SIGNALING MECHANISMS THAT SUPPRESS THE ANABOLIC RESPONSE OF OSTEOBLASTS AND OSTEOCYTES TO FLUID SHEAR STRESS

Julia M. Hum

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Fredrick M. Pavalko, Ph.D., Chair

_____
Joseph P. Bidwell, Ph.D.

Doctoral Committee

_____
Richard N. Day, Ph.D.

August 13, 2013

_____
Jeffrey S. Elmendorf, Ph.D.

_____
Alexander G. Robling, Ph.D.

DEDICATION

This dissertation is dedicated to my family. I could not have made this journey through graduate school without their continued support. I would like to thank my husband, Houston, for his love, encouragement, and patience throughout my graduate career. To my parents, thank you for instilling in me the values necessary to succeed in all areas of life, both academic and personal. Thank you for championing all my dreams and aspirations. To my two brothers, thank you for serving as reminder of what is really important and always bringing more humor into my life. I also want to thank my extended family and friends for their encouragement throughout my academic journey.

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ABSTRACT

Julia M. Hum

SIGNALING MECHANISMS THAT SUPPRESS THE ANABOLIC RESPONSE OF OSTEOBLASTS AND OSTEOCYTES TO FLUID SHEAR STRESS

Bone is a dynamic organ that responds to its external environment. Cell signaling cascades are initiated within bone cells when changes in mechanical loading occur. To describe these molecular signaling networks that sense a mechanical signal and convert it into a transcriptional response, we proposed the mechanosome model. “GO” and “STOP” mechansomes contain an adhesion-associated protein and a nucleocytoplasmic shuttling transcription factor. “GO” mechanosomes functions to promote the anabolic response of bone to mechanical loading, while “STOP” mechanosomes function to suppress the anabolic response of bone to mechanical loading. While much work has been done to describe the molecular mechanisms that enhance the anabolic response of bone to loading, less is known about the signaling mechanisms that suppress bone’s response to loading. We studied two adhesion-associated proteins, Src and Pyk2, which may function as “STOP” mechanosomes. Src kinase is involved in a number of signaling pathways that respond to changes in external loads on bone. An inhibition of Src causes an increase in the expression of the anabolic bone gene osteocalcin. Additionally, mechanical stimulation of

osteoblasts and osteocytes by fluid shear stress further enhanced expression of osteocalcin when Src activity was inhibited. Importantly, fluid shear stress stimulated an increase in nuclear Src activation and activity. The mechanism by which Src participates in attenuating anabolic gene transcription remains unknown. The studies described here suggest Src and Pyk2 increase their association in response to fluid shear stress. Pyk2, a protein-tyrosine kinase, exhibits nucleocytoplasmic shuttling, increased association with methyl-CpG-binding protein 2 (MBD2), and suppression of osteopontin expression in response to fluid shear stress. MBD2, known to be involved in DNA methylation and interpretation of DNA methylation patterns, may aid in fluid shear stress-induced suppression of anabolic bone genes. We conclude that both Src and Pyk2 play a role in regulating bone mass, possibly through a complex with MBD2, and function to limit the anabolic response of bone cells to fluid shear stress through the suppression of anabolic bone gene expression. Taken together, these data support the hypothesis that “STOP” mechanosomes exist and their activity is simulated in response to fluid shear stress.

Fredrick M. Pavalko, Ph.D., Chair

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ABBREVIATIONS

AP1	Activator protein-1
ATP	Adensosine triphosphate
BMP	Bone morphogenetic protein
Cox-2	Cyclooxygenase-2
ECFP	Enhanced cerulean fluorescent protein
ECM	Extracellular matrix
ERK	Ras-extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FA	Focal adhesions
FAT	Focal adhesion targeting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FERM	4.1-ezrin-radixin-moesin
FLIM	Florescent lifetime imaging microscopy
FP	Fluorescent protein
FRET	Förster resonance energy transfer
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
IκB	Inhibitor κB
ILK	Integrin-linked kinase
IP_3	Inositol triphosphate

JNK	c-Jun NH_2-terminal kinase
Lef1	Lymphoid enhancer-binding factor 1
LRP5/6	Low-density lipoprotein receptor-related protein 5
p130Cas	Crk-associated substrate p130
MAPK	Mitogen activated protein kinase
MBD2	Methyl-cpG binding protein 2
MC3T3	Mouse calvarial 3T3
MCOB	Mouse calvarial osteoblasts
MeCP1	Methyl CpG binding protein 2
MEM-α	Minimum essential media alpha
MLO-Y4	Murine long bone osteocyte-Y4
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NES	Nuclear export sequence
NLS	Nuclear localization sequence
NMP4	Nuclear matrix protein 4
OCL	Osteocalcin
OFSS	Oscillatory fluid shear stress
OPN	Osteopontin
PGE_2	Prostaglandin E2
PI-3K	Phosphoinositide 3-kinase
PR	Proline rich
PTH	Parathyroid hormone
Pyk2	Proline-rich tyrosine kinase 2

qRT-PCR	Quantitative real-time polymerase chain reaction
RANKL	Receptor activator of nuclear factor-κB ligand
SH	Src homology
SH1	Src homology 1
SH2	Src homology 2
SH3	Src homology 3
SH4	Src homology 4
SSRE	Shear stress response element
TNFα	Tumor necrosis factor α

Chapter I

INTRODUCTION

Mechanical Regulation of Bone

Bone Development

The skeleton serves to protect, support, and act as a reservoir for metabolic activity. It must be rigid enough to protect the vital organs of the body, lightweight to support mobility, yet also capable of adapting to its external environment (Ehrlich and Lanyon, 2002). Bone is a highly specialized form of connective tissue and a dynamic organ which develops through two different types of formation, intramembranous and endochondral (Miller et al., 2007). Intramembranous bone formation is direct bone synthesis mediated by the inner periosteal osteogeneic layer. Endochondral bone formation results in indirect synthesis of bone from a cartilage scaffold and is responsible for the development of long bones in the longitudinal direction. Bone development begins with a phase called modeling, a metabolic activity involving the deposition of mineralized tissue where a cartilage equivalent existed (Raisz, 1999). During modeling, endochondral bone formation systematically replaces cartilage with bone. Remodeling follows the modeling phase, while remodeling begins in early fetal development, it is the primary metabolic activity occurring in a fully formed skeleton. In general, bone remodeling is a finely tuned cellular process that involves the concerted efforts of osteoblasts, which secrete bone matrix, and

osteoclasts, which resorbs the bone matrix. If the balance of bone remodeling is slightly shifted it can lead to a metabolic diseases such as osteoporosis or osteopetrosis (Boyce et al., 1992).

More specifically, remodeling is a highly regulated, multi-step process initiated by the interactions of cells from the hematopoietic osteoclastic and mesenchymal osteoblastic lineages (Miller et al., 2007). Remodeling begins when these two types of precursor cells interact, causing the formation of large multinucleated osteoclasts. Osteoclasts function to resorb bone by attaching directly to the surface of bone and secreting enzymes and hydrogen ions to degrade the bone matrix. Osteoclasts form resorption pits on the surface of bone, subsequently increasing the local calcium concentration and signaling the reversal stage. During the reversal stage mononuclear cells release growth factors and deposit proteoglycans on the surface of bone. Bone is formed during the final formation stage, in which osteoblasts line the resorption pit and deposit a mineralizable matrix. During the formation phase some osteoblasts become entombed in the new bone matrix and mature into osteocytes. Osteocytes account for 90-96% of mature bone tissue and are responsible for the maintaining the bone matrix (Schaffler and Kennedy, 2012). Each osteocyte resides in a lacuna within the bone matrix, and extends processes through the canaliculi to connect with processes from adjacent osteocytes. This creates a large osteocyte communication network, made up of osteocytic processes within the bone matrix. In summary, while the skeleton may appear to be a rigid

structure, the bone tissue comprising our skeletal system undergoes dynamic remodeling which can be initiated in different ways.

Regulation of bone formation and remodeling

Two of the main mechanisms by which new bone formation occurs, include hormonal regulation and mechanical loading (Miller et al., 2007; Sheffield et al., 1987). Both vitamin D and parathyroid hormone (PTH) function to regulate calcium and have a major effect on bone remodeling (Blair et al., 2002; Suda et al., 2003). In general, vitamin D serves to reduce bone formation, while PTH serves to promote bone formation. Briefly, vitamin D regulates phosphorous and calcium transport and promotes osteoclast differentiation (Bikle, 2012). Both vitamin D and PTH increase osteoclast formation indirectly through increased production of the receptor activator of NF-κB ligand (RANKL) in osteoblasts. Osteoclast differentiation and maturation occur when RANKL binds to RANK receptors on the surface of osteoclast precursors (Blair et al., 2002; O'Brien et al., 1999; Suda et al., 2003). When serum calcium levels are low PTH is secreted and activates both osteoclast and osteoblast differentiation (Blair et al., 2002).

