzed, averaged and normalized to the loading control. (F) Densitometric analysis of active (48 kDa) form of MMP-13 from three replicate Western blots, normalized to  $\beta$ -actin levels shows it to decrease significantly with the HRNF task. \*  $p < 0.05$ , \*\*  $p < 0.01$ , compared to FRC rats; ns = not significant.

### **3.3.3 HSP72 Increases in Muscles and Co-localizes with OA in Sarcolemma of HRNF Rats**

ELISA analysis showed significant increased HSP72 protein in 6-week HRNF muscles ( $p < 0.05$ ), compared to controls (Fig. 3-4A). This result was supported by Western blot analysis that confirmed the expression and increase of the HSP72 protein in 6-wk HRNF rat muscles, compared to controls (Fig 3-4B). We also evaluated the expression of OA in relationship to the expression of HSP72. Double labeling immunohistochemistry of 6-week flexor digitorum muscle fibers demonstrated increased expression of HSP72 (in red) and OA (in green), compared to FRC muscles (Fig. 3-4C-I). In those myofibers in which the levels of the two proteins were increased, they co-localized to the sarcolemma surrounding the fibers (Fig. 3-4G-I). No DAPI labeled cell bodies were visualized within the center of any of the myofibers at this 6-week HRNF time point (data not shown), indicating that the myofibers were not injured.



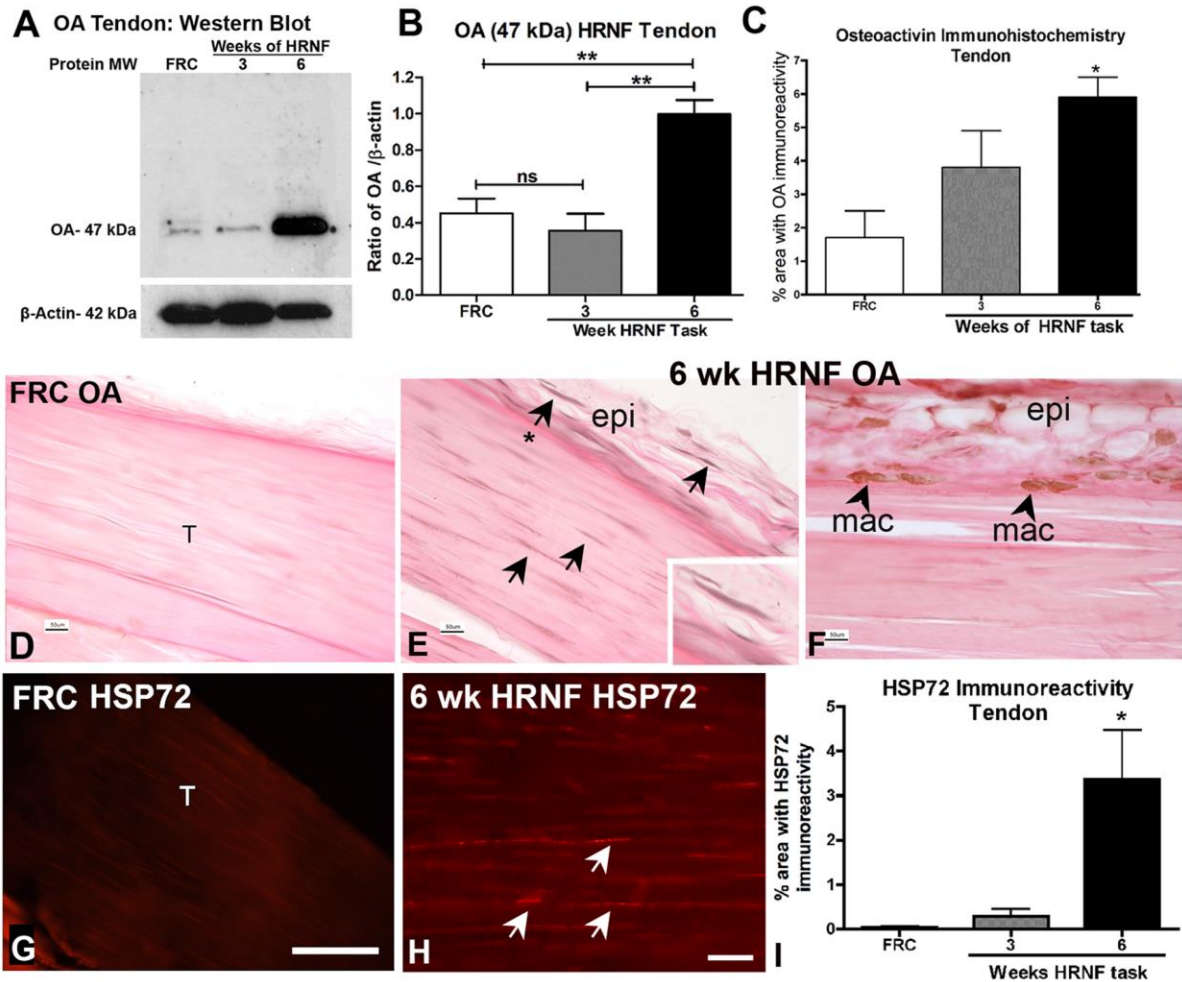
Osteoactivin and HSP72: Flexor digitorum longus muscle



**Figure 3-4. HSP72 increases in flexor digitorum muscles of HRNF rats and co-localizes with OA in the sarcolemma.** (A) ELISA of HSP72 protein levels in FRC and HRNF rat muscle lysates. Nanograms of protein were normalized to  $\mu\text{g}$  of total protein. (B) Representative Western blot of muscle homogenates probed with anti-HSP72, used to confirm the molecular weight of the HSP protein recognized by the antibody. (C, D) Double-labeled immunostained images of HSP72 (red) and OA (green) in muscle from a FRC rat (a proximal portion of the muscle was resected and cut transaxially, and shown). (E-G) Double-labeled immunostained images of HSP72 (red), OA (green) and merged, respectively, in muscle from an 8-week HRNF rat (a proximal portion of the muscle was cut transaxially, and is shown). Arrows indicates HSP72 and OA co-localization in myofiber sarcolemma. The insets in E-F show enlarged regions of these images to illustrate co-localization of HSP72 and OA in myofiber sarcolemma. (H) Quantification of HSP72-positive immunostaining as a percent of total tissue area. \*  $p < 0.05$ , compared to FRC rats. Scale bars in C-G = 50  $\mu\text{m}$ .

### **3.3.4 Osteoactivin and HSP72 Increase in Tendons with HRNF Task**

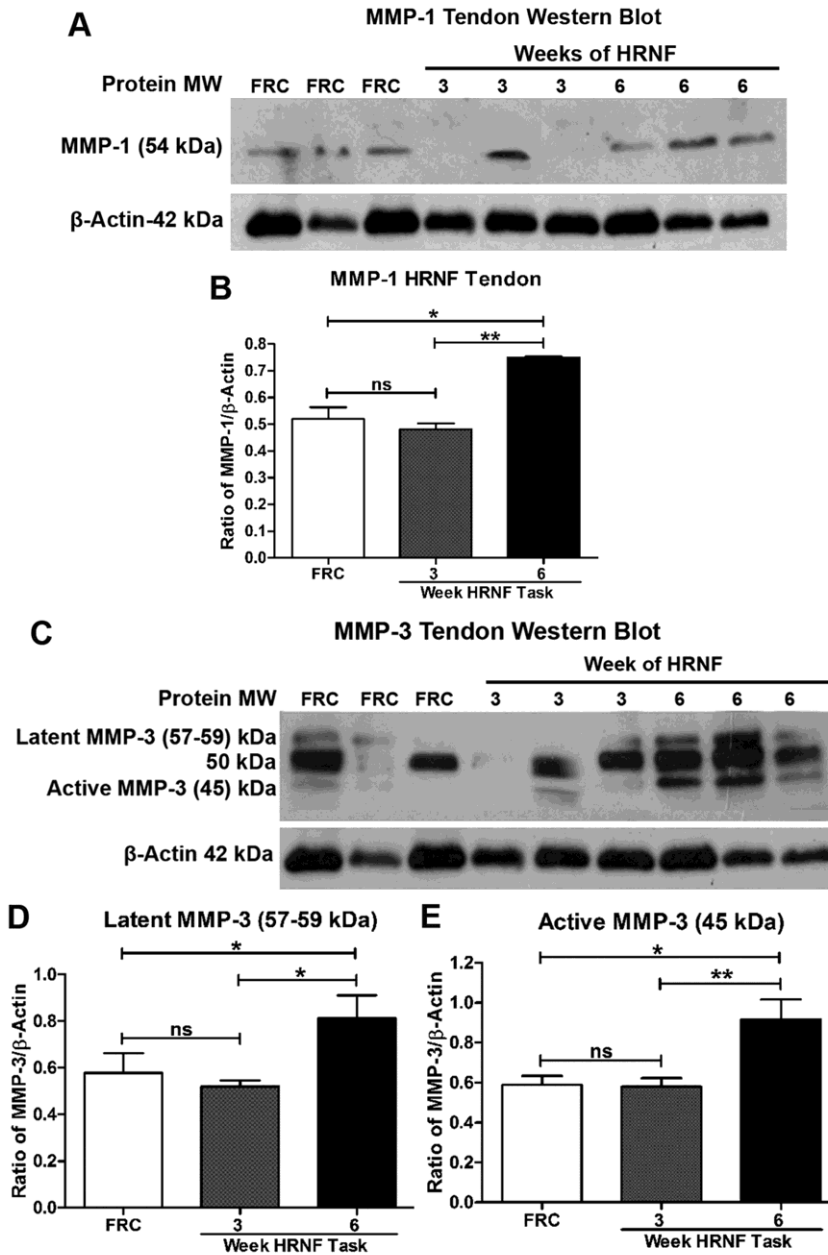
Next, we examined the expression of OA and HSP72 in flexor digitorum tendons. Western blot analysis showed that only the 47 kDa form of OA was increased in tendons of 6-week HRNF task performance ( $p < 0.01$ ), compared to FRC rats (Fig. 3-5A and B). Quantification of immunoreactive OA expressed in the tendons showed significantly increased OA immunostaining in 6-week HRNF tendons ( $p < 0.05$ ), compared to control rat tendons (Fig. 3-5C-E). OA was localized to tenocytes within 6-week HRNF tendons, and to fibroblast- and macrophage-like cells located within the surrounding connective tissue (epitendon), but was absent in control tendons and epitendon (Fig. 3-5 D-F). Similarly, immunoreactive HSP72 was significantly increased in tenocytes located within 6-week HRNF tendons, compared to controls (Fig. 3-5G-I).



**Figure 3-5. Flexor digitorum tendons show increased osteoactivin and HSP72 by six weeks of HRNF task performance.** (A) Representative Western blot of tendon homogenates probed with anti-OA. Band detected is the 47 kDa MW.  $\beta$ -actin used as a loading control (at 42 kDa). (B) Densitometric analysis of the 47 kDa OA band in tendon from three replicate Western blots, normalized to  $\beta$ -actin levels. (C-F) Immunostaining and quantification of OA in FRC and HRNF rat tendons (T). A tendon region located proximal to the wrist, and cryosectioned longitudinally, is shown. Arrows indicate representative OA-immunopositive cells, many of which are tenocyte-like in appearance. Fibroblast-like cells were also observed in the epitendon (epi) (one indicated with an asterisk in panel E and enlarged in the inset), as well as macrophage-like cells (mac) (arrow heads). (G-I) Immunostaining and quantification of HSP72 in FRC and HRNF rat tendons (T). Arrows indicate representative HSP72-immunopositive cells that appear tenocyte-like. \*  $p < 0.05$ , \*\*  $p < 0.01$ , compared to FRC rats. Scale bars in D and E = 50  $\mu$ m; Scale bar in F and G = 100  $\mu$ m.

### **3.3.5 MMP-1 and -3 Increase in Tendons with HRNF Task**

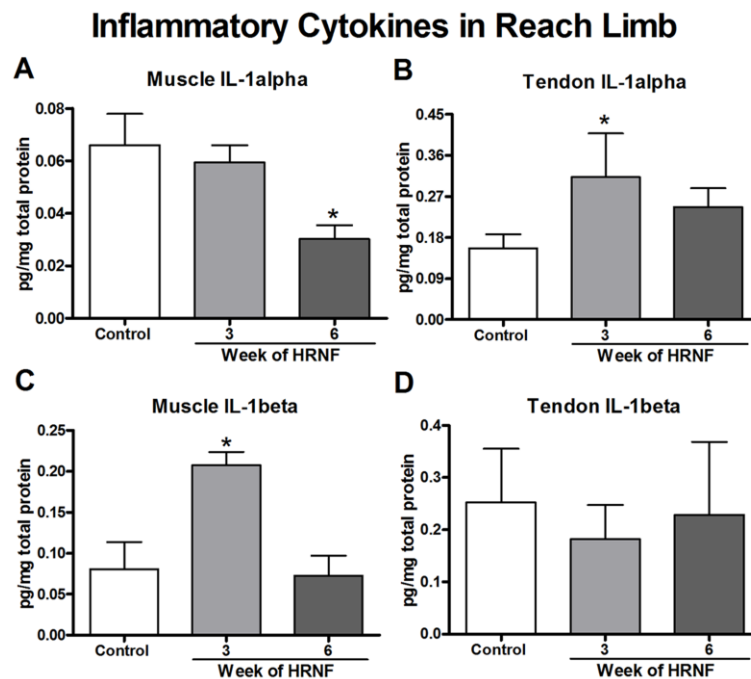
Western blot analysis showed the level of MMP-1 (54 kDa) was increased in 6-week HRNF rat tendons ( $p < 0.05$ ), compared to 3-week HRNF and FRC rat tendons (Fig. 3-6A and B). Note the variability of MMP-1 in the 3-week HRNF tendons (Fig. 3-6A). This was a consistent finding in this group. The latent (57-59 kDa) and active (45 kDa) forms of MMP-3 were increased significantly in 6-week HRNF rat tendons ( $p < 0.05$ ), compared to 3-week HRNF and FRC rats (Fig. 3-6C-E). Again, a variable expression of MMP-3 was observed in the FRC and 3-week HRNF tendons, while MMP-3 was consistently increased in the 6-week HRNF tendons.



**Figure 3-6. MMP-1 and -3 protein levels increase in flexor digitorum tendons with HRNF task.** (A) Representative Western blot of tendon homogenates probed with anti-MMP-1. Bands are at the expected molecular weight of 54 kDa.  $\beta$ -actin used as a loading control (at 42 kDa). (B) Densitometric analysis of three replicate Western blots, showing the ratio of MMP1 band, normalized to  $\beta$ -actin levels. (C) A representative Western blot of muscle homogenates probed with MMP-3. Bands are at the expected molecular weights of latent and active forms of MMP-3 (approximately 57-59, and 45).  $\beta$ -actin used as a loading control. (D, E) Densitometric analysis of latent (57-59 kDa) and active (45 kDa) forms of MMP-3 from three replicate Western blots, normalized to  $\beta$ -actin levels. \*  $p < 0.05$ , \*\*  $p < 0.01$ , compared to FRC rats.