In contrast to their effects on osteoclasts, vitamin D and PTH oppose each other's effects on osteoblast differentiation. PTH promotes osteoblast survival *in vitro* and increases osteoblast differentiation by activating the Runx2 transcription factor (Jilka et al., 1999; Krishnan et al., 2003; Merciris et al., 2007; Selvamurugan et al., 2000). Runx2 was the first osteoblast specific transcription

factor identified (Ducy et al., 1997; Otto et al., 1997) and is considered the "master" regulator for bone formation (Ducy et al., 2000; Franceschi, 1999; Komori et al., 1997). Overall PTH functions to increase bone formation by osteoblasts and mineral degradation by osteoclasts (Blair et al., 2002). Alternatively, vitamin D functions to inhibit osteoblast differentiation by negatively regulating Runx2 and type-I collagen, but is required for bone degradation and mineralization (Blair et al., 2002; Ducy et al., 1997; Suda et al., 2003). Hormonal signals target all three kinds of bone cells to cause changes in gene transcription, resulting in either enhanced or reduced bone formation via the remodeling process.

Mechanical loading of the skeleton also induces bone formation, through the regulation of the bone remodeling process. It has long been established that bone responds to changes in its external environment and new bone formation occurs in response to mechanical loading (Goodship et al., 1979; Lanyon, 1984; Lanyon and Rubin, 1984; Wolff, 1892). Reducing mechanical load, for instance during space flight or prolonged bed rest, causes bone loss (Collet et al., 1997; Vogel and Whittle, 1976). Bone responds differently to the magnitude and rate of strain. High strain changing at fast rates induces more bone formation than low strain or slow rate of strain (Honda et al., 2001; Mosley and Lanyon, 1998; O'Connor et al., 1982; Rubin et al., 1987). This finding explains why high impact exercises result in more bone formation than low impact activities (Nordstrom et al., 1998a; Nordstrom et al., 1998b). Additionally, bones have a greater osteogenic response to repetitive bouts of mechanical loading with rest periods

than persistent loading cycles (Robling et al., 2000). The external environment of bone has a major impact on its architecture. The following sections will explain the structure of bone, its biomechanical properties, and the major signaling cascades that mediate the response to mechanical loading.

Structural and Mechanical Properties of Bone

In order for the skeleton to carryout its primary functions it must maintain an architecture that is both strong and lightweight. Bone accomplishes this structural feat by being curved, allowing bones to withstand remarkable amounts of strain, but remain lightweight (Turner and Burr, 1993). Throughout its development and growth, bone is adapting its shape to the external demands placed on the skeleton (Balling et al., 1992). As previously described, bone modeling and remodeling allow for maintenance of its unique architecture, as well as adapt to its external environment (Robling et al., 2002).

The two main types of bone, cortical and trabecular, play distinctly different structural and functional roles. Cortical bone is made up of a network of highly organized, densely packed collagen fibrils forming concentric lamellae (Marks and Hermey, 1996). Within cortical bone Haversian canals form channels that contain the bone's supply of blood vessels and nerves. Functionally, cortical bone provides strength, protection, and mechanical support. Cortical bone is found primarily in the long bones of the skeleton, for instance the arms and legs. In contrast, trabecular bone is loosely organized and composed of a porous matrix, allowing it to be adaptable and serve its metabolic functions primarily at

the ends of bone. Wolff first observed that trabecular bone was present at the sites of maximum stress on bones, while areas of minor stress lacked trabecular bone (Wolff, 1892). Frost elaborated further on Wolff's observation in his description of the mechanostat theory (Frost, 1987). Frost's theory states that an ideal strain range exists for bone. When increased strain is experienced by an area of bone it responds by depositing new bone matrix in an effort to lower the amount of strain. Likewise, if the strain experienced by bone is below the ideal range, bone will be resorbed in an effort to revert back to the preferred range.

Over the years research has focused on explaining the molecular mechanisms that bone uses to respond to its external environment. Studies have measured the amount of strain on bone *in vivo* and methods have been developed to investigate the effects of mechanical loading on bone (Hert et al., 1971; O'Connor et al., 1982). These findings lead to the expansion of the field of bone cell mechanotransduction.

Bone Cell Mechanotransduction

Mechanotransduction is the conversion of mechanical signals into cellular biochemical responses (French, 1992). Examples of mechanotransduction in the human body include, the role of blood flow in vascular tone (Davies, 1995), hair cells' ability to detect and amplify sound (LeMasurier and Gillespie, 2005), and bone responding to changes in its external environment through remodeling (Turner and Pavalko, 1998). The external environment of bone produces tension and compression forces, causing bones to bend, resulting in fluctuating interstitial

fluid pressure throughout the lacuno-canalicular system. Changes in interstitial fluid shear stress are seen across the surface of osteoblasts and osteocytes. Mathematical models have estimated the physiologic range of fluid shear stress within the lacunae and canalicular spaces to be between 8 – 30 dynes/cm^2 (Weinbaum et al., 1994). To further investigate mechanotransduction in bone cells numerous models have been designed to mimic a mechanical stimulus on bone cells, including hydrostatic pressure (Burger et al., 1992; Ozawa et al., 1990; Shelton and el Haj, 1992), substrate distension (Meikle et al., 1984; Murray and Rushton, 1990; Somjen et al., 1980) or bending (Bottlang et al., 1997; Pitsillides et al., 1995), and fluid shear stress (Dewey, 1984; Frangos et al., 1985; Sakai et al., 1999). While all of the aforementioned models have significantly contributed to our understanding of mechanotransduction, bone cells appear to be more responsive to fluid shear stress models than strain models (Owan et al., 1997; Smalt et al., 1997). Further discussion of fluid shear stress models will occur in a subsequent section.

Both osteoblasts and osteocytes are exposed to changes in interstitial fluid flow (Hillsley and Frangos, 1994; Turner and Pavalko, 1998). However, the osteocyte is thought to be the primary bone cell responsible for responding to mechanical loads. Known as the “great communicator” osteocytes detect strain from within their lacunae entombed in bone matrix (Bonewald, 2011; Cowin, 1998). A communication network is set up among osteocytes throughout the bone matrix by their processes. The processes of osteocytes transmit mechanical signals through their network via gap junction linkages (Cheng et al.,

2001a; Cheng et al., 2001b; Jiang and Cheng, 2001). Osteocytes' role in mechanical loading was highlighted in a seminal study in which osteocytes were targeted for ablation leading to defective mechanotransduction in mice (Tatsumi et al., 2007). One key difference between the response of osteocytes and osteoblasts to mechanical loading is the secretion of sclerostin. A protein product of the SOST gene, sclerostin is only secreted by osteocytes and is a powerful inhibitor of bone formation (Brunkow et al., 2001). SOST null mice exhibit increased bone formation and strength (Li et al., 2008). In response to mechanical loading, osteocytes reduce sclerostin secretion, whereas the secretion is increased under reduced loading (Robling et al., 2008). Thus it appears that sclerostin secretion is a central mechanism by which osteocytes control local osteogeneis. Mice, rats, and nonhuman primates treated with a sclerostin-neutralizing antibody demonstrated increased anabolic bone formation (Li et al., 2009; Li et al., 2010; Ominsky et al., 2011; Ominsky et al., 2010). Sclerostin, a suppressor of anabolic bone formation, is proving to be a promising target for pharmacological intervention. This type of osteogenic suppression is the focus of this dissertation project.

Besides the secretion of sclerostin, osteocytes and osteoblasts otherwise respond to mechanical loading by initiating similar signaling cascades. Many complex signaling cascades work in concert to ensure bone responds properly to its external environment. The main signaling cascades utilized by osteoblasts and osteocytes to respond to changes in their external environment include calcium, prostaglandins, MAPK, Wnt, growth factors, PTH, TNFα, and focal

adhesion (FA) integrin-mediated signaling cascades (Liedert et al., 2006; Thompson et al., 2012). In general, most of these pathways lead to in changes in bone cell proliferation, differentiation, metabolic activity, and survival.

A rapid increase in intracellular calcium (Ca^{2+}) is one of the first cellular responses to fluid shear stress (el Haj et al., 1999). Ca^{2+} channels on the plasma membrane are mechanosenstive and open in response to fluid shear stress (Iqbal and Zaidi, 2005). A change in the plasma membrane's potential is triggered by the increased Ca^{2+}, which causes the voltage-sensitive Ca^{2+} channels to open, further increasing intracellular Ca^{2+} (el Haj et al., 1999). Subsequently, the cell releases adenosine triphosphate (ATP), which acts in an autocrine/paracrine fashion, binding to the purigenic P2X receptors to cause further extracelluar Ca^{2+} entry into the cell (Li et al., 2005). Additionally, phospholipase C is activated and cleavage of phosphoinositol-4,5-bisphosphate into diacyglycerol and inositol trisphosphate (IP_3) results from ATP binding to P2Y receptors (Genetos et al., 2005). Intracellular stores of Ca^{2+} are then released when IP_3 then binds to its receptor on the endoplasmic reticulum

Prostaglandin release is a rapid and continuous occurrence throughout the duration of fluid shear stress exposure (Bakker et al., 2001). As intracellular Ca^{2+} levels increase, PKC is activated and induces phospholipase A_2 to cleave arachidonic acid from the plasma membrane (Kudo and Murakami, 2002; Murakami and Kudo, 2002). Arachidonic acid is converted into prostaglandin-G_2 (PGH_2) and H_2 by the rate limiting enzyme cyclooxygenase 2 (Cox-2) (Herschman, 1994). Next, PGH_2 is converted into various eicosanoids including

prostaglandin E_2 (PGE_2) and PGI_2 (Dubois et al., 1998). When prostaglandins are secreted from a bone cell they are capable of autocrine and paracrine signaling. The significance of prostaglandins on bone was demonstrated when treatment with PGE_2 stimulated osteoblast differentiation (Zhang et al., 2002), bone formation (Jee et al., 1985; Jorgensen et al., 1988), and increased release of the important growth factor, insulin-like growth factor 1 (McCarthy et al., 1991; Zaman et al., 1997). Additionally, the importance of Cox-2 was demonstrated by showing that load-induced bone-formation was blocked by an inhibitor, NS-398 (Forwood, 1996). In response to fluid shear stress, an increase in Cox-2 expression is recognized as an indication of the anabolic response of bone cells (Pavalko et al., 1998b).

Downstream of Ca^{2+} increase and prostaglandin release, fluid shear stress results in the activation MAPK signaling cascade, which functions to increase bone cell proliferation and survival (Liedert et al., 2006). Periods of fluid shear stress result in the activation of the extracellular signal-related kinase (ERK), p38, mitogen activated protein kinases (MAPKs), and c-jun N-terminal kinase (JNK) (Liedert et al., 2006; Martineau and Gardiner, 2001). All of these signaling molecules target the upregulation of *c-fos* and *c-jun* expression, the two components of the activator protein-1 (AP1) transcription factor. AP1 can bind to the promoter region of many mechanoresponsive genes, affecting their transcription in response to mechanical loading (Franceschi, 2003). Shear stress response element (SSRE) is another significant transcription factor-binding site activated in response to mechanical loading (Nomura and Takano-Yamamoto,

2000). A SSRE was found in several genes including the aforementioned COX-2. In MC3T3 osteoblastic cells exposure to fluid shear stress induces an increase in Cox-2 expression, mediated by AP-1, CCAAT/enhancer-binding protein β, and cAMP-response element-binding protein (CREB) (Ogasawara et al., 2001).

Mechanical stimulation leads to increased activity of the canonical Wnt signaling pathway. During active signaling, a Wnt family member (Wnt 3, 5, or 7) bind co-receptors Frizzled and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), causing the accumulation of β-catenin (Lin and Hankenson, 2011; Monroe et al., 2012). The central signaling protein of the Wnt signaling pathway, β-catenin, translocates to the nucleus and associates with the transcription factor LEF1 to activate transcription of target genes. When Wnt is unbound to its co-receptors, β-catenin's accumulation is prevented by glycogen synthase kinase-3's phosphorylation, targeting β-catenin for degradation. Fluid shear stress induces β-catenin nuclear translocation in osteoblasts and osteocytes (Case et al., 2008; Kamel et al., 2010; Norvell et al., 2004). In the bone field, intense research interest has surrounded the canonical Wnt signaling pathway due to recent major advances. Mutations in the LRP5 receptor result in dramatic changes in bone mass. Activating mutations in LRP5 cause high bone mass phenotype (Boyden et al., 2002; Little et al., 2002; Qiu et al., 2007), while inactivating mutations cause a low bone mass phenotype (Gong et al., 2001; Qiu et al., 2007). Furthermore, β-catenin is a central signaling component in bone differentiation, formation, and maintenance. A conditional β-catenin knockout in

mesenchymal progenitor cells caused disrupted chondrocyte formation and limited osteoblast differentiation (Day et al., 2005). Mice expressing an osteoblast-specific β-catenin mutation had an osteopenic phenotype and an abundance of osteoclasts (Holmen et al., 2005). β-catenin's ability to serve as an important component within fluid shear stress-induced mechansomes will be discussed in a later section.

Upregulation of growth factors including insulin-like growth factor (IGF) I and II, transforming growth factor (TGF) β1, vascular endothelial growth factor, and bone morphogenetic protein (BMP) 2 and 4 occurs in response to mechanical stimulation (Franceschi and Xiao, 2003; Papachroni et al., 2009). These growth factors act through autocrine and paracrine mechanisms, via their tyrosine and serine/threonine kinase receptors, and activate PI3K, MAPK, and SMAD signaling cascades (Farhadieh et al., 2004; Hughes-Fulford, 2004; Mikuni-Takagaki, 1999). For example, the induction of the BMP-2 pathway increases the expression of the three most pivotal osteogenic transcription factors Runx2, osterix, and Dlx5 (Lee et al., 2003).

Additionally, PTH signaling is also stimulated in response to mechanical loading. PTH signals in bone cells through the G protein-coupled receptor (GPCR) at the plasma membrane, inducing the activation of adenylate cyclase. Consequently protein kinase A phosphorylates the CREB transcription factor, which binds to the Cox-2 promoter (Ogasawara et al., 2001). Many of the signaling pathways reviewed here lead to increased bone cell proliferation and differentiation. However, it has been estimated that more than 70% of

osteoblasts at sites of bone formation undergo apoptosis (Jilka et al., 1998). Therefore, it has been proposed that inhibiting osteoblast apoptosis pathways might be an important mechanism by which new bone formation could be increased.

Osteoblasts treated with tumor necrosis factor-alpha (TNFα), an apoptosis-inducing agent, and exposed to fluid shear stress experienced less apoptosis than the static control osteoblasts treated with TNFα (Pavalko et al., 2003a). This study demonstrated that phosphorylation and activation of the pro-survival protein Akt was increased in response to fluid shear stress. Next, Akt inactivates proteases that initiate the apoptotic pathway. In addition, Akt phosphorylates the inhibitor of kappa B (IκB), promoting its degradation and permitting the nuclear translocation of nuclear factor-κB (NF-κB) (Chen and Goeddel, 2002). NF-κB is a transcription factor that controls the expression of many pro-survival genes. Additionally, fluid shear stress causes a reduction in the amount of TNFα receptor at the plasma membrane and decreased TNFα-induced interleukin 8 promoter activity (Wang et al., 2011). Fluid shear stress causes bone cells to be less apoptotic, and seemingly promote a larger osteoblast population capable of producing more bone. In summary, in response to fluid shear stress, many signaling cascades are initiated that result in changes in gene transcription and ultimately effect the bone remodeling process. While many of the pathways involved in bone cells' response to fluid shear stress have been elucidated, some molecular details from each signaling cascade are unknown. For example, it is not known whether a molecule or protein complex

functions to directly convert the mechanical signal into a change in gene transcription. The following section will review the mechanosome hypothesis and the signaling molecules that may function within them.