### 3.3.6 IL-1alpha and IL-1beta alter across time in muscles and tendons with HRNF Task

We observed a significant decrease in IL-1alpha in 6-week HRNF muscles, compared to controls (Fig 3-7A). We also observed significant yet transient increases of IL-1alpha in 3-week HRNF tendons (Fig. 3-7B), and significant yet transient increases of IL-1beta in 3-week HRNF muscles (Fig. 3-7C). No increase was observed for IL-1beta in tendons (Fig. 3-7D). TNF-alpha and IL-10 did not alter significantly with task performance, in either tissue, compared to controls (data not shown).



**Figure 3-7. ELISA of inflammatory cytokines in flexor digitorum muscles and tendons of reach limb.** Data is shown for (A, B) IL-1alpha and (C, D) IL-1beta in FRC and 3- and 6-week HRNF rats. \*  $p < 0.05$ , compared to FRC rats.

### 3.4 Discussion

For the first time to our knowledge, we show in a rat model of limb overuse that OA expression increases progressively in forearm muscles and tendons with prolonged

performance of an upper extremity high repetition negligible force (HRNF) task for up to 6 weeks. Immunostaining also showed localization of OA to myofiber sarcolemma, macrophage-like cells fibroblast-like cells and tenocytes. MMP-1, -2 and -3 protein levels also progressively increased, whereas MMP-13 decreased, in forearm muscles with HRNF task performance. MMP-1 and -3 levels progressively increased in forearm tendons. HSP72 protein levels were increased only in 6-week HRNF muscles and tendons, compared to controls, with HSP72 co-localizing with OA in the sarcolemma of 6-week HRNF muscles.

We have previously shown modest muscle and tendon injury, followed by a low-grade but transient inflammatory response, with performance of this HRNF reaching and food retrieval task (Barbe et al., 2003; Barbe et al., 2008). Since inflammation occurring in injured tissues as a consequence of overuse is thought to be related to repair processes (Song et al., 2012), we extended our past studies to examine repair and remodeling factors in this current study. Repair of musculoskeletal tissues may be driven by a number of anabolic factors. We examined OA because it has been identified as an anabolic growth factor in long bones (Safadi et al., 2001), and found that it was increased in muscles and tendons of HRNF rats, with the greatest increases in parallel with a return of inflammatory cytokines to baseline levels. The increase in OA was concomitant with increased MMP and HSP72, proteins known to participate in repair and remodeling processes, thus further supporting a role for OA as an anabolic molecule. Several studies have shown that OA regulates cell proliferation, adhesion, differentiation and synthesis of extracellular matrix proteins in various cell types under normal and pathological conditions (Abdelmagid et al., 2008; Abe et al., 2007; Chung et al., 2007b; Haralanova-

Ilieva et al., 2005; Nakamura et al., 2007; Ogawa et al., 2005; Onaga et al., 2003; Rich et al., 2003; Safadi et al., 2001; Shikano et al., 2001). In skeletal muscle, OA increases after denervation or distraction osteogenesis injury (Furochi et al., 2007a; Furochi et al., 2007b; Ogawa et al., 2005; Tonogai et al., 2015), and even up-regulates MMP-3 and -9 expression in fibroblasts infiltrating denervated skeletal muscle (Ogawa et al., 2005). Extracellular fragments of OA produced by ectodomain shedding induce MMP-3 expression in myofibroblasts after unloading (Furochi et al., 2007a).

Tissue injury also induces the expression of matrix-related genes, including MMPs (Guerin and Holland, 1995; Kherif et al., 1998; Kherif et al., 1999; Urso et al., 2010). Therefore, we examined their expression and observed that performance of the HRNF task for 6 weeks increased MMP expression. Specifically, MMP-1 (collagenase), -2 (gelatinase), and -3 (stromelysin-1) were increased in flexor digitorum muscles, while MMP-1 and -3 were increased in tendons. We have previously shown that MMP-2 increases in serum, forearm muscles and tendons of rats performing a high repetition low force lever-pulling task for 18 weeks (Gao et al., 2013). MMPs are zinc-dependent proteases that regulate cell-matrix composition, modulate ECM turnover, and are produced by fibroblasts, mesenchymal cells and macrophages (Mandal et al., 2003; Massova et al., 1998; Visse and Nagase, 2003; Woessner, 1991). The expression of most MMPs is low in normal steady-state tissues and are induced only when ECM remodeling is needed (Nagase et al., 2006), such as for musculoskeletal tissue adaptability to loading and training (Cawston, 1995; Kjaer, 2004; Murphy et al., 1994). Their expression is transcriptionally controlled by growth factors, cell-cell and cell-matrix interactions (Delany and Brinckerhoff, 1992; Nagase and Woessner, 1999; Overall et al., 1991), and

inflammatory cytokines (Cawston, 1995; Murphy et al., 1994). Examination of an experimental model of muscle regeneration [cardiotoxin (CTX) injection] shows a tightly regulated time course of MMP activation and resolution of tissue damage (Goetsch et al., 2003; Kherif et al., 1999; Zimowska et al., 2008). Also, generalized MMP inhibition impairs muscle repair (Bellayr et al., 2013), further supporting their requirement in resolution of muscle damage (Lei et al., 2013).

In tendons, MMPs are involved in collagen catabolism, and show increased activity with prolonged periods of high or low mechanical loading, or during periods of tendon repair (De Mello Malheiro et al., 2009; Kamekura et al., 2005; Pap et al., 1998). Studies have shown increased MMP-1 or MMP-2 with chronic loading of tendons (Asundi and Rempel, 2008), in tendons with signs of overuse injury (Fu et al., 2002; Riley, 2004), and in flexor tendosynovial tissues collected from patients with carpal tunnel syndrome (Hirata et al., 2005). In agreement with our data, a synergistic effect of mechanical stretch and presence of inflammatory cytokines induced MMP-1 and -3 expressions in tendon cells more than stretch alone (Archambault et al., 2002; Kawai et al., 1996; Ngan et al., 1990). Thus, the increased MMPs with HRNF task performance are likely responding to the continued loading and transient increases of IL-1alpha in tendons and IL-1beta in muscles, and contributing to tissue remodeling for repair.

Since previous studies suggest that heat shock proteins (HSPs) play a role in skeletal muscle repair after high-force eccentric exercise and that elevated HSP70 and its inducible form (HSP72) protects skeletal muscle against further injury (McArdle et al., 2004; Paulsen et al., 2007), we examined its expression and found that this stress-inducible protein was increased in 6-week HRNF muscles, compared to controls. HSP72

plays a role in skeletal muscle remodeling and adaptation processes in response to exercise and stress (Goto et al., 2004; Naito et al., 2000). Exercise training increases the expression of HSP72 a few days after the onset of training (Locke et al., 1995; Noble et al., 1999; Paroo et al., 2002), while prolonged exercise training, using intermittent high-intensity treadmill running for 8 weeks, induces long-term enhancement of HSP70 expression in skeletal muscle (Ogata et al., 2009). Interestingly, Sjogaard et al. showed in human subjects that performance of repetitive tasks increased HSP72 in muscles, while prolonged exercise training decreased its basal levels (Sjogaard et al., 2013). Activation of HSP72 may play a dual role in inflammation (Noble and Shen, 2012), inhibiting the release of inflammatory cytokines, including IL-1beta (Cahill et al., 1996; De et al., 2000; Kim et al., 2005; Pockley et al., 2009; Wieten et al., 2007). This interaction between HSP72 and inflammatory cytokines may explain our findings of only low levels of IL-1alpha and beta by 6 weeks of task performance.

As mentioned above, since HSP72 stimulates anti-inflammatory cytokines and inhibits the release of some inflammatory cytokines, we next examined the muscles and tendons for presence of inflammatory cytokines. We observed that HRNF task performance for 3 weeks induced small but significant increases in IL-1alpha in tendons and IL-1beta in muscles, yet declined to baseline or even below baseline levels by 6 weeks of task performance. Since pro-inflammatory cytokine expression are induced simultaneously at early time points during tissue repair (Karlmark et al., 2010), we suggest that the 3 week time point is the peak of inflammatory phase, and that the 6-week time point is the beginning of the repair proliferative phase, with HSP72 and OA mediating tissue repair and adaptation.

### **3.5 Conclusions**

These findings suggest that performance of a HRNF task for 6 weeks induces a repair response that might be mediated, at least in part, by OA, MMPs and HSP72.

Further research is needed to determine if these proteins can be targeted to enhance this repair process. Although little is known about the role of OA in tissue repair, this study suggests that pharmaceutical regulation of OA may be useful in the treatment of overuse injuries.

## CHAPTER 4

# OSTEOACTIVIN EXPRESSION IS MODULATED WITH TUMOR NECROSIS FACTOR TREATMENT IN MUSCULOSKELETAL TISSUES IN A RAT MODEL OF REPETITIVE OVERUSE

### 4.1 Introduction

Bone is a dynamic tissue that maintains its material and structural properties by undergoing continuous remodeling process to adapt to the demands that are placed on the skeleton (Feng and McDonald, 2011). Bone remodeling is triggered by different factors including mechanical loading (muscular and/or cyclical), growth, injuries and various local or systemic cytokines, chemokines and hormones (Burr et al., 2002; Hsieh et al., 2001; Robling et al., 2002; Sample et al., 2010; Schriefer et al., 2005; Srinivasan et al., 2002; Warden et al., 2007). Of these, mechanical loading of bones plays a key role in the development and maintenance of bone mass and strength (Kannus et al., 1996). In response to loading demands during active remodeling, old or damaged bone is resorbed by osteoclasts and replaced by new bone formed by osteoblasts (Feng and McDonald, 2011).

Physical activity, mechanical stress, or pathological states may influence muscle and bone simultaneously (Tagliaferri et al., 2015). The effects of disuse or overuse on both muscle strength and bone mass are well known, with disuse causing muscle wasting and bone loss, and overuse increasing muscle strength and bone mass (Burr, 1997). However, in subjects with normal or elevated bone density, it has been hard to determine the effect of muscle strength on bone mass due to indistinct correlations between muscle

strength and bone mass (Burr, 1997; Frost, 1987). The extent to which mechanical loading dominates the processes of bone gain versus loss over nonmechanical factors appears related to the combination of other variations, including cytokine levels which are altered with mechanical loading of the skeleton (Frost, 1987; Rodan, 1991; Westerlind et al., 1997).

Osteoactivin (OA) is a type I glycoprotein that is expressed in most adult tissues under normal conditions. OA is involved in many biological processes, including cell differentiation, tissue regeneration and inflammation (Rose and Siegel, 2010), and plays a significant role under conditions of injury and repair of the musculoskeletal system. In bone, OA was initially identified by its overexpression in osteopetrotic rat femurs

(Safadi et al., 2001). In an animal fracture model, OA was expressed during the healing process, further supportive of a role for OA in bone formation and repair (Abdelmagid et al., 2010). In skeletal muscle, OA increases after denervation or distraction osteogenesis injury, indicating that OA contributes to muscle regeneration (Furochi et al., 2007a; Furochi et al., 2007b; Ogawa et al., 2005; Tonogai et al., 2015). Possible mechanisms for OA's contribution to muscle regeneration include increased expression of anti-fibrotic genes (e.g., increased glypican-1 and decorin-1 mRNA in muscles after denervation in OA transgenic mice) (Furochi et al., 2007b), or as an inducible feedback repressor of inflammation that acts at every level of the inflammatory cascade (Ripoll et al., 2007). A more recent study reported that the inhibition of OA expression by OA siRNA dramatically suppressed the expression of pro-inflammatory factors TNF- $\alpha$  and IL-1 $\beta$  in activated microglia, showing involvement of OA in brain inflammatory responses (Shi et al., 2014). However, the expression of OA in association

with upper extremity overuse injuries has been examined in only one study to date that is still under review for publication (Chapter 3; Frara et al, submitted).

Cyclical loading and high force loads are both known to affect bone quality (Gross and Srinivasan, 2006; Robling et al., 2002; Rubin et al., 2002). A small number of studies have examined changes occurring in upper extremity bones as a consequence of occupational tasks. Increased incidence of hand/wrist osteoarthritis and reduced bone mass has been identified in female dentists and teachers with heavy or one-sided hand workloads (Ding et al., 2010; Solovieva et al., 2005; Vehmas et al., 2005). Bone scans of patients with upper extremity musculoskeletal disorders show increased blood flow and pooling in affected forearm bones (al-Nahhas et al., 1997; Amorim et al., 2006). Premenopausal women with carpal tunnel syndrome, a type of upper extremity musculoskeletal disorder, have significantly decreased bone mineral density in the distal radius, ulna, and metacarpal bones, compared to control subjects (Erselcan et al., 2001). Such changes can increase osteopenia and fracture risk, and effective treatments need to be developed. The 2014 National Occupational Research Agenda emphasizes the need for research to develop effective preventions of work-related musculoskeletal disorder (WMSD) induced deterioration of tissues (OSHA, 2014).