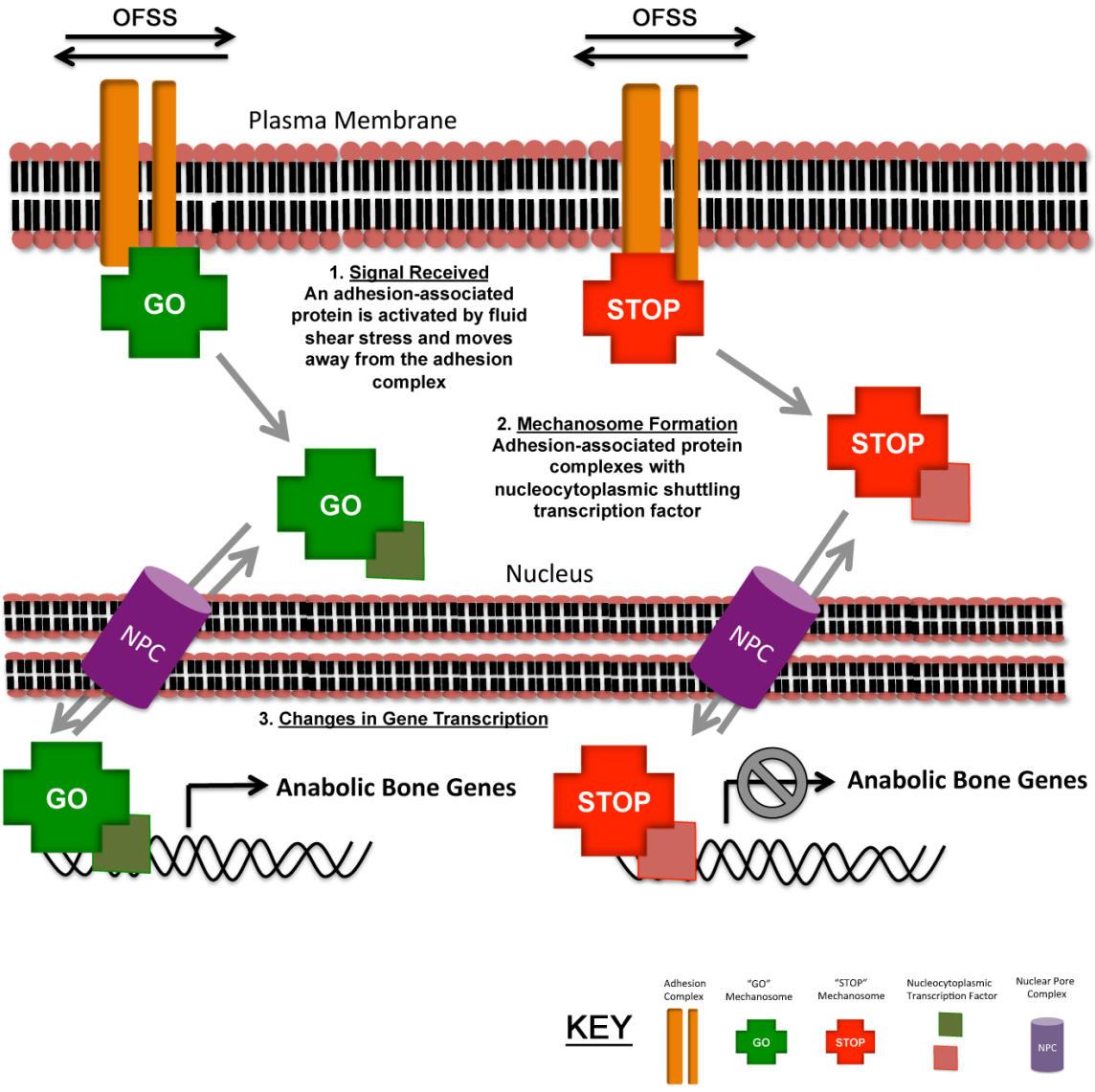
Signaling Through Focal Adhesions

The Mechanosome Model

We have proposed the mechanosome model to describe how mechanical stimuli sensed at the plasma membrane, result in changes in gene transcription (Bidwell and Pavalko, 2010; Bidwell and Pavalko, 2011; Pavalko et al., 2003b). A mechanosome consists of an adhesion-associated protein and a nucleocytoplasmic shuttling transcription factor. There are two forms of mechanosomes, a “GO” mechanosome and a “STOP” mechanosome. A “GO” mechanosome functions to promote the anabolic response of bone to mechanical loading, while a “STOP” mechanosome functions to suppress the anabolic response of bone to loading (Figure 1). β-cateinin and Lef1 are an example of a “GO” mechanosome. In response to fluid shear stress β-catenin moves away from its structural role at the plasma membrane and translocates to the nucleus to bind the transcription factor, lef1, to change gene transcription (Norvell et al., 2004; Yang et al., 2010). Nuclear matrix protein 4 (NMP4) and 130 kD Crk-associated substrate (p130Cas) function as a “STOP” mechanosome (Childress et al., 2010). NMP4 is a nucleocytoplasmic shuttling protein that

inhibits bone anabolism through its function as a trans-acting protein and can antagonize β-catenin/Lef1 “GO” mechanosome launching (Hino et al., 2007; Morinobu et al., 2005; Robling et al., 2009; Thunyakitpisal et al., 2001; Yang et al., 2010). Another component of this “STOP” mechanosome, p130Cas, is an adhesion-associated protein known to be mechanosensor (Geiger, 2006; Sawada et al., 2006). Additionally, the Pilz group has recently described a mechanosome made up of protein kinase G, Src and Src homology 2 domain-containing tyrosine phosphatase 1 and 2 (Rangaswami et al., 2010). The subsequent sections will review one of the launching sites of mechanosomes, focal adhesions and some key molecules that may function as part of a mechanosome.

Figure 1. The Mechanosome Hypothesis



The "GO" and "STOP" mechanosome model in response to OFSS. Mechanosomes form in three basic steps in response to OFSS. First, OFSS induces the activation of an adhesion-associated protein found sites of adhesion near the plasma membrane. Second, a mechanosome is formed when it complexes with a transcription factor. Finally, the mechanosome either promotes ("GO") or suppresses ("STOP") gene transcription. Modified figure from Bidwell and Pavalko, 2011.

Focal Adhesions

One of the two components of a mechanosome is an adhesion-associated protein. In bone cells, numerous adhesion-associated proteins are found on the cytoplasmic side of focal adhesions (FA's). First described as small extended regions of the ventral plasma membrane, FA's tightly join cells to the substrate (Abercrombie and Dunn, 1975; Abercrombie et al., 1971; Izzard and Lochner, 1976; Izzard and Lochner, 1980). FA's have two distinct roles, to function in the detection of mechanical signals and structurally link the extracellular matrix contact (ECM) to the cytoskeleton (Abercrombie and Dunn, 1975; Burridge and Chrzanowska-Wodnicka, 1996; Geiger and Bershadsky, 2001; Geiger and Bershadsky, 2002). FA's are formed in clusters at the cell periphery and composed primarily of ECM-binding integrins, but also contain bundles of actin stress fibers, structural proteins and cytoplasmic associated signaling proteins (Hynes, 1992). Integrins are large, heterodimeric transmembrane proteins composed of varying α and β subunits and classified into families by their β subunit (Hynes, 1992). Integrins bind the ECM through their large extracellular domain, while most of the small intracellular domains bind FA associated proteins (Liu et al., 2000). Integrins are uniquely suited to play a structural and signaling role in bone cells. It was demonstrated, using RGD peptides to disturb integrin-ECM interactions, that integrins play a significant signaling role in osteoblasts in response to oscillatory fluid shear stress (OFSS) (Ponik and Pavalko, 2004).

Disruption of integrin-ECM interactions caused Cox-2 protein levels and PGE_2 secretion to decrease in response to OFSS. Since integrins do not contain any intrinsic kinase activity they rely on other signaling molecules, including adhesion-associated proteins, to convey mechanical signals to the nucleus (Alahari et al., 2002; Burridge and Chrzanowska-Wodnicka, 1996). While integrins are the main protein in the FA site, other membrane proteins localize to FA's including glycosaminoglycan receptors (Bono et al., 2001; Borowsky and Hynes, 1998), dystroglycans (Belkin and Smalheiser, 1996), proteoglycans (Woods and Couchman, 1994; Zimmermann and David, 1999), and signaling molecules (Myohanen et al., 1993; Tang et al., 1998; Wei et al., 1999). The type of integrins found in FA's is determined by the ECM to which the cell is adhered (Dejana et al., 1988; Fath et al., 1989). FA formation requires the transmembrane domain of integrin, but the α and β subunits of the cytoplasmic domains of integrins are also functionally important. While the β subunit of the cytoplasmic domain targets integrins to FA sites (Geiger et al., 1992; LaFlamme et al., 1992), the α subunit of the cytoplasmic domain can prevent the association of FA's (Briesewitz et al., 1993). Ligand binding induces a conformational change in the cytoplasmic tails allowing the β cytoplasmic subunit to bind other FA associated proteins. The cytoplasmic portion of integrins are involved in an array of functions. There functions can be classified into three categories: signaling proteins, actin-binding proteins, and proteins of other functions (Liu et al., 2000). The importance of the α and β subunits was revealed in a study where point mutations were introduced in integrins that lead to the disruption of

cytoplasmic integrin tail mediated signaling (Hughes et al., 1996). After the cytoplasmic integrin tails undergo a conformational change they are free to either bind directly or indirectly with FA associated proteins. Proteins that can bind FA are grouped into the following categories: tyrosoine kinases, serine/threonine kinases, cytoskeletal proteins, modulators of small GTPases, tyrosine phosphatases, and other enzymes (Zamir and Geiger, 2001). We have outlined how FA's are structurally capable of supporting the association of cells to the ECM, next we will discuss the signaling capacity of FA's.

Cell Signaling through Focal Adhesions

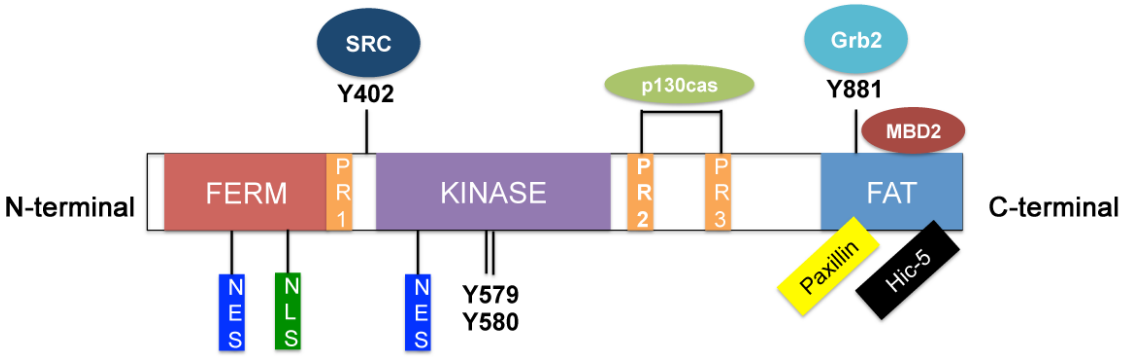
As briefly mentioned above, one of the roles of FA's is to participate in cell signaling cascades. FA's serve to induce signaling cascades and amplify growth factor signals. Furthermore, FA's have demonstrated the ability to signal through growth factor receptors and affect ion channel activation (Miyamoto et al., 1995; Moro et al., 1998). Studies have shown that FA's and ECM proteins both reorganize in response to fluid shear stress (Davies et al., 1994; Pavalko et al., 1998a). Many signaling molecules associate with FA's and are responsible for activating downstream signaling cascades. Signaling proteins including p130Cas (Nojima et al., 1995; Polte and Hanks, 1995; Vuori and Ruoslahti, 1995), integrin linked kinase (ILK) (Li et al., 1999; Tu et al., 1999), paxillin (Burridge et al., 1992), zyxin (Reinhard et al., 1995), phosphoinosotide-3 kinase (PI-3K) (Chen and Guan, 1994), focal adhesion kinase (FAK) (Hanks et al., 1992; Schaller et al., 1992), and Src (Nigg et al., 1982; Rohrschneider, 1980) localized to FA's.

FAK is a widely studied adhesion-associated protein found FA. A non-receptor tyrosine kinase, FAK, is the principal kinase in FA's and responds to the clustering of integrins or adhesion (Burridge et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992; Lipfert et al., 1992). Additionally, our group and others have also shown that FAK is activated in response to mechanical stimuli or fluid shear stress (Ishida et al., 1996; Li et al., 1997; Takai et al., 2006; Young et al., 2009). Upon activation FAK autophosphorylates at tyrosine 397 (Calalb et al., 1995), exposing binding sites for Src and Fyn, which in turn phosphorylate additional sites on FAK and result in increased FAK activity (Schaller et al., 1994a; Xing et al., 1994). Additionally, the activation of FAK also exposes binding sites for PI3-K, paxillin, talin, and p130Cas. FAK's activation and association with these signaling molecules initiates the PI3-K pathway, ERK-pathway, and the c-Jun NH_2-terminal kinase (JNK)-pathways (Schaller et al., 1992). Therefore FAK, through its association with other signaling molecules, has an effect on cell cycle progression, early-gene expression, and apoptosis (Clark and Brugge, 1995; Schwartz et al., 1995). Moreover, Src can phosphorylate FAK at Y576 and Y577, leading to increased FAK activity (Schlaepfer and Hunter, 1996). We have reported FAK to be crucial for OFSS-induced mechanotransduction in osteoblasts (Young et al., 2009). Osteoblasts lacking FAK fail to show either early (5-30 minutes) or mid-late responses (2-24 hours) to mechanical stimulation. Two less understood signaling components of FA signaling in response to fluid shear stress are proline-rich tyrosine kinase 2 (Pyk2) and Src.

Proline-rich tyrosine kinase 2

Pyk2 is a closely related family member to FAK, sharing approximatly 45% sequence identity (Herzog et al., 1996; Inazawa et al., 1996). Both FAK and Pyk2 contain an N-terminal 4.1, ezrin, radixin, moesin (FERM) domain, three proline-rich regions, a kinase domain, and a C-terminal focal adhesion targeting (FAT) domain (Figure 2) (Ceccarelli et al., 2006; Hayashi et al., 2002; Hiregowdara et al., 1997; Schaller et al., 1992; Schlaepfer et al., 1999). While structurally similar, there are important differences between FAK and Pyk2. FAK is widely expressed across many cell types, while Pyk2 is highly expressed primarily in brain cells, fibroblasts, platelets, and bone cells. Interestingly, FAK null cells overexpress Pyk2 in what appears to be a compensatory mechanism (Lim et al., 2008b; Sieg et al., 1998; Weis et al., 2008). FAK is principally activated through its interaction with integrins at sites of FA, but Pyk2 can also be activated through increases in intracellular calcium (Astier et al., 1997; Avraham et al., 2000; Lev et al., 1995; Tokiwa et al., 1996). Finally, the intracellular distribution of Pyk2 differs from FAK. While both are found to associate with integrins at sites of FA, Pyk2 is more evenly distributed throughout the cell and often found to be concentrated in the perinuclear region (Klingbeil et al., 2001; Schaller and Sasaki, 1997). Pyk2, similar to FAK, is autophosphorylated at Y402, which leads to association with Src and focal adhesions (Figure 2). Unlike FAK, Pyk2 can interact with and phosphorylate paxillin, a focal adhesion-associated protein (Hiregowdara et al., 1997; Schlaepfer et al., 1999).

Figure 2. Pyk2's Binding Domains and Important Phosphorylation Sites



Pyk2 contains three distinct domains that meditate protein-protein interactions. Pyk2's FERM domain contains both a nuclear export sequence (NES) and a nuclear localization sequence (NLS). Pyk2's activation depends on autophosphorylation at tyrosine 402 (Y402), which then allows Src to bind Pyk2 via its SH2 domain. The kinase domain of Pyk2 has a second NES and two tyrosine sites for Pyk2's inactivation (Y579, Y580). Three proline rich (PR) regions span Pyk2. PR2 and PR3 mediated the assocation of Pyk2 with p130Cas, while the focal adhesion targeting (FAT) domain mediates the interaction of paxillin, Hic-5, and MBD2 with Pyk2.

In bone, Pyk2 is involved in remodeling (Avraham et al., 2000; Boutahar et al., 2004; Gil-Henn et al., 2007; Guignandon et al., 2006; Hall et al., 2011). Global Pyk2 knockout mice exhibit a phenotype charaterzied by elevated bone mass (Gil-Henn et al., 2007; Okigaki et al., 2003). There is a controversy in the bone field as to the reasons for the high bone mass phenotype. One report indicates the phenotype results from defective osteoclast function implicating Pyk2's role in osteoclast driven bone resorption (Gil-Henn et al., 2007), while another contends it is a result of increased osteoblast differentiation (Buckbinder et al., 2007). As previously reviewed, Pyk2's more well-known family member, FAK, serves as an important positive regulator of mechanical stimuli in osteoblasts (Young et al., 2009). Pyk2's role in mediating the response of bone cells to mechanotransduction is less well known, but is suggested to be different than FAK's (Young et al., 2011). Additionally, reciprocal phosphorylations occur, with Src phosphorylating both FAK and Pyk2, while FAK and Pyk2 also associate and phosphorylate Src (Calalb et al., 1995; Frame et al., 2002; Schaller et al., 1994a; Xing et al., 1994). Unknown is whether Src is dependent on FAK and/or Pyk2 to transmit intracellular signals in response to mechanical loading. While FAK and Pyk2 are in prime position to relay signals from the external enviroment to bone cells, recent studies have proposed a role for FAK and Pyk2 in the nucleus. For example, it was reported that FAK and Pyk2 may play a direct role in regulating gene transcription in muscle and nerve cells, respectivley (Luo et al., 2009; Mei and Xiong, 2010). Methyl-CpG binding domain protein 2 (MBD2) was found to associate with FAK through the N-terminal region of MBD2 and the

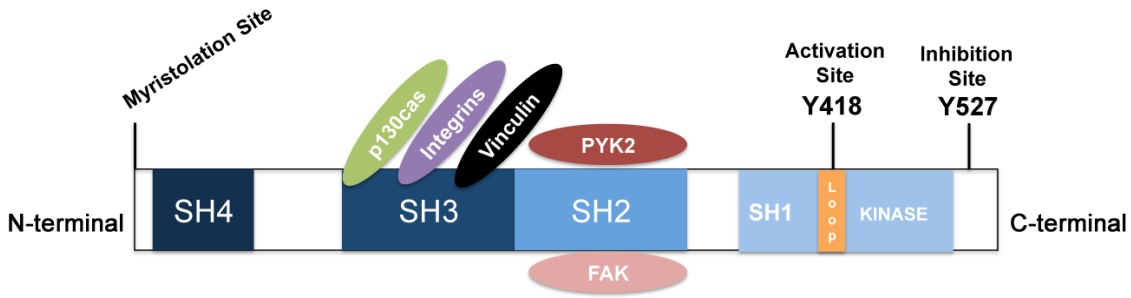
C-terminal focal adhesion-targeting domain of FAK (Luo et al., 2009; Mei and Xiong, 2010). Their interaction promotes regulation of myogenin expression and differentiation in muscle cells (Luo et al., 2009; Mei and Xiong, 2010). These reports suggest MBD2 binds either FAK or Pyk2 in the nucleus to effect target gene transcription, indicating that MBD2 might be a component of a mechanosome containing Pyk2 and/or Src kinase. MBD2 is a family member of the methyl CpG-binding domain containing proteins, which functions to suppress gene transcription (Wade, 2001). MBD2 aids in repressing transcription within the methyl-CpG binding protein, MBD1) MeCP1 complex (Bird and Wolffe, 1999; Leonhardt and Cardoso, 2000). MBD2 interacts with heterochromatin by its association with methylated DNA at CpG islands. MBD2 then recruits silencing complexes and histone deacetylases (HDAC), resulting in condensed heterochromatin (Bird and Wolffe, 1999; Boeke et al., 2000; Hendrich and Bird, 1998; Ng et al., 1999). Undetermined is the interaction of Pyk2 or Src with MBD2 in response to fluid shear stress and the target genes of such a mechanosome.