Barbe and Barr have previously developed a voluntary rat model of repetitive reaching and grasping that permits the examination of both tissue and behavioral responses to non-weight bearing muscular loads on bone and mechanisms of naturally occurring inflammation in upper limb tissues (Barbe et al., 2008; Barbe et al., 2013; Barbe et al., 2015; Barr et al., 2003; Driban et al., 2010; Jain et al., 2014). Rats performing a high repetition high force (HRHF) task for 2 hours/day, 3 days/week for 18

weeks showed transient increases in TNF- $\alpha$  that peaked in serum and muscles at 6 weeks (Fisher et al., 2015) (See Fig. 4-1 for muscle findings). TNF- $\alpha$  is a key pro-inflammatory cytokine known to promote tissue catabolism (Feldmann et al., 1994; Grounds and Torrisi, 2004; Williams et al., 2000), and may be one contributor to forelimb bone resorption and muscle strength declines reported in 12-week HRHF rats (Barbe et al., 2015; Jain et al., 2014). A two week treatment of rats performing a HRHF task with an

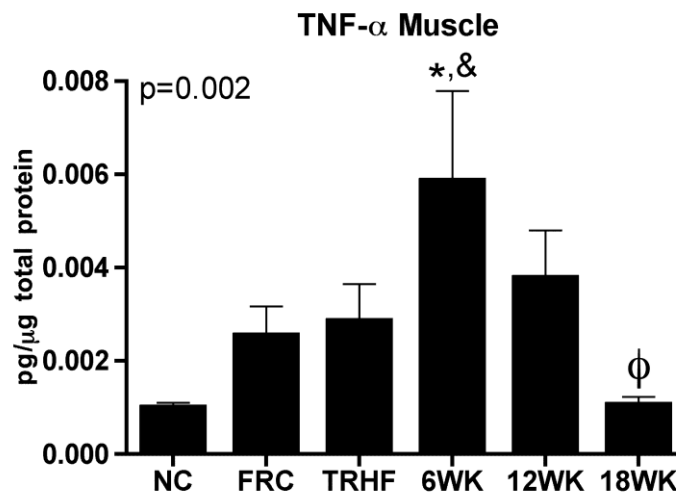
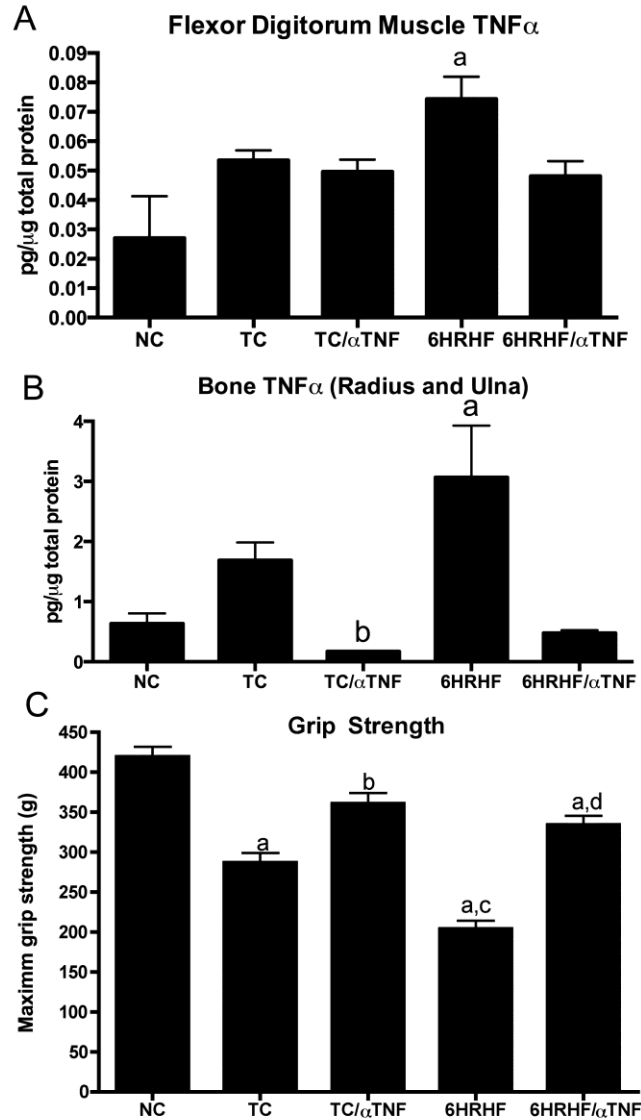


Figure 4-1. Levels of the pro-inflammatory cytokine, TNF $\alpha$ , in forearm flexor digitorum muscles, analyzed using ELISA. Data is shown for normal control (NC), food-restricted controls (FRC), trained only (TRHF), and 6-, 12-, and 18-week high repetition high force (HRHF) rats. ANOVA value shown. \*:p<0.05 compared to NC; &;p<0.05 compared to FRC;  $\phi$ : p<0.05 compared to 6-week HRHF rats. Used by permission from Fisher et al, 2015.

anti-rat TNF- $\alpha$  drug lead to decreased protein levels of TNF- $\alpha$  in forelimb bone and muscles by week 6, and partially rescued concomitant grip strength declines (Rani et al., 2010) (Fig 4-2). Based on the previously published data shown in Figures 4-1 and 4-2 , we hypothesized that provision of the anti-rat TNF- $\alpha$  drug for 4 weeks rather than two

weeks, and in a time frame that included the 6 week peak of TNF- $\alpha$ , might be an effective intervention in this model.



**Figure 4-2. Anti-TNF intervention for 2 weeks reduced tissue TNF $\alpha$  levels and partially rescued grip strength declines in high repetition high force (HRHF) task animals.** Data is shown for normal controls (NC), trained controls (TC) with or without anti-TNF treatment (TC/ $\alpha$ TNF and TC, respectively), and 6-week HRHF rats with or without anti-TNF treatment (6HRHF/ $\alpha$ TNF and 6HRHF, respectively), provided in weeks 5 and 6 before euthanasia. Levels TNF $\alpha$  in flexor digitorum muscles (A), and distal radius and ulna (B), analyzed using ELISA. (C) Forelimb grip strength. <sup>a</sup>p<0.001 compared to NC; <sup>b</sup>p<0.01 compared to TC; <sup>c</sup>p<0.001 compared to TC; <sup>d</sup>p<0.001 compared to 6-week HRHF rats. Used by permission from Rani et al, 2010.

Therefore, in this study, we evaluated motor strength, tissue morphometry and the presence of OA and inflammatory cytokines in forelimb muscles and bones of young adult rats that had performed a HRHF task for 11 weeks, with or without treatment with either an anti-TNF- $\alpha$  drug or task cessation (i.e. rest) provided in task weeks 4-7, compared to saline treated HRHF rats. We hypothesized that adaptive remodeling in musculoskeletal tissues undergoing prolonged repetitive loading at high force loads is modulated by a superimposed inflammatory response, specifically an increased TNF- $\alpha$  response known to enhance catabolic tissue changes. We further hypothesized that anti-TNF- $\alpha$  treatment or task cessation (rest) provided in weeks 4-7 of an 11-week HRHF task paradigm would alter the balance towards anabolic responses, such as increased OA production and tissue growth. We also expect these interventions to relieve the forelimb motor strength declines that may also be contributing to the bone loss.

## **4.2 Materials and Methods**

### **4.2.1 Animals**

All experiments were approved by the Temple University Institutional Animal Care and Use Committee (Temple University IACUC) in compliance with NIH guidelines for the humane care and use of laboratory animals. Rats were housed individually in the central animal facility in transparent plastic cages in a 12 hour light: 12 hour dark cycle with free access to water. Studies were conducted on 56 young adult (3.5–4 months of age at onset of experiments), female, Sprague-Dawley rats (See Fig. 4-3). All rats were weighed once to twice per week, provided regular rat chow daily, and

allowed to gain weight over the course of the experiments, since they were young adult rats. All rats were provided cage enrichment toys (chew bones and tunnels) across the weeks of the experiment.

#### ***4.2.2 Training, Behavioral Task and Treatment***

##### *4.2.2.1 Behavioral apparatuses*

Sixteen custom-designed behavioral apparatuses were used for these experiments. The apparatus consisted of a open field box from Med Associates (St. Albans, VT) with plexiglas walls and top, and rounded bars on the bottom on which the rats stood, located 2 mm above a solid plastic base. This box was placed into a sound proof chamber (Med Associates). The front of the box was outfitted with a customizable metal region with a portal located at the rats' shoulder height (Med Associates). The rats reached through the shoulder height portal and isometrically pulled on a 1.5 mm in girth metal bar, termed a force lever, that was attached to a load cell (Futek Advanced Sensor Technology, Irvine, CA) positioned 2.5 cm outside of the chamber wall. The bar was oriented vertically. The load cell output was interfaced with a signal conditioner (Analog Devices, Norwood, MA), which amplified and filtered the signal before it was sampled digitally at 100 Hz with Force Lever software (Med Associates). The load cell was interfaced with custom written Force-Lever software that allowed us to choose a set force level before a food reward was provided (version 1.03.02, Med Associates). Every 15 seconds, a series of auditory indicators (Stimulus Clicker; Med Associates) lasting 5 seconds cued the animal to attempt a reach. Auditory and light indicators cued the reaching rate (defined below). During this period, the animal had to grasp the force lever bar and pull toward the

chamber wall with a graded effort of a percentage of the maximum grip strength of control rats (rats not included in this study) for at least 50 milliseconds. If reach and force criteria (defined below) were met within a 5 second cueing period, a 45 mg food pellet was dispensed into a trough located at floor height for the animal to lick up.

#### *4.2.2.2 Training to learn the high force task*

Prior to the initiation of the experiments, all rats were handled for 10 minutes/day for 1 week to acclimate rats to the handlers. Rats were initially food-restricted for 7 days to no more than 10-15% less than their naive weight or the weights of age-matched normal control rats with free access to food, to initiate interest in food reward pellets. After that week, they were given extra rat chow to gain weight quickly back to only  $\pm 5\%$  than age-matched normal control rats.

Thirty-two HRHF rats underwent an initial training period in which they learned the task. During the training period, the rats moved through several stages of training. First, in week 1 of training, they were placed in a plastic box outfitted with a piece of Plexiglas with a portal located at shoulder height connected to a small plastic trough. In this chamber, the rats were introduced to the 45 mg food pellets that served as food reward (a 1:1 mix of grain-based and banana-flavored food pellets). Using calibrated force transducers, we estimate that retrieval of the 45 mg food pellets required a negligible amount of force ( $< 5\%$  of the rats' maximum grasping force). When the rats learned to reach (without a specified reach rate) into a trough for the food pellets, a time period of typically 3 days, they were moved to the custom-designed operant conditioning chambers (Med Associates) described above. In the operant chambers, rats learned with

the aid of auditory and light cueing to reach through a shoulder-height portal within the apparatus to isometrically pull the force lever attached to a force transducer. Using a magazine style training, in which the force lever started within the chamber for the remainder of week 1, the rats learned to grasp the force lever bar and exert an isometric pull of approximately 1% of their maximum voluntary force, without any specified repetition rate for a food reward. In week 2, the force lever bar was moved to 2.5 cm outside of the chamber and the rats were required to grasp the force lever and exert an isometric pull of 0.10 Newton's (11 grams; approximately of their maximum voluntary pulling force) without any specified repetition rate for a food reward. In week 3, they were required to pull at 0.29 Newton's (30g; approximately 15% of their maximum voluntary pulling force), without any specified repetition rate for a food reward, and in week 4, at 0.58 Newtons (60g; approximately 30% of their maximum voluntary pulling force). By the end of the 5 week training period, the rats were able to perform the high repetition high force (HRHF) task of four reaches/min at 1.93 Newtons (110g; approximately 45-55% of their maximum voluntary force). All rats used their forelimbs in a bilateral manner during this training period.

#### *4.2.2.3 HRHF task regimen*

The thirty-two trained rats went on to perform the HRHF task regimen for 2 hrs/day, 3 days/week for up to 11 weeks. The task was divided into 4, 0.5-hr sessions separated by 1.5 hours in order to avoid satiation. HRHF rats were cued to reach at a rate of 8 reaches/min and to grasp the force handle at a target force effort of  $55\% \pm 5\%$  (which equals 120 to 128 grams, and 1.02 to 1.12 Newtons). HRHF rats had to grasp the

force handle and exert an isometric pull at the target level for at least 50 milliseconds to receive a food reward, as described previously (Barbe et al., 2013).

Trained observers tracked changes in spontaneous behaviors occurring during each period of HRHF task performance (30 min/task period; 4 times/day beginning at 9:30 am; 3 days/week). Bilateral pulling of the lever bar was recorded upon occurrence, as was a switch in forearm used to pull the lever bar. All rats used their forelimbs in a bilateral manner during this HRHF task period, either by switching the limb used to pull from week to week (except during the rest period of course), or performed bilateral pulling with both forelimbs simultaneously.

#### *4.2.2.4 Secondary Treatments Provided During the Task Regimen*

As shown in Figure 4-3, after three weeks of HRHF task performance, the HRHF rats were divided into 4 groups of n=8/group. Two groups of rats continued to perform the HRHF task for the entire 11 weeks, with one receiving five intraperitoneal injections of saline (HRHF+Veh) across weeks 4-7, to parallel its partner group (HRHF+anti-TNF) that received anti-rat-TNF $\alpha$  antibody (CNTO 1081; generously provided by Janssen R&D) as five injections across weeks 4-7, as described previously (Rani et al., 2010). Briefly, this drug was dissolved in saline and then injected intraperitoneally (i.p., 15 mg/kg body wt, the recommended dose) into HRHF rats in the middle of task week 4, at the end of task week 4, and then an injection at mid-task week for the next 3 weeks (for a total of 5 injections over 4 weeks). The other two groups of HRHF rats rested during weeks 4-7. One group received five intraperitoneal injections of saline (HRHF+Veh/Rest) to parallel its partner group (HRHF+anti-TNF/Rest) that received anti-rat-TNF $\alpha$  antibody treatments as described above for HRHF+anti-TNF rats. After

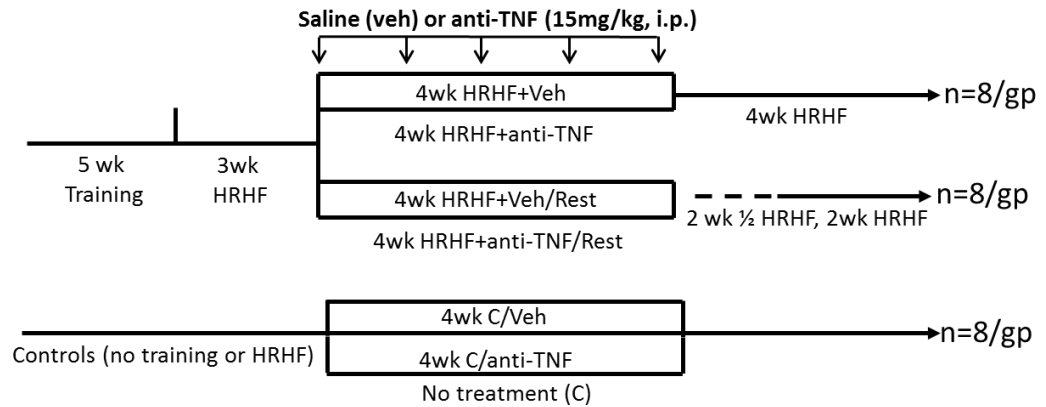
this 4 week intervention period, the HRHF+Veh/Rest and HRHF+anti-TNF $\alpha$ /Rest groups performed the HRHF task for two sessions per day (i.e., a reduced 1/2 work day) for two weeks, and then the HRHF task for four sessions per day (the full work load) for the remaining 2 weeks. All rats were euthanized at matched time points of HRHF week 11. One animal in the HRHF+anti-TNF group died approximately 10 min after the last anti-TNF injection and was removed from further analysis.

Twenty-four rats that were not trained and that did not perform the HRHF task regiment served as age-matched controls for this second experiment. Eight received 5 injections of saline across 4 weeks (C+Veh, n=8), at the same time points as HRHF+Veh rat. Eight received 5 injections of anti-TNF $\alpha$  injections at the same doses and time points as HRHF+anti-TNF rats (C+anti-TNF, n=8). The remaining control rats were not treated.