Src Kinase

As briefly described above, Src is involved in integrin-mediated signaling, but it also participates in numerous signaling cascades and cellular functions including growth, movement, differentiation and cell adhesion (Brown and Cooper, 1996; Thomas and Brugge, 1997). A broad range of substrates have been shown to be tyrosine phosphorylated by Src, including platelet-derived growth factor, epidermal growth factor, macrophage colony stimulating factor 1,

FAK, Pyk2, and vinculin (Hunter and Cooper, 1985; Parsons and Parsons, 1997). Src is broadly expressed in many different cell types and it is localized to different subcellular domains. A member of the Src family of nonreceptor tyrosine kinases, Src is one of nine family members including Fyn, Yes, Frk, Blk, Fgr, Hck, Lck, and Lyn (Parsons and Parsons, 2004). The Src family kinases share similar structural features that include Src homology (SH) domains (Boggon and Eck, 2004). Specifically, Src kinase is made up for four different SH domains (Figure 3). Src is localized to the plasma membrane where it participates in the integrin-mediated signaling response via an N-terminal myristoylation site (Boggon and Eck, 2004; Resh, 1994). Src switches between a myristoylated and nonmyristolated form through the use of a hydrophobic pocket in the SH1 kinase domain (Cowan-Jacob et al., 2005). Additionally, the SH4 domain of Src is required for membrane attachment. Src's SH3 and SH2 domains are responsible for mediating intramolecular and intermolecular binding partners that regulate both Src kinase activity and signaling cascades (Figure 3) (Koch et al., 1991; Pawson, 1988; Pawson and Gish, 1992). The SH3 domain binds many FA-associated proteins including integrins (Arias-Salgado et al., 2003), paxillin (Weng et al., 1993), and p130Cas (Nojima et al., 1995). The SH2 domain is highly conserved and mediates many protein-protein interactions including FAK and Pyk2 (Pawson and Nash, 2003; Schaller et al., 1994b). The SH1 domain is the cataylictic or kinase domain within Src. To control the specific binding of Src's numerous partners Src has two distinct conformations. For full catalytic activity of Src, autophosphorylation occurs at tyrosine 418 (Y418) within the

Figure 3. Src's Binding Domains and Important Phosphorylation Sites



Src mediates many of its protein-protein interactions via SH domains. Src contains an N-terminal myristolation sites for anchoring itself in plasma membrane. The SH3 and SH2 domains mediate the binding of FA-associated proteins, including p130Cas, integrins, vinculin, Pyk2, and FAK. The SH1 domain, also known as the kinase domain, contains Src's phosphorylation site (Y418) for activation within the activation loop. A C-terminal inhibition site is found at tyrosine 527.

activation loop of the SH1 domain (Smart et al., 1981). In an active confirmation, the SH2 and SH3 domains are readily accessible for substrate binding. When Src is in an inactive state the autoinhibitor phosphorylation site (Y527) in the C-terminal tail is phosphorylated (Cooper et al., 1986) and the SH2, SH3, and SH1 domains bind to one another to form an autoinhibited conformation (Sicheri and Kuriyan, 1997; Williams et al., 1997; Xu et al., 1997). C-terminal Src kinase (Nada et al., 1991) and CSK-homologous kinase (Davidson et al., 1997; Hamaguchi et al., 1996) usually carry out the inactivating phosphorylation at Y527. Alternatively, protein tyrosine phosphatases including SH2 domain-containing protein tyrosine phosphatases 1 and 2 (SHP1, SHP2) are capable of activating Src by dephosphorylating Y527 (Chiang and Sefton, 2001; Rangaswami et al., 2010).

In bone Src helps maintain the balance of normal bone remodeling. Mice lacking Src exhibit an osteopetrotic phenotype (Soriano et al., 1991). More specifically, Src null mice exhibit incisor eruption failure, thickened growth plate, perseverance of the endochondral primary spongiosa, reduced bone marrow tissue, and overall small size (Soriano et al., 1991). The high bone mass phenotype of Src null mice is caused by malfunctioning osteoclasts and osteoblasts. Src null mice display increased numbers of inactive osteoclasts. These osteoclasts lack a ruffled border; therefore they cannot attach to the surface of bone and promote bone resorption (Boyce et al., 1992; Horne et al., 1992; Lowe et al., 1993). Osteoblasts lacking Src contribute to the high bone mass phenotype by overexpressing Runx2, alkaline phosphatase, PTH/PTHrP,

and osteocalcin (Marzia et al., 2000). Finally, as the Src null mice age their bone mass continues to increase. Therefore, a loss of Src activity causes an increase in bone mass (Amling et al., 2000). Recent studies of Src inhibitors have lead to clinical trials exploring their use for treating low bone mass (Hannon et al., 2010; Missbach et al., 1999), however it is imperative to understand the implications of Src inhibition on bone remodeling in response to mechanical loading.

Fluid shear stress causes an increase in Src activation in osteoblasts and osteocytes, as well as endothelial cells and colon cancer cells (Jalali et al., 1998; Okuda et al., 1999; Plotkin and Bellido, 2001; Rangaswami et al., 2010; Rangaswami et al., 2012; Takahashi and Berk, 1996; Thamilselvan et al., 2004). Due to its location and numerous binding partners, Src is in prime positioned to propagate cellular signals in bone cells after exposure to fluid shear stress. The Pilz group has described a new pathway of Src activation in response to unidirectional fluid flow, in which a NO/cGMP/PKGII/SHP-1 pathway leads to Src activation via association with integrins (Rangaswami et al., 2010).

In Vitro Methods to Assess Signaling in Response to Fluid Shear Stress

Bone Cell Culture Models

Commonly, researchers have isolated primary cells to study the cellular properties and signaling cascades of osteoblasts. A traditional source of primary osteoblasts is isolated from the calvaria of newborn mice. Mouse calvarial

osteoblasts (MCOB) exhibit high alkaline phosphatase expression and increase its activity in response to treatment with ascorbic acid and β-glycerophosphate (Lynch et al., 1995). Additionally, MCOB's respond to calcitonin and mineralize in culture (Binderman et al., 1974). While MCOB's are a phenotypically ideal model, primary osteoblasts can be difficult to work with in culture and their proliferative capacity is limited.

To overcome some of the limitations of MCOB, several types of bone cell lines have been developed to investigate bone cell physiology. In establishing bone cell lines researchers wanted to develop models that mimicked important characteristics of primary osteoblasts and osteocytes. For immortalized osteoblastic cell lines some of the sought after hallmarks included alkaline phosphatase activity, mineralization, expression of collagen type 1, production of collagenase, release of prostaglandins, responsive to parathyroid hormone and prostaglandin E2, and expression of the receptors for of 1,25(OH)-vitamin D3, parathyroid hormone and epidermal growth factor. The cell line MC3T3 is a commonly used osteoblastic cell line originally derived from the calvaria of newborn mice (Sudo et al., 1983). These cells can differentiate into osteoblasts and osteocytes, can deposit mineral into bone matrix, and express high amounts of alkaline phosphatase (Sudo et al., 1983).

The murine long bone osteocyte Y4 (MLO-Y4) was derived from a single colony isolation, derived from long bone mice expressing SV40 T Large antigen, driven by the osteocalcin promoter (Kato et al., 1997). MLO-Y4 osteocytes exhibit osteocyte-like dendritic morphology, expression of connexin 43, and

secretion of osteocalcin. Establishing immortalized osteoblast and osteocytes cells lines was an important step to enable *in vitro* investigations into the signaling mechanisms induced by fluid shear stress. Only recently has successful isolation of primary osteocytes from the long bones been described (Stern et al., 2012).

Models of Fluid Flow

In vivo models of mechanical loading and unloading of bone have been developed to study broad changes in bone in architecture, as well as, alterations in the microarchitecture. Two common models include ulnar loading (Torrance et al., 1994) and hindlimb suspension (Morey, 1979). To investigate changes in cell signaling networks in response to mechanical loading *in vitro* models of fluid shear stress have been designed. On a cellular level, osteoblasts and osteocytes experience mechanical loading via pressure changes in interstitial fluid flow (Hillsley and Frangos, 1994). Therefore, researchers have designed numerous methods for replicating the interstitial fluid shear stress experienced by osteoblasts and osteocytes.

A model of unidirectional fluid flow in a parallel plate flow chamber was first described by Frangoe and colleagues to study the metabolic response of endothelial cells to steady and pulsatile shear stresses (Frangos et al., 1988). The unidirectional model of fluid shear stress was used by many researchers in the bone field until oscillatory fluid flow models were developed, and thought to be more physiologically similar to the flow pattern of interstitial fluid. Our group

demonstrated unidirectional and oscillatory fluid flow profiles produce different responses from osteoblasts and osteocytes (Ponik et al., 2007). Oscillatory fluid flow is produced within parallel plate flow chambers that house a glass slide on which bone cells are plated. Hard-walled tubing connects the parallel plate flow chamber to the oscillatory pump, which controls the adjustable flow rate (0-25 dynes/cm^2) of media across the surface of the bone cells. The main advantage of using of an oscillatory pump to induce fluid shear stress is the controllable and uniform flow pattern that it produces across bone cells. Three prominent disadvantages are the limited length (< 12 hours), non-repetitive nature of flow exposure and the dissimilar set-up for static samples.

A newer method for inducing fluid shear stress across the surface of bone cells uses an orbital shaking platform. Use of an orbital shaking platform overcomes some of the significant limitations in the experimental design of the parallel plate flow chamber model (Inoue et al., 2004; Kido et al., 2009; Sakai et al., 1999; Young et al., 2011). Two of the major advantages of using an orbital shaking platform include the opportunity for long (> 24 hours) and/or intermittent periods of fluid flow and easier collection of static samples for analysis. Inducing fluid flow via an orbital shaking platform rotating at ~200 rpm produces approximately 1.5-2.5 Pa (~15-25 dynes/cm^2) of shear stress force at the outer radius of the dish. Shear stresses toward the center of the dish are lower in magnitude. (Sakai et al., 1999). While still a new method to the field of bone biology, the orbital shaking platform has been used in a number of studies. Interleukin-11 expression during osteoblast differentiation was successfully

examined in response to mechanical stimulation via orbital platform shaking (Kido et al., 2009; Sakai et al., 1999). Additionally, orbital shaking platform induced fluid shear stress led to increased FosB/ΔFosB expression in osteoblasts (Inoue et al., 2004). Finally, osteoblasts respond similarly to fluid shear stress induced by either the oscillatory pump or the orbital platform shaker (Young et al., 2011). In summary, while the oscillatory pump produces oscillatory fluid flow and the orbital shaking platform generates dynamic fluid flow, both produce physiologically relevant fluid shear rates.

Live Cell Imaging

The field of microscopy encountered a revolutionary change with the cloning of green florescent protein (GFP) from the jellyfish, *Aequorea Victoria*. GFP was discovered by Shimomura and collogues who noted that "a protein giving solutions that look slightly greenish in sunlight through only yellowish under tungsten lights, and exhibiting a very bright, greenish fluorescence in the ultraviolet of a Mineralite" (Shimomura et al., 1962). This description of GFP is still correct; it is in the green portion of the visible spectrum. The jellyfish GFP's main excitation peak is at a wavelength of 395 nanometers, with a minor peak at 475 nanometers, and emission peak at 509 nanometers (Johnson et al., 1962). GFP is composed of 238 amino acids and consists of eleven β-sheets with six alpha helices and contains a covalently bonded chromophore buried in the center of the cylinder (Morise et al., 1974; Prendergast and Mann, 1978). In research, GFP has been used as a tracer of cell lineage, a reporter of gene expression,

and protein tag to monitor localization in living cells (Chalfie and Kain, 2006). The jellyfish GFP was limited by low brightness, complicated photoisomerization, near UV excitation, a substantial delay between protein synthesis and fluorescent development. Mutagenesis has allowed researchers to expand the spectral characteristics of GFP by shifting the excitation and emission wavelengths. The engineering of the jellyfish GFP, along with cloning and optimization of similar proteins from a variety of marine organisms, have yielded a whole spectrum of fluorescent proteins (FP) allowing researchers to monitor biological events.

The field of Förster Resonance Energy Transfer (FRET) microscopy has benefitted tremendously from genetically encoded FPs. FRET is named for the German scientist, Theodor Förster, who first provided a quantitative understanding of the process (Förster, 1948). FRET is a distance dependent process by which energy is directly transferred from a donor fluorophore to a nearby acceptor via near-field electromagnetic dipole interactions. When FRET occurs the donor's emission signal is quenched. For efficient transfer of energy from donor to acceptor there are three essential requirements (Förster, 1965; Stryer, 1978). First, the donor and acceptor probes must be in close proximity. FRET can only take place over a distance of less than 10 nanometers, making it a highly sensitive technique for investigating biological activities. Second, the donor and acceptor dipole-dipole alignment must be favorable. Third, there must be significant overlap between the donor emission and acceptor excitation spectra. Spectral overlap leads to spectral bleedthrough, a pitfall that must be corrected when detecting FRET signals. There are two components to spectral

bleedthrough. The first is the direct excitation of the acceptor at the wavelength used to excite the donor (acceptor cross talk). The second is the donor emission that is detected in the acceptor (FRET) channel (Day and Davidson, 2012). When the three requirements for FRET are fulfilled quantification of FRET signals can offer Ångstrom level measurements of the distance between the donor and acceptor fluorophores within living cells.

There are a number of different ways of measuring FRET signals. The most common methods for assessing FRET are spectral bleed-through correction, spectral imaging, acceptor photobleaching, and fluorescent lifetimes (Day and Davidson, 2012). Spectral bleedthrough correction is a general computer algorithm-based method that can be applied to any type of microscopy. Spectral imaging of FRET signals entails acquiring a wide range of emission wavelengths to produce a lambda stack which allows for analysis of spectral characteristics of the fluorescent signal of each pixel (Dickinson et al., 2001; Zimmermann et al., 2003). Spectral imaging of FRET is a specific type of microscopy that spectral bleedthrough correction can be applied to. A major advantage of spectral imaging is that it is acquired more quickly than other methods. Acceptor photobleaching measures the quenched donor population first, and then the acceptor fluorophores are deliberately photobleached, eliminating them from participating in the FRET process (Bastiaens et al., 1996; Day et al., 2001; Kenworthy, 2001). Removing the acceptor fluorophore causes the donor molecule to dequench, increasing the fluorescence emission from the donor. Importantly, after photobleaching the acceptor is not susceptible to