#### ***4.2.3 Motor Function Behavioral Assay***

Reflexive grip strength of the forelimbs was measured on the day of euthanasia in all rats, bilaterally, at baseline (the naïve time point), after training (the 0 week time point of the HRHF task), and every 6 weeks thereafter, as described previously (Barbe et al., 2003; Clark et al., 2004; Clark et al., 2003; Elliott et al., 2010). Briefly, rats were lifted gently by the tail and allowed to grasp a rigid bar attached to a force transducer and digital display unit (Stoelting Dale, Staelting Co. Wood Dale, IL). When the first signs of active grasp were observed, the rats are pulled upward slowly by the tail with increasing firmness until their grasp was overcome. The peak force was recorded as maximum grip strength. The test was repeated 3–5 times/limb, and maximum grip strength per trial included in the statistical analysis.

## Intervention Dosing Design



**Figure 4-3. Experimental design.** Experimental design showing onset of food restriction. Over a 5 week period, the percentage of maximum pulling force required to pull a force lever for a food pellet reward was increased each week as indicated. These rats went on to perform a high-repetition, high-force (HRHF) task for 3 weeks. Then, animals were randomized into four intervention groups: 4 weeks of HRHF plus saline (vehicle) injections (i.p.; 5 times over 4 weeks), 4 weeks HRHF plus anti-TNF $\alpha$  antibody (15 mg/kg body weight, i.p.; 5 times over 4 weeks), 4 weeks of rest plus i.p. saline injection, 4 weeks rest plus anti-TNF $\alpha$  antibody injections. After return to work, rest intervention rats performed the task for 2 sessions/day (1/2 day work) for 2 weeks, followed by 2 weeks at full workload (4 sessions/day). The other two HRHF groups performed the task at full workload in the last 4 weeks. Control rats were either treated with saline (vehicle) or anti-TNF $\alpha$  antibody in the same time period, or were left untreated. Muscle and bone tissue was collected from all rats at euthanasia and assayed using biochemical or histological methods.

### 4.2.4 MicroCT Imaging and Analysis

Animals were euthanized by lethal overdose (sodium pentobarbital, 120 mg/kg body weight) 36 hours after their last task session. Since all HRHF rats in the study used their limbs bilaterally (either switching limbs to pull on the lever bar, or pulling with both forelimb bilaterally), the left forelimb was removed from both HRHF and control rats prior to perfusion for biochemical analysis. Then rats were perfused transcardially with 0.9% saline and then fixed with 4% paraformaldehyde in 0.1M PO $_4$  buffer, pH 7.4, for

microCT (all right limbs, n=7-8/group). Twenty-four hours prior to the micro-CT analysis, forelimb bones from the right limbs were collected, cleaned of soft tissues, and stored in phosphate buffered saline (PBS) with sodium azide until micro-CT analysis of the radius, before being processed for histological examination. Skyscan volume rendering software (CTVox) and analysis software (CTAn) was used to render the 3D models and transaxial sections. Micro-CT analysis was performed according to recent guidelines (Bouxsein et al., 2010) and as previously described (Barbe et al., 2013; Jain et al., 2014). The radius and ulna bones were scanned from their distal ends proximally towards the elbow, to a diaphyseal site that was 10 mm proximal from the growth plate, using a SkyScan 1172, 12 megapixel, high-resolution cone-beam micro-CT scanner (Bruker, Kontich, Belgium), using the following settings: a pixel resolution size of 5.89  $\mu\text{m}$ , x-ray source spot size of 300 nm, Al 0.5mm filter, voltage of 59 kV, current of 167  $\mu\text{A}$ , rotation step of 0.40°, frame averaging of 5. Each scan was approximately 45 minutes per set of forelimb bones. During reconstruction of the images using cone-beam reconstruction software based on the Feldkamp algorithm (Skyscan NRecon), a ring artifact correction of 10, and a beam hardening correction of 60% were applied to all samples. This process yielded more than 2700 tomographic sections, 5.89  $\mu\text{m}$  in thickness, in the axial plane, for each set of forelimb bones. Using the Skyscan CT Analyzer (CTAn) software, two regions of interest (ROI) of the radius were delineated using a region of interest tool, and then binarized separately. The trabecular bone ROI for the distal radial metaphysis was chosen for analysis as we have reported previously that it undergoes more morphological changes than the ulnar bone (Barbe et al., 2015; Jain et al., 2014). This radial ROI was defined from 1.5 mm below the center of the growth plate

and extending proximally for 1mm. The volume of interest (VOI) for the trabecular microarchitecture variables was a consistent circle shape within a few pixels of the endocortical margin. The saved data sets were segmented into binary images. Because of a low noise and the relative high resolution of the data sets, we used simple global thresholding methods. For trabecular bone, an upper threshold of 255 and a lower threshold of 95 were used.

Trabecular morphometric traits of the distal radial metaphyses were then computed from binarized images using direct 3D techniques that do not rely on prior assumptions from the underlying structures. Trabecular bone volume per total volume (BV/TV), mean trabecular thickness (Tb.Th), mean trabecular number (Tb.N), and mean trabecular separation (Tb.Sp) indices were measured in 3 dimensions (3D) where 0 represent isotropic organization. The person carrying out the microCT analyses was blinded to treatment and age.

#### ***4.2.5 Muscle and Bone Histomorphometry***

Flexor digitorum muscles were collected from the same limbs as for micro-CT (n=7-8 rats/group). After transcardial fixation and then immersion fixation for at least 24 hours, a 3 mm thick cross-sectional piece of the flexor digitorum muscle was cut, with a scalpel, from the proximal end of the muscle mass. The muscles were cryosectioned into 12  $\mu\text{m}$  sections, placed onto charged and coated slides (Fisher Scientific, Tissue Path Superfrost Plus Gold Slides), dried, and stained with hematoxylin and eosin (H&E). The cross-sectional area and circumference of the cross-sectional piece of the flexor digitorum muscle was quantified using a Nikon microscope interfaced with a digital

camera and image analysis system (Bioquant Osteo 2012, v12.1), and is reported in micrometers or micrometer<sup>2</sup>.

Bones assayed for micro-CT were used for histomorphometry according to the recommendations of the American Society for Bone and Mineral Research (Dempster et al., 2013; Parfitt et al., 1987). Forearm bones from each group were decalcified in a 14% acid free EDTA solution), then underwent paraffin embedding as previously described (Jain et al., 2014). Bones were sectioned into 5  $\mu\text{m}$  longitudinal sections, placed onto charged and coated slides (Fisher Scientific, Tissue Path Superfrost Plus Gold Slides), and dried at 60°C overnight. Slides were stained with Goldner's Trichrome for counting osteoblasts, or for tartrate resistant acid phosphatase (TRAP; Sigma kit **387A**) for counting osteoclasts. For all histomorphometric analyses of the trabecular bone described below, we targeted the same area as analyzed with microCT. This area was the distal radial metaphysis located 100  $\mu\text{m}$  below the chondro-osseous junction of the secondary spongiosa using a 20x objective, a Nikon E800 microscope interfaced with a Q-Imaging digital camera, and an image analysis system (Bioquant Osteo 2012, v12.1). Osteoblast numbers per bone surface (N.Ob/B.S), osteoblast volume per bone volume (Ob.V/BV), and osteoblast surface per bone surface (Ob.S/B.S) on trabecular surfaces was determined in Trichrome stained sections, and numbers of osteoclasts (TRAP+) per bone surface (N.Oc/B.S) and osteoclast surface per bone surface (Oc.S/B.S) were counted in TRAP stained sections, on trabecular surfaces using the same image analysis program as above. Only multinucleated osteoclasts on the bone surface with 3 or more nuclei were counted. The person carrying out the histomorphometry was blinded to treatment.

#### ***4.2.6 Tissue Biochemical Assays***

Since all HRHF rats in the study used their limbs bilaterally (either switching limbs to pull on the lever bar, or pulling with both forelimb bilaterally), the left forelimb was removed prior to perfusion, and the flexor digitorum muscles were separated from the bone, tendon and nerve tissues with a scalpel, and flash frozen in liquid nitrogen before storage at -80°C until homogenization. Muscles were homogenized as previously described (Barbe et al., 2008). Muscle homogenates were assayed using customized multiplex bead-based analysis kits (LXSARM, Luminex R&D Systems, Minneapolis, MN) for CINC-2ab, CINC-3, GM-CSF, IL-1 $\alpha$ , IL-6, IL-18, VEGF, IFN $\gamma$ , IL-1 $\beta$ , IL-10, and TNF $\alpha$ .

At the time of tissue collection, the metaphysis of each rat's left forelimb bones (radius and ulna) was separated from the diaphysis using bone scissors (Tough Cut Surgical Scissors, Fine Science Tools), and flash frozen in liquid nitrogen before storage at -80°C until homogenization. Bone segments were powdered and homogenized, separately, and were assayed using a customized multiplex bead-based analysis kit (RECYTMAG-65K, Millipore Corporation, Billerica, MA) for monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNF- $\alpha$ ), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1 $\alpha$ ), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-18 (IL-18), interferon gamma (IFN $\gamma$ ), interferon gamma-induced protein 10 (IP-10), macrophage inflammatory protein 1 alpha (MIP-1 $\alpha$ ), macrophage inflammatory protein 2 (MIP-2), lipopolysaccharide-inducible CXC chemokine (LIX), and keratinocyte chemoattractant (GRO/KC). Bone homogenates were also assayed for OA using

commercially available DuoSet ELISA Development kit (DY2330, R&D Systems, Minneapolis, MN). ELISA was conducted using the manufacturer's protocol.

Each sample was run in duplicate in a blinded manner. The optical absorbance of the solution was measured at 450 nm using a micro-plate reader (iMark, Bio-Rad). The concentration of each protein present in the bone homogenates was quantified from the slope standard curve estimation and normalized to  $\mu\text{g}$  of total protein which was determined using a bicinchoninic acid (Pierce BCA Protein assay #23225, Thermo Scientific, MA).

#### ***4.2.7 Statistical Analyses***

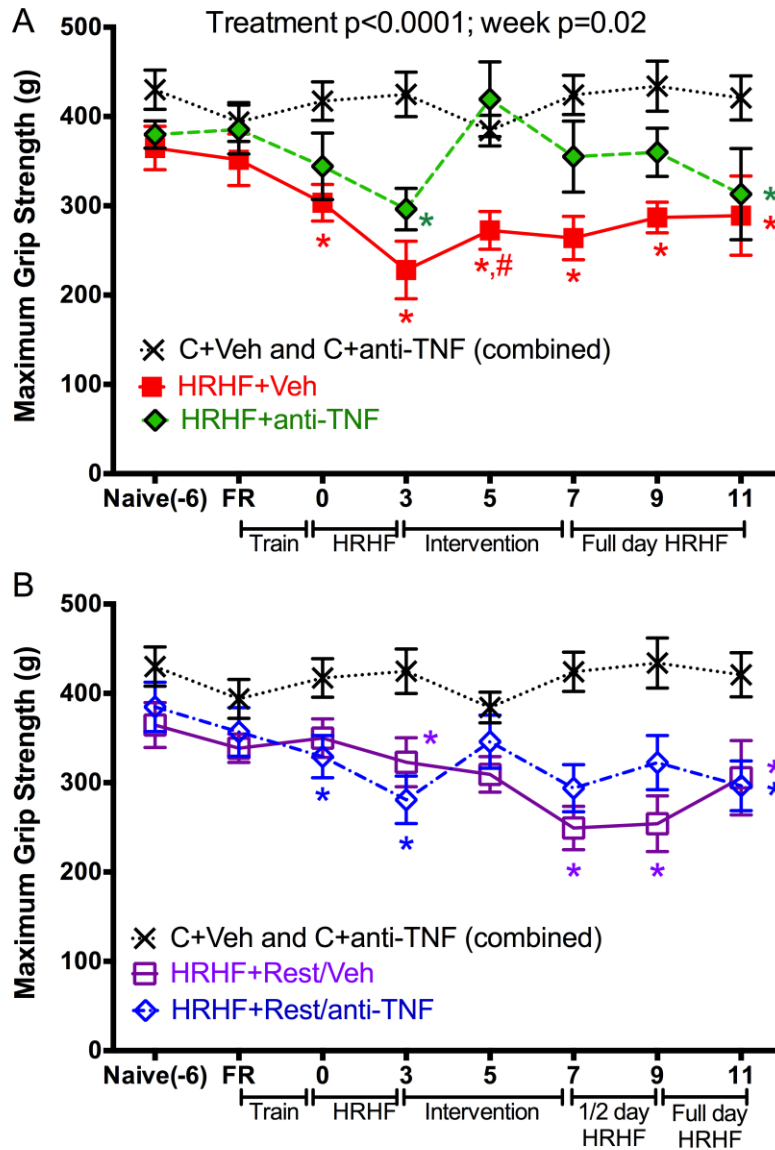
GraphPad PRISM v.6.02 was used for the statistical analyses. All data are expressed as mean  $\pm$  SEM. P values of  $< 0.05$  were considered significant for all comparisons. Grip strength was analyzed using two-way ANOVA and the factors week and intervention type. One-way ANOVA was used to compare results from the muscle and bone multiplex cytokine analysis. During the post-hoc ANOVA analyses for between group differences, the Bonferroni correction method was used to reduce the chances of obtaining false-positive results (type I errors) when multiple pair wise tests are performed on a single set of data.

### **4.3 Results**

#### ***4.3.1 Anti-TNF Treatment Maintains Grip Strength throughout Task Performance***

Motor behavior was assessed throughout the study and revealed decreased grip strength in HRHF+Veh rats immediately post training (week 0) and thereafter, compared

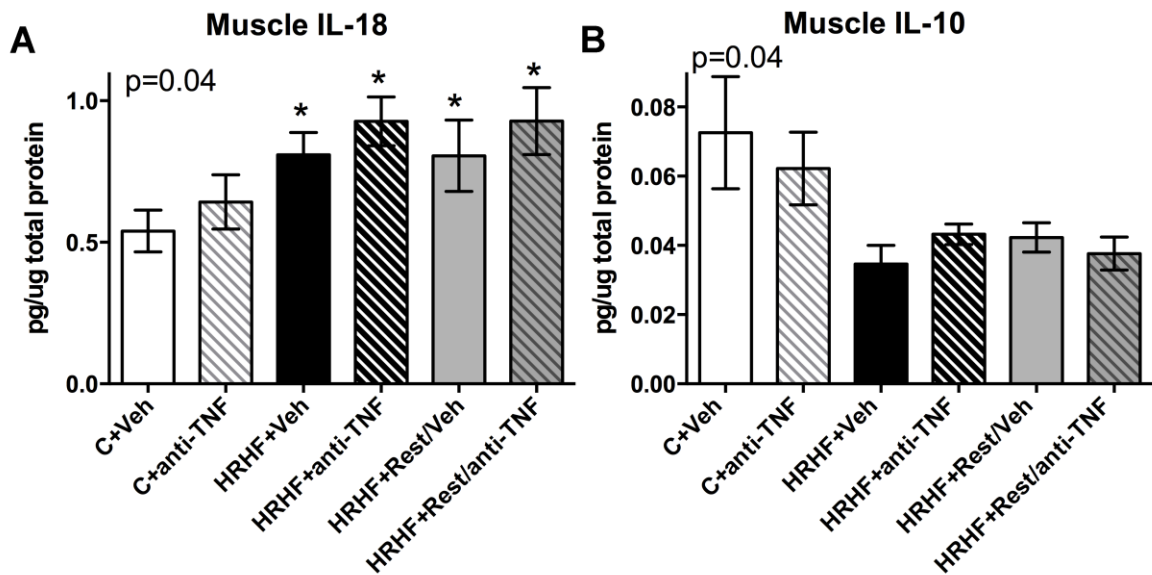
to controls (Fig. 4-4A). In contrast, rats treated with the anti-rat-TNF $\alpha$  antibody in task weeks 4-7 (HRHF+anti-TNF) maintained their grip strength at levels indistinguishable from age-matched control rats until week 11 at which point they declined again. Rats allowed 4 weeks of rest plus saline treatment (HRHF+Rest/Veh) or rest plus anti-rat-TNF treatment (HRHF+Rest/anti-TNF) also did not exhibit significant grip declines in week 5 (Fig. 4-4B). However, this rescue was not maintained in the HRHF+Rest/Veh rats by week 7. Similar to anti-TNF treated only rats, HRHF+Rest/anti-TNF rats maintained their grip strength at levels indistinguishable from age-matched control rats until week 11 at which point they declined again. These data indicate that the 4-week anti-TNF- $\alpha$  treatment prevents grip strength declines in rats performing a HRHF task, that this treatment extends maintenance of grip strength near control levels through week 9, despite no further treatment after week 7 (as shown in the design slide, Fig. 4-3). Rest alone did not improve grip strength.



**Figure 4-4. Grip strength changes across weeks of task performance.** (A) Maximum reflexive grip strength is shown for control rats (C+Veh and C+anti-TNF treated rats were combined, as there were no significant differences between these groups), HRHF rats that received saline (HRHF+Veh), and HRHF rats that received anti-TNF treatment (HRHF-anti-TNF). (B) Maximum reflexive grip strength is shown for control rats, HRHF rats treated with rest and saline (HRHF+Rest/Veh), or HRHF rats treated with combined treatments (HRHF+Rest/anti-TNF). Grip strength is reported at native (-6 weeks), after the 1<sup>st</sup> week of food restriction (FR), post training (0 weeks), at end of task week 3 (which was 1 day after first anti-TNF $\alpha$  injection), after the treatments (task week 7), week 9 (end of 1/2 work days for rest groups) and week 11 (end of experiment). \*= $p < 0.05$  and \*\*= $p < 0.01$ , compared to C+Veh; #= $p < 0.05$  compared to HRHF+anti-TNF rats.

### 4.3.2 Muscle IL-18 Levels Increase in all Task Performing Groups, Regardless of Treatment

To examine for muscle cytokine changes, homogenates of flexor digitorum muscle from the reach limb were analyzed using multiplex ELISA (Fig. 4-5). Muscle levels of IL-18 were elevated in each HRHF treatment group, compared to the control groups (Fig. 4-5A). In contrast, muscle levels of the anti-inflammatory IL-10 were decreased in all groups, compared to control (Fig. 4-5B).

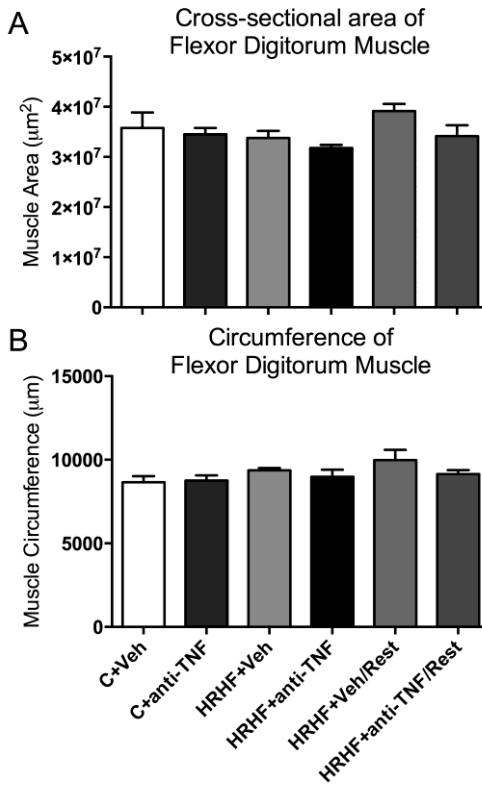


**Figure 4-5. Effectiveness of anti-TNF intervention on cytokine levels in flexor digitorum muscles.** Muscle levels of (A) Interleukin (IL)-18 and (B) Interleukin (IL)-10 were evaluated using multiplex ELISA. \* $p < 0.05$ , compared to C+Veh.

### 4.3.3 HRHF Loading, with and without Intervention, has no Effect on Size of Flexor Digitorum Muscle

We investigated the area and circumference of the flexor digitorum muscles in an effort to determine if muscle growth were occurring as a consequence of prolonged task

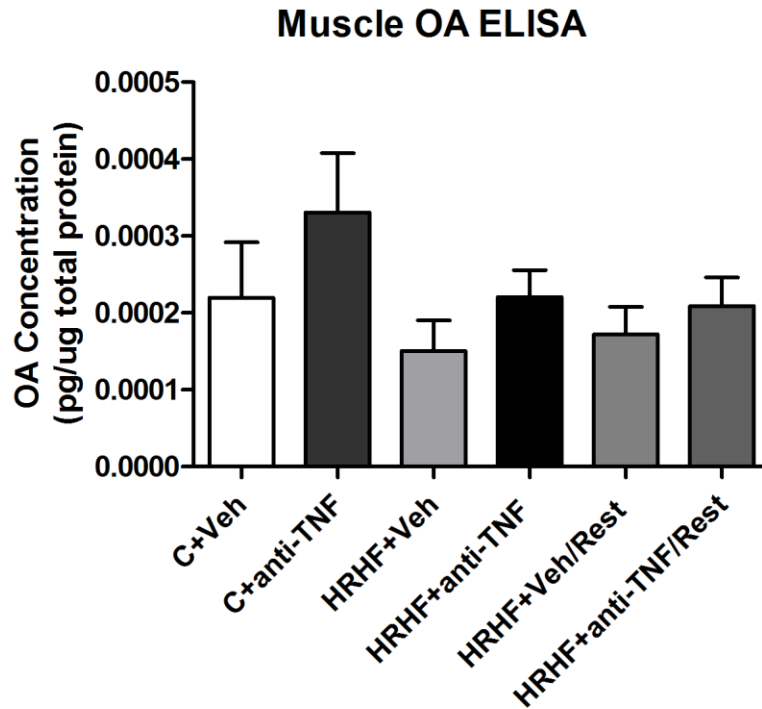
performed. The cross-sectional size and circumference of this muscle, at its widest point (near the elbow after being cut into a cross-sectional slice) was similar in all tested groups (Figure 4-6A and B).



**Figure 4-6. Flexor digitorum muscle size** (A) Mean cross-sectional area and circumference of the flexor digitorum muscle, quantified proximally near the elbow at its widest point was measured using an image analysis system in all experimental groups of rats. No difference was observed between the groups.

#### ***4.3.4 The Prolonged Task Performance and Anti-TNF Treatment Have No Effect OA Levels in Reach Limb Muscle***

ELISA analysis of flexor digitorum muscles showed no task-induced changes in OA protein level, compared to controls (Fig. 4-7).

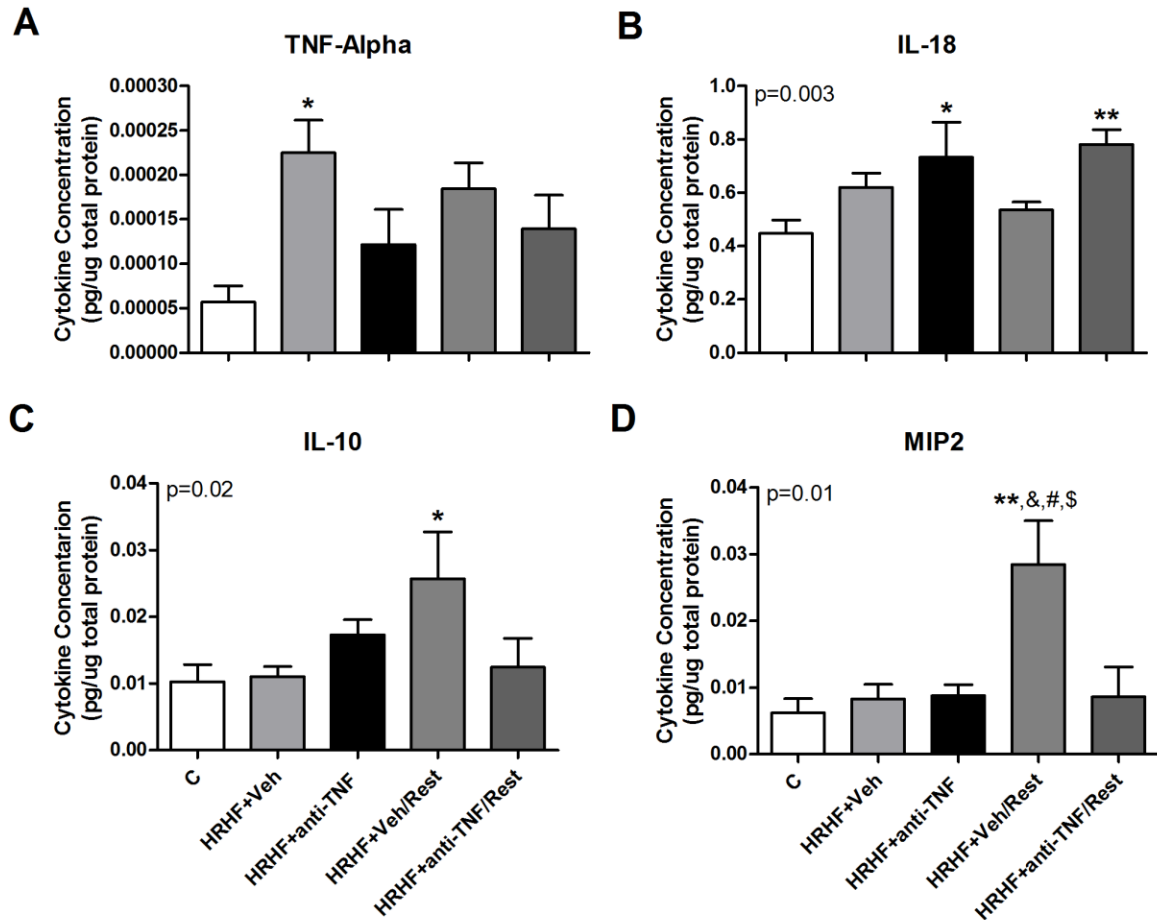


**Figure 4-7. Osteoactivin (OA) levels in muscles.** OA levels in flexor digitorum muscles of all groups with or without intervention were tested using ELISA. No difference was observed between the groups.

#### ***4.3.5 Bone Cytokine levels Increase Differentially with Treatment Paradigm***

To examine for bone cytokine changes, forelimb bone tissue homogenates were analyzed using multiplex ELISA (Fig. 4-8). Levels of TNF- $\alpha$  were increased in HRHF+Veh rats, compared to control rats (C+Veh) (Fig. 4-8A). In contrast, levels of IL-18 were increased in HRHF+anti-TNF and HRHF+anti-TNF/Rest rats, compared to control rats (Fig. 4-8B). Interestingly, bone levels of IL-10 and MIP2 were elevated only in HRHF+Veh/Rest rats, compared to controls and to the other task groups, respectively (Fig. 4-8C and D).

## Bone Cytokines

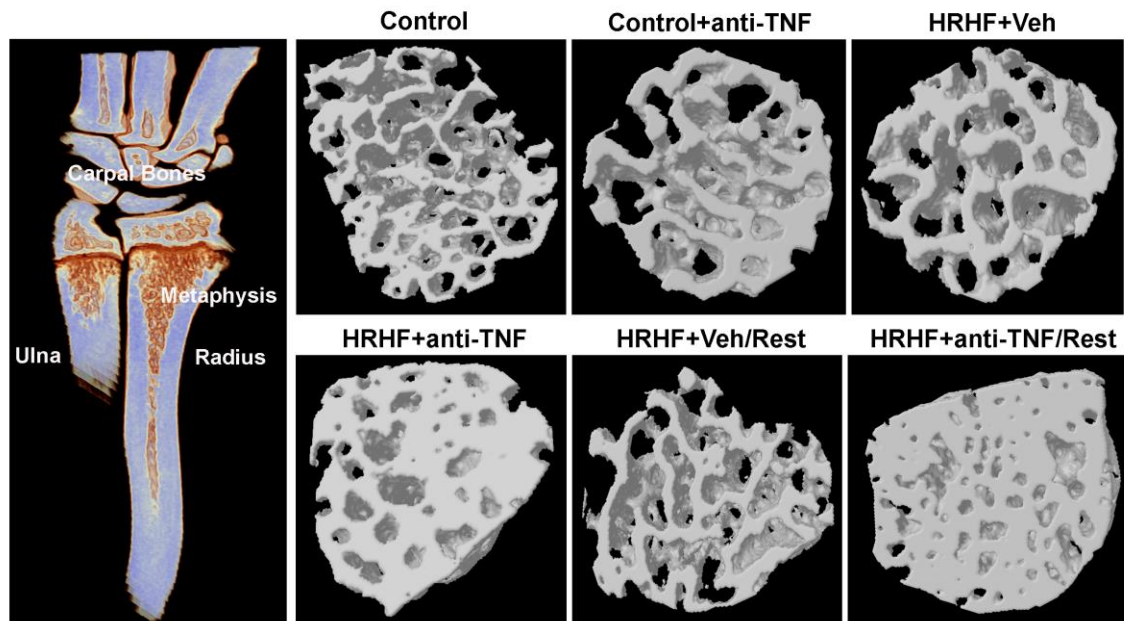


**Figure 4-8. Effectiveness of secondary interventions on cytokine levels in forelimb bones.** Bone levels of: (A) TNF-Alpha, (B) IL-18, (C) IL-10, and (D) MIP2 were assayed using multiplex ELISA. \* $p < 0.05$  and \*\* $p < 0.01$ , compared to Control, respectively; & $p < 0.05$ , compared to HRHF+Veh; # $p < 0.05$ , compared to HRHF+anti-TNF; \$ $p < 0.05$ , compared to HRHF+anti-TNF/Rest.

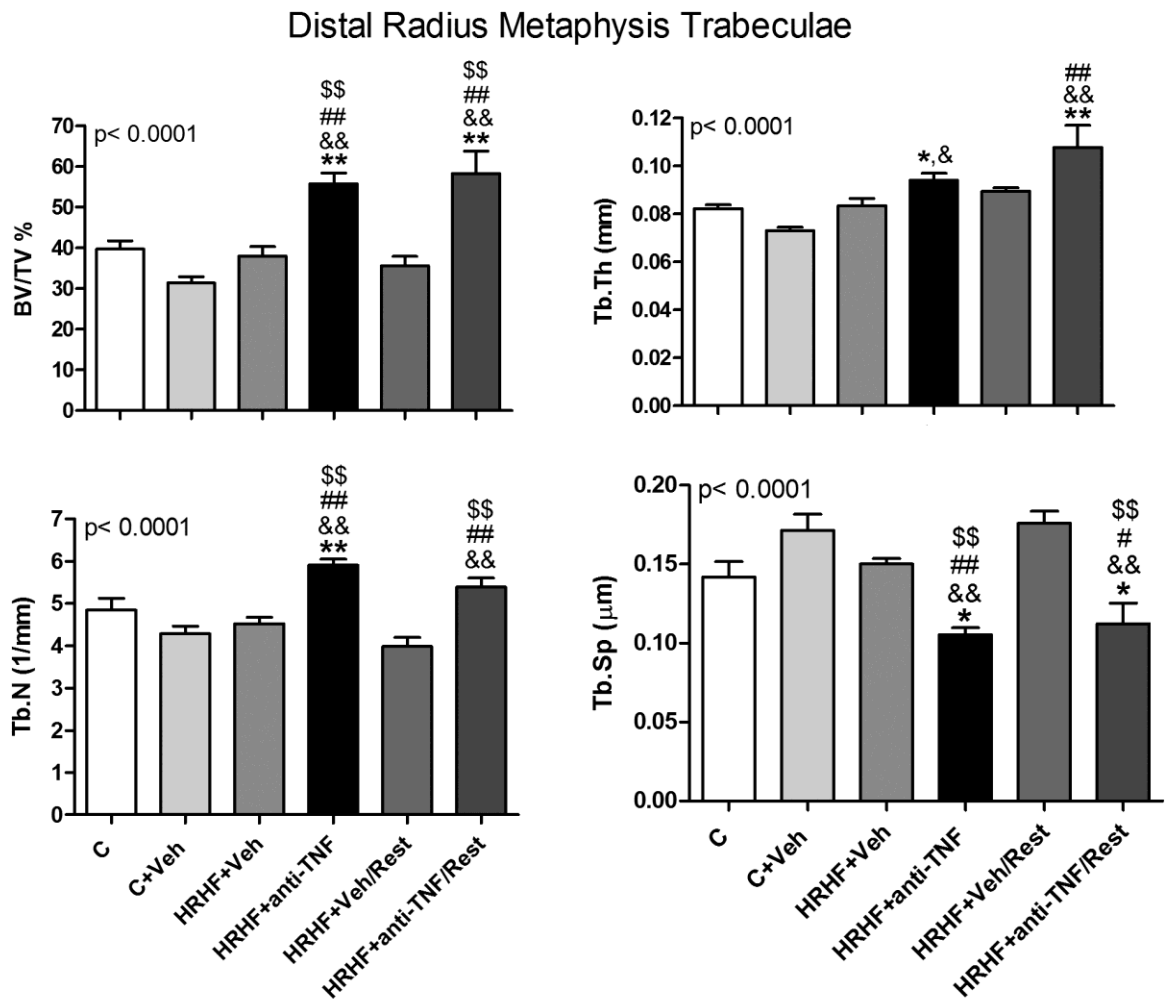
### *4.3.6 Anti-TNF- $\alpha$ Treatment Combined With Long Term Loading at HRHF Loads Leads to Increased Bone Mass of Distal Radial Metaphyseal Trabeculae*

Possible changes in the distal radial trabecular microarchitecture that could be caused by bone adaptation due to performing the HRHF task for 11 weeks were evaluated

using micro-CT (Fig. 4-9 and 4-10). Micro-CT images of distal metaphyseal regions of anti-TNF treated HRHF group showed increased bone, compared to other groups, whether or not that is combined with rest (Fig. 4-9). We observed increased trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Th.N) in anti-TNF treated HRHF rats, with or without rest, compared to controls or HRHF rats without treatments (Fig. 4-10). In contrast, there was a reciprocal reduction in trabecular separation (Tb.Sp) in the treated group, compared to controls or untreated rats.



**Figure 4-9. Micro-CT images of distal radius and ulna.** At left is a representative 3D model of the distal radius, cut sagittally, showing the metaphyseal region assay (labeled metaphysis). Representative 3D models of transaxial micro-CT slices through the metaphyseal region of the radius of a control, control+anti-TNF, HRHF+Veh, HRHF+anti-TNF, HRHF+Veh/Rest, and HRHF+ anti-TNF/Rest rats, with the surrounding cortical bone segmented away from the cortical bone in order to show the trabecular architecture. The transaxial reconstructions are located from 1.0 to 2.0 mm proximal to the respective growth plates, and are viewed from the bottom looking towards the growth plate.

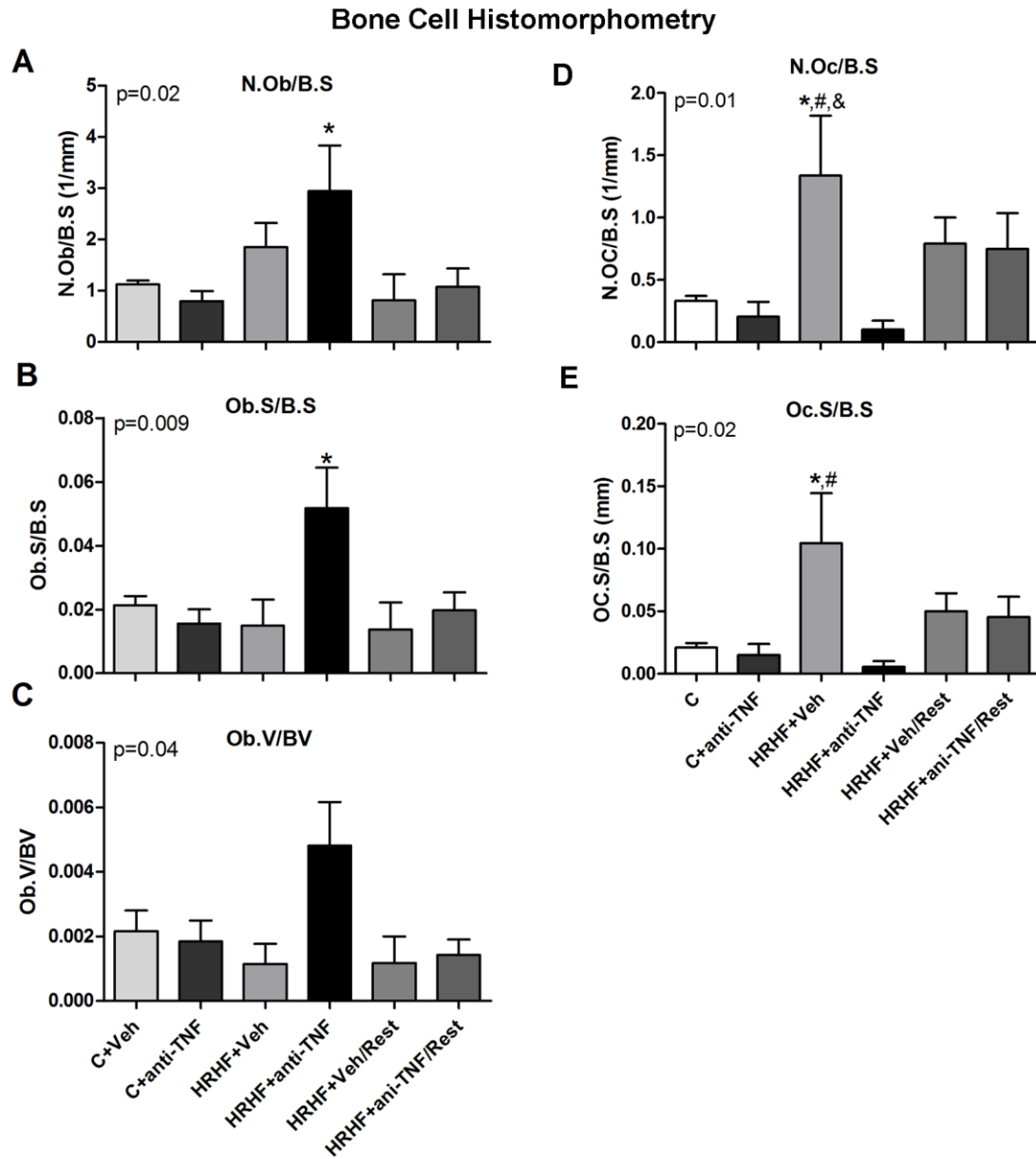


**Figure 4-10. Micro-CT analysis of trabeculae of distal radial metaphyses.** Results for trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N.), and trabecular separation (Tb.Sp.) are shown. \*:p<0.05 and \*\*:p<0.01, compared to control rats, respectively; &:p<0.05 and &&:p<0.01, compared to C+Veh; #:p<0.05 and ##:p<0.01, compared to HRHF+Veh; \$:p<0.05 and \$\$:p<0.01, compared to HRHF+Veh/Rest rats.

#### ***4.3.7 Anti-TNF Treatment Ameliorated HRHF Loading-Induced Increases in Osteoclasts in Distal Metaphyseal Trabeculae and Enhanced Osteoblast Numbers***

To determine if the higher bone mass in anti-TNF treated rats was due to an increase in bone formation or a decrease in bone resorption, we investigated the distal

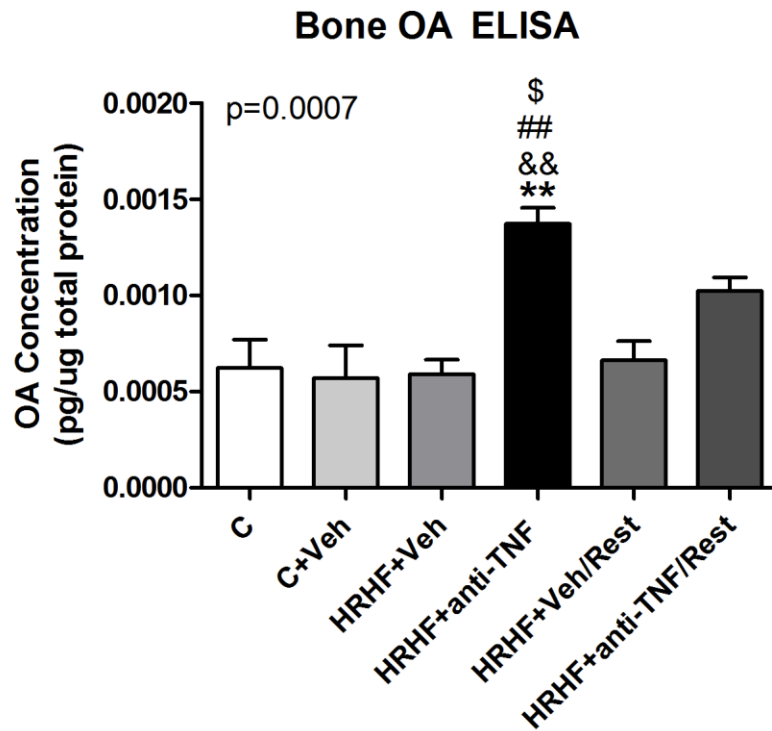
radial metaphyses for changes in osteoblast and osteoclast numbers. Histomorphometric analysis following Masson's Trichrome staining showed an increase in osteoblast numbers per bone surface (N.Ob/B.S) and osteoblast surface per bone surface (Ob.S/B.S) in HRHF+anti-TNF rats, compared to the control groups (Fig. 4-11A and B). Although, osteoblast volume per bone volume (Ob.V/BV) was increased in the HRHF+anti-TNF group, that increase was not statistically significant (Fig.4-11C). Histomorphometric analysis following TRAP staining showed an increased in osteoclast numbers per bone surface (N.Oc/B.S) and osteoclast surface per bone surface (Oc.S/B.S) in HRHF+Veh rats compared to controls (Fig. 4-11D and E). These findings suggest that the anti-TNF treatment both increased bone formation and decreased in osteoclast activity. Unfortunately, these bones were processed for paraffin sectioning rather than plastic sectioning, and dynamic bone formation parameters that could be garnered from calcein labeling could not be assessed.



**Figure 4-11. Bone cell histomorphometry in distal radial trabeculae.** Density of osteoblasts (N.Ob.) and osteoclasts (N.Oc.) normalized to bone surface (B.S), of distal radial metaphyseal trabeculae. Osteoblast surface per bone surface (Ob.S/B.S), osteoblast volume per bone volume (Ob.V/BV), and osteoclast surface per bone surface (Oc.S/B.S) were measured at the distal radial metaphysis of reach limb. \* $p<0.05$ , compared to Control; # $p<0.05$ , compared to C+anti-TNF; & $p<0.05$ , compared to HRHF+anti-TNF.

#### 4.3.8 Bone OA Levels Increase by Task Performance for 11 weeks only with Anti-TNF Treatment

ELISA analysis of forelimb bones showed task-induced increases in OA protein level, compared to controls (Fig. 4-12). These data suggest that the anabolic activity of OA in bone was enhanced by the down-regulation of TNF-alpha signaling.

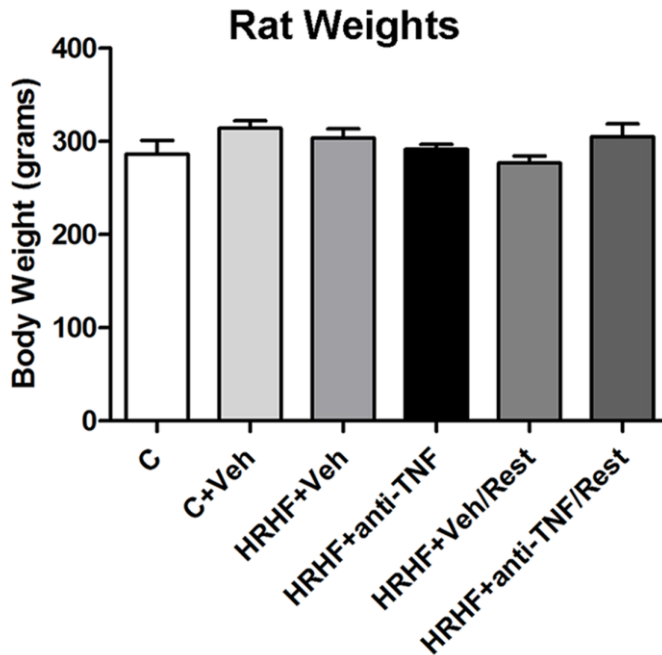


**Figure 4-12. Osteoactivin (OA) level in high repetition high force (HRHF) preferred limb bones.** OA levels in forelimb bones of all task groups with or without intervention, were tested using ELISA. \*\*p<0.01, compared to Control (C); &&p<0.01, compared to C+Veh; ##p<0.01, compared to HRHF+Veh; \$p<0.05, compared to HRHF+Veh/Rest.

#### 4.3.9 Rat Weights

A two-way ANOVA indicated that there were no differences in weight were observed between task performing groups, compared to control rats at the day of

euthanasia (Fig. 4-13B). Thus, any changes observed in bone structure were not due to changes in body weight.



**Figure 4-13. Rat body weights over time.** Rats were weighed at the time of euthanasia. Body weight measured in grams (g). No differences were observed.

#### 4.4 Discussion

Since TNF- $\alpha$  is a key cytokine that regulates inflammation (Guo et al., 2008; Kaneki et al., 2006; Rani et al., 2010; Williams et al., 2000), we examined the effects of blocking TNF- $\alpha$  in our model. In this manner, we extended our prior studies using our rat model of work-related upper extremity musculoskeletal disorders to examine behavioral, inflammatory, morphological, and OA expression changes following an 11 week high repetition high force (HRHF) loading paradigm, in combination with anti-inflammatory drug, task cessation (rest), or both provided as secondary interventions in task weeks 4-7.

Grip strength declines in HRHF+Veh rats were prevented by the anti-TNF- $\alpha$  treatment, alone or in combination with rest. Muscle levels of IL-18 were increased in all task groups, but were not affected by the interventions. No muscle hypertrophy, no gain in grip strength above control levels, and no increase in OA were observed in any HRHF treatment group, compared to controls. In contrast, distal forelimb bones showed great responsiveness to the 11 weeks of intensive loading, rest and anti-TNF- $\alpha$  treatment, with regards to inflammatory cytokine levels, morphology and OA expression. For example, an expected decrease in TNF- $\alpha$  and increase in OA was observed in anti-TNF- $\alpha$  treated rats, while an unexpected increase in IL-18 was observed in all HRHF groups, regardless of treatment. Micro-CT analysis showed that rats receiving anti-TNF- $\alpha$  treatment, with or without rest, had increased bone mass, compared to the other groups. Histomorphometry showed increased osteoblasts and decreased osteoclasts in HRHF+anti-TNF rats, compared to HRHF+Veh rats, indicative of increased bone anabolism with anti-TNF- $\alpha$  treatment. This is our first study to identify an intervention that significantly rescued HRHF-induced bone loss. These data suggest that the anabolic activity in bone of OA may be enhanced by the down-regulation of TNF- $\alpha$  signaling.

Behavior motor assays revealed decreased grip strength in HRHF rats immediately post training (HRHF task week 0), presumably due to cytokine-enhanced inflammatory pain since the grip strength declines were rescued by the anti-TNF- $\alpha$  treatment. These results are in line with previous studies from our group reporting that increased muscle inflammatory cytokines, including TNF- $\alpha$ , correlate with declines in grip strength (Barbe et al., 2008; Coq et al., 2009). The four-week treatment with anti-TNF- $\alpha$  in this study more successfully rescued grip strength to control levels than in a

prior study in which this same drug was provided for only two weeks (Rani et al., 2010). The observed bone tissue inflammation may also have contributed to the grip strength losses, since forearm muscles and tendons involved in performing this reaching and task are attached to distal forearm bones. Since no changes in muscle girth were observed across the groups, we assume that increased inflammation, but not changes in muscle size, are contributing to the grip strength declines. This is supported by findings that treatment of subjects with subcutaneous TNF- $\alpha$  receptor before unaccustomed exercise improves muscle strength (Rice et al., 2008).

A modulated effect of the task or treatment on inflammatory cytokines in limb muscles and muscles was detected at 11 weeks of HRHF task performance. The observed increase of muscle and bone inflammatory cytokines in rats performing the HRHF task is consistent with prior studies from our lab examining the effect of this or related tasks on forearm tissues (Gao et al., 2013; Jain et al., 2014; Rani et al., 2010). We have also previously shown that a 2-week treatment with anti-TNF- $\alpha$  and an 8-week treatment with ibuprofen reduced HRHF-induced increases in muscle and bone inflammatory cytokines (Barbe et al., 2015; Driban et al., 2011; Jain et al., 2014; Kietrys et al., 2011; Rani et al., 2010). It has been reported that the integrated cytokine response to injury is complex and that tissue responses depend not only on absolute concentrations of TNF- $\alpha$ , but also on the simultaneous presence of naturally occurring cytokine inhibitors and anti-inflammatory cytokines (Ostrowski et al., 1999). The local response to tissue injury involves the production of cytokines released at the site of inflammation. These cytokines facilitate an influx of monocytes and other cells, which participate in the clearing of antigens and healing of tissue.

Micro-CT data indicated that rats performing the HRHF task for 11 weeks, without anti-TNF- $\alpha$  treatment, showed no changes in the distal radius metaphysis trabeculae, indicative of no bone adaptation. The increased osteoclast numbers in the HRHF+Veh group of rats is likely due to the increased TNF- $\alpha$  expression in this group. Increased bone TNF- $\alpha$  is known to stimulate osteoclastogenesis and activity, and to alter the balance between bone formation and bone resorption in favor of bone resorption (Huang et al., 2003; Kulkarni et al., 2012; Nanes, 2003; Ochi et al., 2010; Yamazaki et al., 2009; Zhang et al., 2001).

We observed a decrease in interleukin (IL)-10 in reach limb muscles of all task groups, compared to controls. However, forelimb bones showed increased IL-10 only in the HRHF+Veh/Rest group. These data are suggestive of inflammatory process in bones, and that the systemic anti-inflammatory treatment prevented HRHF-induced increases in bone IL-10. This is consistent with previous reports of IL-10 as an anti-inflammatory cytokine with the ability to inhibit the release of TNF- $\alpha$  and other pro-inflammatory cytokines (Chernoff et al., 1995). Ostrowski et al. also reported that IL-10 has multiple effects in inflammation and its production increases immediately after prolonged exercise to balance exercise-induced increases in pro-inflammatory cytokines, such as TNF- $\alpha$  (Ostrowski et al., 1999).

We also observed increases in IL-18 in 11-week HRHF muscles, regardless of treatment, yet increases in bone IL-18 were only detected only in HRHF rats that had received the anti-TNF- $\alpha$  treatment. IL-18 is a cytokine that is secreted by a variety of cell types, including macrophages, antigen presenting cells, chondrocytes, osteoblasts and fibroblasts (Nakanishi et al., 2001). IL-18 is secreted by activated macrophages to

regulate the promotion of T cell (Th1) immune responses (Okamura et al., 1995). It has potent immunomodulatory effects which can be pleiotropic, complex, and paradoxical in various disease processes (Reddy, 2004). Patients with upper extremity soft tissue disorders showed increased serum IL-18 at early stages (Rechardt et al., 2011). It has been reported that IL-18 expression increases in patients with rheumatoid arthritis and osteoarthritis (Shao et al., 2009), and Marotte et al found that TNF- $\alpha$  can regulate the expression of IL-18 in rheumatoid arthritis synovial fibroblasts (Marotte et al., 2010). IL-18 has been shown to promote smooth muscle cell migration, such as in angiogenesis (Amin et al., 2010; Park et al., 2001), and to play an important role in cutaneous wound healing (Kampfer et al., 1999). While the increase in IL-18 in our study was unexpected, its increase in muscles and bones of anti-TNF- $\alpha$  treated rats supports a role for IL-18 in repair processes in this model.

Others have reported that bone responds to loading along a continuum ranging from anabolism to catabolism, depending on the magnitude, frequency and duration of loading (Bentley et al., 2007; Gross et al., 2010; Gross and Srinivasan, 2006; Srinivasan et al., 2002). Significant losses in bone mass (observed in decreased BV/TV, Tb.Th. and Tb. N.) have been reported in our model after 12 weeks of HRHF task performance (Barbe et al., 2013; Barbe et al., 2015; Jain et al., 2014). Here, examination of trabecular bone parameters, using micro-CT methods, showed an increase in bone mass in rats that had received the anti-TNF- $\alpha$  treatment, either alone or combined with rest. TNF- $\alpha$  is known to stimulate bone resorption and inhibit bone formation, with inflammatory bone loss partly due to inhibition of osteoblasts (Kaneki et al., 2006); therefore, one would expect to see increased bone formation when TNF signaling is blocked. This result was

confirmed by histomorphometric analyses that showed increases in the number of osteoblasts in the anti-TNF- $\alpha$  treated group. However, no significant differences in the trabeculae were detected between saline-treated HRHF rats versus controls, at the time of tissue collection at week 11 (4 weeks after provision of the interventions), despite an increase in osteoclast numbers. The 11-week end point used in this study may be the onset of these catabolic losses. The increased bone mass in the anti-TNF treated rats may also be linked to the increase in OA levels, as it is known as an osteogenic factor that enhances bone formation (Abdelmagid et al., 2014; Frara et al., 2015; Moussa et al., 2014).

Increases in OA expression levels also were detected in forelimb bones after anti-TNF treatment. Our group has shown that OA acts in bone tissue as an osteogenic factor that enhances bone formation and osteoblast differentiation (Abdelmagid et al., 2008; Frara et al., 2015; Safadi et al., 2001). OA has also been associated with repair and inflammation (Abdelmagid et al., 2010; Ripoll et al., 2007). Perhaps OA is acting as a feedback regulator of inflammation by upregulating IL-18 production and consequently limiting other pro-inflammatory cytokines and contributing to bone formation and repair.

Because muscle and bone are biomechanically linked (Edwards et al., 2013; Gross et al., 2010), it is often assumed that greater physical strength is associated with a greater load on the bone, and corresponding increases in bone mass (Daly et al., 2004; Schoenau and Frost, 2002). Therefore, we investigated the size of the flexor digitorum muscles, and we found that its cross-sectional area and circumferences were similar in all tested groups, regardless the treatment. This indicates that a change in muscle size (either hypertrophy or atrophy) was not a contributing factor to the observed bone changes. One

study showed that factors other than muscle size accounted for 12-16% of the variance in differences in bone traits, such as bone mass, such as factors associated with loading that are distinct from muscles (Daly et al., 2004). Our results suggest that inflammatory cytokine and OA expression changes occurring with HRHF loading are contributing to the observed bone changes in the radius.

There are several limitations in this study. One limitation of this study is that we did not examine other bony sites of the upper extremity for bone changes. The effectiveness of these intervention should also be repeated in more mature female rats (we studied young adult female rats to the point of musculoskeletal maturity (7 months of age), and in male rats. Lastly, we used an operant rat model of overuse. The effectiveness of these secondary interventions should be repeated in human subjects.

#### **4.5 Conclusion**

This is our first study to identify an intervention that significantly rescued HRHF-induced bone loss. A strong role for task-induced inflammatory processes in muscle and bone tissues, as well as motor declines, is supported in our model. TNF- $\alpha$  suppresses bone formation through upregulation of inflammatory cytokines in our rat model, where bone signaling pathways mediated possibly by other proteins such as OA are activated during the course of inflammation and loading.

## CHAPTER 5

### CONCLUSIONS AND FUTURE DIRECTIONS

As reviewed in Chapter 1, our group has reported that osteoactivin (OA) is expressed in normal bone, bone growth and remodeling and increased in osteoblasts during fracture healing (Abdelmagid et al., 2010; Abdelmagid et al., 2008a; Safadi et al., 2001a; Singh et al., 2010). High levels of OA expression in bone were initially described in a rat model of osteopetrosis in which its expression was increased during osteoblast differentiation (Safadi et al., 2001). We showed localized OA protein as associated predominately with osteoblasts lining trabecular bones *in vivo*, and indicated that local injection of recombinant OA increased bone mass in a rat model (Abdelmagid et al., 2010; Singh et al., 2010). In another study, recombinant OA supported bone regeneration and formation in a rat critical-size calvarial defect model (Bateman et al., 2012). Our *in vitro* studies documented the novel role of OA in osteoblast differentiation and function. We showed that OA expression has a temporal pattern during osteoblast differentiation, being highest during matrix maturation (Abdelmagid et al., 2008; Owen et al., 2003; Safadi et al., 2001; Selim et al., 2003; Selim et al., 2007). Using loss-of-function and gain-of-function approaches in osteoblasts, we also showed that OA overexpression increases osteoblast differentiation and function and that OA down-regulation decreases nodule formation, alkaline phosphatase (ALP) activity *in vitro* (Abdelmagid et al., 2008). Our *in vitro* studies further reported on the positive role of OA in mesenchymal stem cell (MSCs) differentiation into osteoblasts (Arosarena et al., 2011). In another study, we

showed that recombinant OA protein induces higher osteogenic potential of fetal-derived MSCs, compared with bone marrow-derived MSCs (Raynaud et al., 2012).

In Chapter 2, as part of my dissertation work, I and collaborators described how the over-expression of OA affects bone mass *in vivo* in a transgenic mouse model, and regulates osteoblast differentiation *ex vivo*, using cells from these transgenic mice. Our aim was to determine if OA overexpression enhanced bone mass and increase osteoblast differentiation. Specifically, we sought to determine if bone mass increased in OA-Tg mice compared to wild type (WT), and if osteoblast differentiation increased in association with enhanced gene expression in OA-Tg osteoblasts compared to WT osteoblasts *in vitro*. We found that OA overexpression enhanced bone formation in the OA-Tg mice. The increased bone formation was due to increased osteoblast proliferation and activity (more bone formation) as well as a decrease in osteoclast numbers and activity. The osteoblast findings were confirmed by *in vitro* studies. The *in vitro* studies also showed that TGF $\beta$  levels and receptors were upregulated with OA overexpression, indicating that TGF $\beta$  signaling may be one reason by which OA enhances bone formation. Additional studies that investigate the downstream signaling proteins, such as Smads are warranted to determine whether the activated levels are altered in the OA-Tg osteoblasts.

Several studies have also postulated that OA is involved in tissue turnover during regeneration and degeneration. As mentioned above, OA increases in tissue matrices during fracture repair (Abdelmagid et al., 2010). It also influences the adhesion and migration of select cell types, including fibroblasts, key cell types involved in tissue

repair (Shikano et al., 2001). Previous studies showed OA expression is upregulated in damaged skeletal muscles (Furochi et al., 2007b). In denervated mouse skeletal muscle OA upregulates MMP-3 and MMP-9 in infiltrating fibroblasts (Ogawa et al., 2005). HSP70/72 another protein that plays a role in skeletal muscle repair or regeneration and adaptation after high-force eccentric exercise (Koh, 2002; McArdle et al., 2004; Paulsen et al., 2007), and increases concomitant with MMP-2 in skeletal muscle following high intensity training (Carmeli et al., 2010), apparently acting together to promote muscle matrix remodeling (Hirunsai et al., 2015).

Thus, Chapter 3 described, for the first time, the temporal and spatial expression of OA, as well as HSP72 (a known repair molecule) and MMPs (remodeling enzymes of ECM) in an *in vivo* model of upper extremity repetitive motion injuries. Our aim was to determine if OA is linked to repair processes in forelimb flexor digitorum muscles and tendons in rats performing a high repetition high force (HRNF) task for 3 to 6 weeks, and if injury/inflammatory processes are present in the tissues at the same time points. We hypothesized that we would observe increased OA expression in the overloaded skeletal muscles and tendons, as well as known mediators of repair, MMPs and HSP72. We found that OA increased concomitantly with HSP72 and a variety of MMPs in musculotendinous tissues in the rat model, supporting our hypothesis.

In addition to its role in injury and repair, OA also functions as an anti-inflammatory regulator by inhibiting the activation of T lymphocytes (Chung et al., 2007b) or by reducing the secretion of proinflammatory cytokines from macrophages (Ripoll et al., 2007). Furthermore, blocking TNF- $\alpha$  with antibodies successfully reduces

inflammation-induced pulmonary and liver fibrosis (Bargagli et al., 2008; Bhalla et al., 2002; Vassallo et al., 2002), and delays muscle breakdown in dystrophic muscle (Grounds and Torrisi, 2004). In our model, we have shown that provision of an anti-rat TNF- $\alpha$  drug during the early stages of inflammation attenuated HRHF task-induced increases of periostin like factor in bone (Rani et al., 2010), and fibrotic tissue responses in muscle (Abdelmagid et al., 2012). Therefore, it is possible that OA produced by muscle and bone tissues acts on immune effector cells and alleviates excessive proinflammatory responses.

Chapter 4 describes our findings regarding OA expression in our *in vivo* model of upper extremity repetitive motion injuries, except in this particular study, a high repetition high force (HRHF) paradigm was used and bone tissue was examined in addition to muscle tissue. Additionally, the effects of three interventions were examined: anti-TNF- $\alpha$ , cessation of work (i.e. rest), or a combined treatment of anti-TNF- $\alpha$  and rest, with each hypothesized to improve tissue repair and function. Our aim was to determine if OA expression is linked to inflammation and repair in a rat model of repetitive overuse in musculoskeletal tissue (muscle and bone) in young adult rats performing a HRHF task. Also, we sought to determine if a rat anti-TNF- $\alpha$  drug or task cessation (i.e. rest) provided in weeks 4-7 of an 11 week HRHF task paradigm would, by decreasing the inflammatory responses, increase OA expression in forelimb bones of rats performing the prolonged task. In bone, the hypothesis that OA may play a role in repair/bone formation processes was maintained. Bone formation increased as OA upregulated and with reduced inflammation. Surprisingly, we did not observe increased OA in muscle, nor did we see any signs of inflammation, injury (other than past signs of muscle damage) or

repair with growth (muscle did not hypertrophy). This is perhaps due to a lower force load used in this particular study (45% maximum loading force), as opposed to the 55% used in prior studies. In contrast to Chapter 3's findings in which OA expression increases progressively in forearm muscles with prolonged performance of HRNF task for up to 6 weeks (Chapter 3, Fig. 1A-G), no task-induced changes in OA protein level were detected by week 11 of HRHF task performance (Chapter 4, Fig. 7). We hypothesize that the peak of injury happened before tissue collection in week 11; presumably, at week 6 since that was the peak of cytokine production (Fisher et al., 2015). We have shown that TNF- $\alpha$ , a potent pro-inflammatory cytokine increases at this time point (i.e. 6 weeks of HRHF task performance) (Fisher et al., 2015). Since the expression of several pro-inflammatory cytokines is induced simultaneously during early time points of tissue repair (Karlmark et al., 2010), we suggest that the 6 week time point is the peak of inflammatory phase, and the beginning of the repair proliferative phase. We also suggest that OA is one of the factors mediating tissue repair and adaptation. Interestingly, we also observed an unexpected increase in IL-18 by week 11 of performance of the HRHF task (Chapter 4, Fig. 5A). Since IL-18 is a known growth factor involved in tissue repair processes, and since OA has been shown to act as a feedback regulator of inflammation, perhaps OA is acting to upregulate IL-18 production and tissue repair before tissue collection in week 11. Only examination of tissues from rats performing this 45% maximum pulling force task at earlier weeks, such as week 6, will answer this hypothesis.

It is important to understand at this point the difference between the food retrieval HRNF task used in Chapter 3 versus the handle-pulling HRHF task used in Chapter 4. An

obvious difference between the two tasks is the force level (< 5% of maximum of maximum grip strength in the HRNF task versus 40-45% of maximum pulling force in the HRHF task), which does not help explain the differences in the findings. A less obvious difference, is that the food retrieval task is perhaps a more difficult task because it requires precision/pinching (Sommerich et al., 2007), while the lever bar pulling task allows that rats to pull on the lever with any finger and keeps the forepaw in a single position, as the lever bar is in a fixed position. Performing a HRNF pinching task for 6 weeks in both rats and humans creates postural hand constraints and has been shown to increase fatigue significantly within forearm and hand muscles. Fatigue can be described as a metabolic change in muscle with increased oxidative metabolites and free radicals, which in turn lead to increased cytokine production. The increase in Hsp72 in the 6-week muscles is supportive of task-induced increased oxidative metabolites and free radicals, as these physiological changes are known inducers of Hsp72 production.

Returning to our Chapter 4, HRHF task induced tissue changes. We have previously reported frank degradation of radial and ulnar trabecular bone in our model, after 12 weeks of performing a HRHF task at 55-60% of the rats' maximum pulling force (Barbe et al, 2013; Jain et al, 2014; Barbe et al, 2015). We did not see such bone degradation in the saline-only treated rats performing a HRHF task at 40-45% of the rats' maximum pulling force for 11 weeks. Nor, did we see any sign of bone anabolism. It has been reported that the response of bone to loading alternates between anabolism and catabolism, depends on the magnitude, frequency and duration of loading (Bentley et al., 2007; Gross et al., 2010; Gross and Srinivasan, 2006; Srinivasan et al., 2002). Our current results, compared to our past results seem to suggest that 55% is beyond the radial bone's

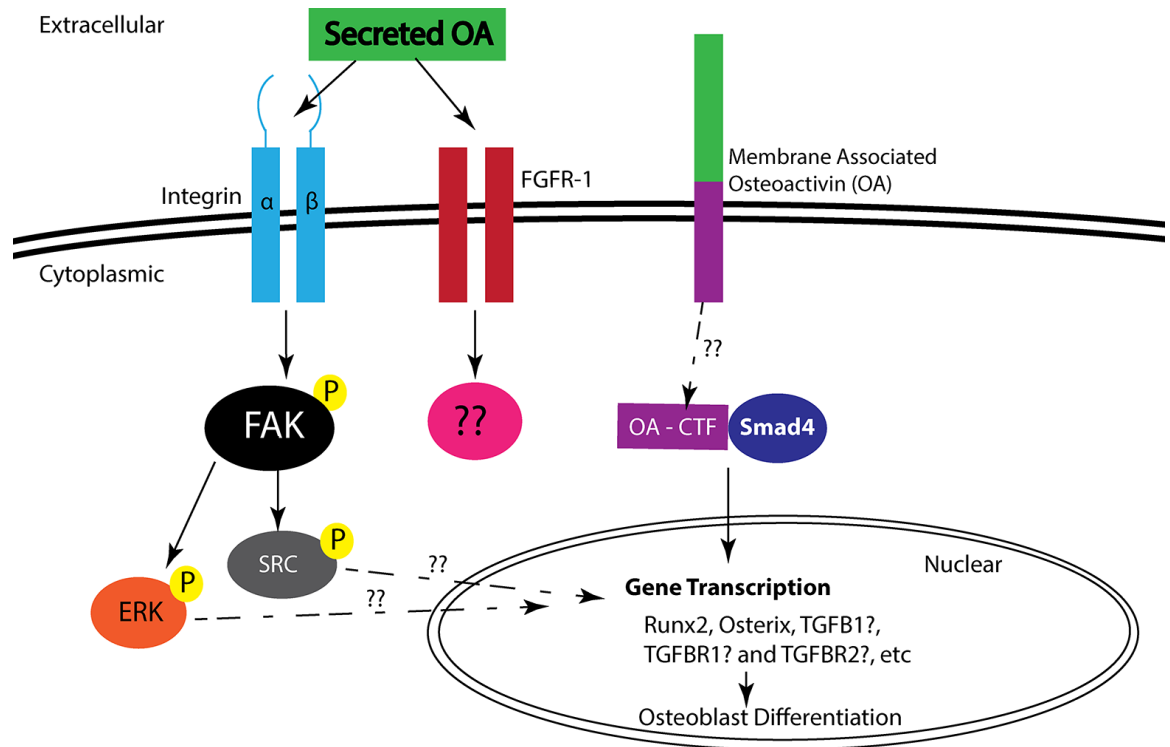
ability to adapt to long term repetitive loading, while the 45% load is within the levels of adaptation.

Chapter 4 also shows that a 4 week anti-TNF- $\alpha$  treatment in rats performing a HRHF task at 40-45% of the rats' maximum pulling force, allows bone anabolism. The mostly likely explanation for the bone anabolism is the anti- TNF- $\alpha$  treatment induced decrease in osteoclast numbers and activity. The anti- TNF- $\alpha$  treatment also increased OA levels, which further enhances bone anabolism through osteoblasts, since, as shown in Chapter 1, an increase in OA expression enhances osteoblast proliferation and differentiation leading to increased bone formation (Frara et al., 2015). The combined decrease in osteoclast numbers and activity, and an increase in osteoblast proliferation and activity apparently altered the balance in bone homeostasis in favor of bone anabolism.

Also in Chapter 4, there were two groups treated with rest, with or without an anti- TNF- $\alpha$  drug. Forelimb bones in the rest-only treated group showed no differences from control levels, perhaps because of a lack of loading for 4 weeks when the rats were at rest. When loading resumed in week 7, osteoblast activity was stimulated again, although apparently, osteoblast activity had returned to homeostatic levels by week 11. Only examination of bones from the mid-point of this loading regimen will answer this question. When rats in which rest was combined with anti-TNF- $\alpha$  treatment in weeks 4-7, bone anabolism was increased above control levels, compared to control rats. Again, this may be due to anti- TNF- $\alpha$  induced reductions in osteoclast activity and an anti- TNF- $\alpha$  induced increase in OA production. Further studies are needed to answer this interesting question.

Thus, our findings clearly demonstrate, through different studies, a novel function of OA as a repair protein and an anabolic factor that induces osteoblast differentiation and bone formation, and as a contributor to muscle repair. The bone findings are consistent with earlier studies suggesting that OA can act to promote bone growth and remodeling and increased in osteoblasts during fracture healing (Abdelmagid et al., 2010; Abdelmagid et al., 2008; Safadi et al., 2001; Singh et al., 2010). Based on the observations made in the current study, we propose a model (Fig. 5-1) in which OA stimulates osteoblast differentiation through the upregulation of specific transcription factors. Here we report that OA upregulated by different factors, such as overexpression and loading stresses was cleaved by proteases at the cell surface and within the cell membrane. As a type I transmembrane protein, OA gets activated by proteolysis on the cell surface (ectodomain shedding), as do several type I transmembrane proteins (Nanba et al., 2003; Arribas and Borroto, 2002). This ectodomain proteolysis results in the formation of intracellular and extracellular OA fragments. The carboxy-terminal fragment of OA (OA-CTF) is cytoplasmic and upon cleavage, possibly, after interacting with smad4, it translocates to the nucleus and acts as a transcriptional regulator of osteoblast specific genes such as Runx-2, Osterix, TGF- $\beta$ 1 and receptors I and II (Nanba et al., 2003; Kovall, 2007). The secreted form which has different molecular weights (as we detected in the western blots) becomes active and modulates gene expression by induction of a complex of transcription initiation proteins. This could possibly offer an explanation into the up regulation of osteoblast differentiation markers, beyond any possible signaling, through integrins (which could also be causing osteoblast differentiation through activation of FAK and consequently, activation of either ERK or

SRC). The other possible mechanism whereby the secreted OA functions is through binding to fibroblast growth factor (FGFR)-1, which has been reported to induce OA expression (Ogawa et al., 2005; Dell’Era et al., 2002), and signals through an unknown pathway to mediate cell proliferation and differentiation and consequently promotes tissue repair. Different studies reported that FGF (1 or 2) and its receptor are to be localized to the muscle–bone interface and periosteum of the mouse forelimb, respectively (Hamrick et al., 2010). It was shown that mechanically induced sarcolemma wound-mediated FGF-2 release is an important autocrine mechanism for transducing the stimulus of mechanical loading to a skeletal muscle growth response (Abraham et al., 1986; Clarke and Feedback, 1996; Clarke et al., 1993), suggesting that FGF-2 could be an osteoinducer factor released by muscle. Intraosseous or intravenous injections of FGF-2 stimulated bone formation in ovariectomized rats (Liang et al., 1999; Nakamura et al., 1998). Recently, Hu et al. reported on the strong phosphorylation signals of FGFR-1 and its downstream signaling molecule ERK1/2 in OA-treated human endothelial cells and bone marrow stromal cells and suggested that FGFR-1 signaling is responsible for OA-induced bone healing. However, how OA interacts with FGFR-1 and whether OA functions as a ligand for FGFR-1 are questions that still need further investigation. He concluded that OA stimulates bone regeneration by inducing osteogenesis and angiogenesis via regulating FGFR-1 signaling. However, it is still unclear if OA activate FGFR-1 signaling directly or through other molecules (Hu et al., 2013).



**Figure 5-1. Proposed model of OA regulating different signaling pathways during tissue growth and repair.** The full length osteoactivin (OA) is a membrane associated protein. OA is cleaved through the ectodomain shedding into two fragments: the intracellular carboxy-terminal fragment (OA-CTF) and the extracellular secreted fragment. The OA-CTF fragment binds Smad4, translocates into the nucleus, and regulates the expression of different genes. The activated, secreted OA fragment binds either to integrins and phosphorylates FAK, ERK and SRC or binds to fibroblast growth factor receptor (FGFR)-1 and signals through unknown downstream signaling pathway to regulate the expression of specific genes involved in tissue growth and repair.

The potential of growth factor-related therapies to augment the early healing process of overuse injuries of upper-extremities has been of great interest to hand surgeons. Therefore, promotion of tissue healing and repair through molecular approaches and seeking potential targets of molecular modulation should be based on a thorough understanding of the activities of all factors involved. Since the factors that initiate and modulate different stages of tissue-repair remain to be elucidated and are areas of active investigation, we believe that future studies are needed to clarify the

interactions between OA and other repair molecules during inflammatory and repair responses underlying musculoskeletal injuries, such as upper extremity musculoskeletal disorders.

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