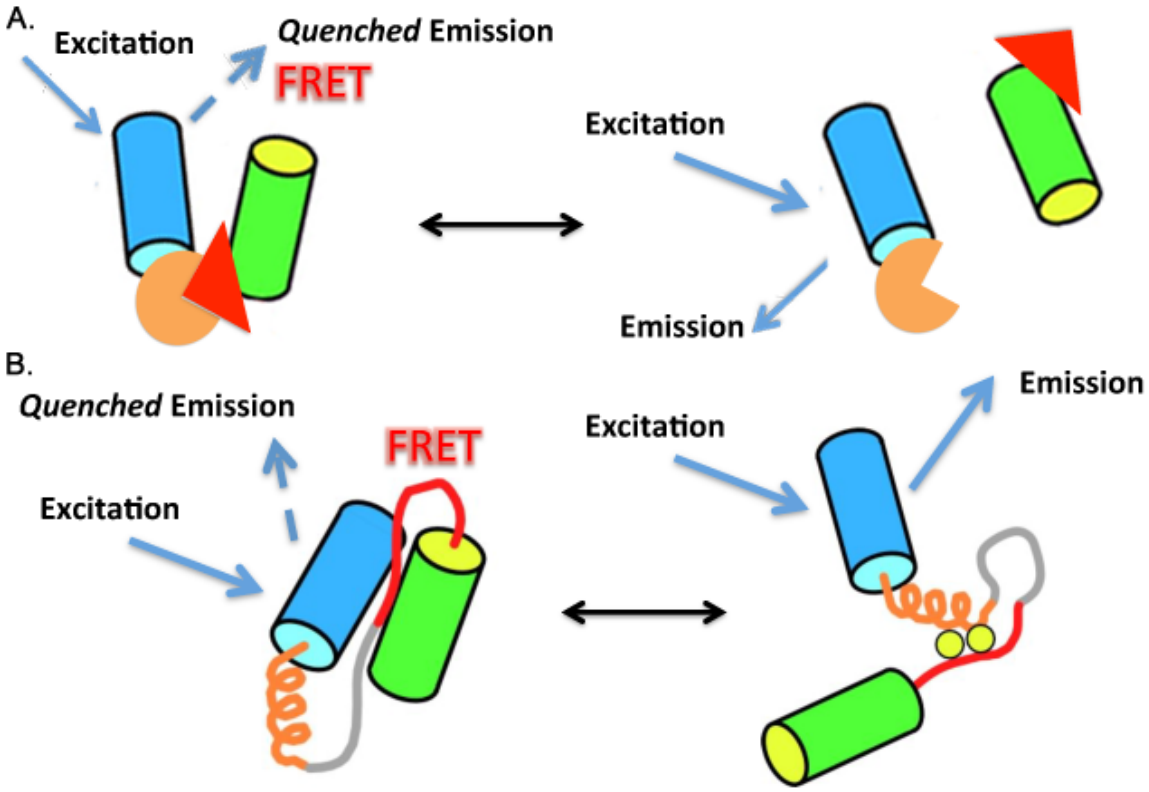
spectral bleed-through due to the elimination of the acceptor's participation in the FRET process. Since photobleaching is not reversible it is an approach that limits the possibility for dynamic FRET measurements. Lastly, measuring the changes in donor fluorescent lifetimes is another method for measuring FRET (Periasamy and Clegg, 2009). Fluorescent lifetime is the average time a molecule spends in the excited-state before relaxing down to the ground state. It is a fundamental property of a fluorophore and range between one to ten nanoseconds. When measuring donor fluorescent lifetimes, spatial distribution of the lifetimes can be mapped using Fluorescence Lifetime Imaging Microscopy (FLIM). A major advantage to this method is that lifetime measurements are independent of deviations in the probe concentration and excitation intensity.

FLIM can be analyzed by two different approaches, time domain and frequency domain (Clegg, 2010). A pulsed-light source is used to excite a fluorophore in the time domain method and then the emission photons are collected at various time points. This information is used to produce a fluorescence decay profile that estimates the fluorescent lifetime. The frequency domain method uses a high frequency modulated light source to excite the fluorophores. Depending on the lifetime of the fluorophores, a modulation frequency is chosen often between 20-140 megahertz. Changes in the phase and amplitude of the emission signal are compared to the excitation source to extract the fluorophore's fluorescence lifetime. A major advantage to using FLIM to assess FRET is that the measurements are made in the donor channel, which is usually unaffected by spectral bleed-through. FLIM is suitable for visualizing

probes that indicate changes such as ion concentration, post-translational modifications or pH. Fluorescent lifetime are sensitive to their environment and would not be appropriate for fixed specimens. Rapid changes in FRET signals (less than a minute) may be missed by the time it takes to acquire fluorescent lifetimes.

Experimentally, there are two forms of FRET, intermolecular and intramolecular. Intermolecular FRET occurs between independent donor fluorophore and acceptor molecule (Figure 4). For example, intermolecular FRET could occur between two different proteins, one labeled with a donor fluorophore and one with an acceptor. Intramolecular FRET occurs between a donor fluorophore and acceptor on the same molecule. Commonly, biosensors are examples of intramolecular FRET. A biosensor usually contains a donor and acceptor fluorophore, along with a phosphorylation substrate used to monitor dynamic changes in kinase activity. For instance, a conformational change within the molecule could cause the fluorophores to move away from one another, diminishing a FRET event. The first biosensor was the cyclic AMP (cAMP) biosensor, which was a genetically encoded sensor of activities mediated by cAMP (Adams et al., 1991). Interesting, the first version of the cAMP biosensor contained organic dyes, rather than fluorophores, which were attached to the catalytic and regulatory subunits of cAMP (Adams et al., 1991). Other examples of FRET biosensors used by investigators include GTPases (Nakamura et al., 2005), second messengers (Herbst et al., 2009), protein kinases (Herbst et al., 2009), and membrane receptors (Lohse et al., 2007).

Figure 4. Intermolecular and Intramolecular FRET



Schematic examples of intermolecular and intramolecular FRET. (A) Example of intermolecular FRET. The donor (blue) fluorophore is fused with a protein (orange) that finds a binding partner in a different protein (red) that is fused to an acceptor fluorophore (green). When the two proteins (orange and red) are bound, a FRET event occurs between the two fluorophores resulting in quenched emission of the donor. (B) Example of intramolecular FRET. The donor (blue) fluorophore and acceptor fluorophore (green) are on the same molecule, usually connected through a short amino acid linker. Figure is adapted from Hum, et al., 2012.

Interestingly many FRET biosensors have been developed to monitor enzymatic modifications, such as protein ubiquitination (Perroy et al., 2004), acetylataion (Sasaki et al., 2009), O-glycosylation (Carrillo et al., 2006), histone methylation (Lin et al., 2004), and phosphorylation/dephosphorylation (Newman and Zhang, 2008; Ni et al., 2006). Kinase activation/activity is a heavily utilized category of FRET biosensors (Ni et al., 2006; Zhang and Allen, 2007). Recently, a RhoA biosensor was utilized to monitor RhoA activation in osteoblasts in response to 1 hour of fluid shear stress (Hamamura et al., 2012). A Src biosensor was capable of detecting an increase in Src activity in response to mechanical stimulation in endothelial cells (Wang et al., 2005). A combination of Src and FAK biosensors were used to demonstrate that Src activity decreases and FAK activity increases during differentiation of human mesenchymal stem cells (Liao et al., 2012). The field of signal transduction requires tools for tracking the dynamic nature of signaling molecules. FRET biosensors can serve this need and are an exciting tool that permits monitoring molecules with great spatiotemporal resolution in their native cellular environment.

Thesis Hypothesis and Specific Aims

Based on the research findings summarized above and the current lack of understanding of the molecular mechanisms that curb the response of bone to mechanical loading, the following central hypothesis was formulated: “STOP” mechanosomes exist to actively suppress the anabolic response of osteoblasts and osteocytes to fluid shear stress. This hypothesis was tested by examining two specific aims: 1) Determine Src’s ability to function as a “STOP” mechanosome in osteoblasts and osteocytes and 2) Characterize the role of Pyk2 in response to fluid shear stress in osteoblasts and osteocytes.

Chapter II

MATERIALS AND METHODS

Cell Culture Conditions. MC3T3 osteoblasts, mouse calvarial osteoblasts (MCOB), and Pyk2 -/- osteoblasts were cultured in minimal essential media alpha (MEM-α, Gibco, Life Technologies, Carlsbad, CA) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Gibco, Life Technologies, Grand Island, NY). MLO-Y4 osteocytes were cultured on collagen-coated plates (rat tail collagen type I, BD Biosciences, San Jose, CA) in MEM-α (Gibco, Life Technologies, Carlsbad, CA) supplemented with 5% FCS, 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Life Technologies, Grand Island, NY). Cells were maintained in 5% CO_2 at 37° C during experiments and imaging.

Fluid Flow Conditions. Two methods were used to induce fluid shear stress over the surface of cells. An oscillatory pump connected to parallel plate flow chambers via hard–walled tubing was used to induce a shear rate of 10 dynes/cm^2 across the surface of cells plated on glass slides. Additionally, a reservoir was created for the movement of fluid and 5% CO_2 exchange using hard-walled tubing attached to the outlet of the parallel plate. Static controls were incubated in the same volume of media. Prior to exposure to fluid flow, cells were serum starved (MEM-α, 0.5% FCS) overnight. During fluid flow

experiments the parallel plate flow chambers and attached hard-walled tubing were placed in an incubator set to 37°C and 5% CO_2.

Similarly, an orbital shaking platform was used to induce fluid flow over the surface of MC3T3 osteoblasts and MLO-Y4 osteocytes. Cells plated in 6 well culture dishes were placed on an orbital shaking platform within an tissue culture incubator set to 37°C and 5% CO_2. Prior to experimentation, cells were grown overnight in low-serum α-MEM media supplemented with 0.5% FCS and antibiotics. Cells were subjected to fluid flow generated by 1 mL of media on an orbital platform shaker rotating at a speed of ~200 rpm (2Hz) producing a estimated shear rate of ~10-25 dynes/cm^2 while inside a tissue culture incubator (Inoue et al., 2004; Kido et al., 2009; Sakai et al., 1999; Young et al., 2011).

Src Inhibitor. MC3T3 osteoblasts and MLO-Y4 osteocytes were treated with Src Inhibitor 1 (Santa Cruz Biotechnology, Santa Cruz, CA) (10μM) for 1 hour before experiments were conducted. Control samples were treated with equal volume of DMSO.

Western Blotting Analysis. Cells exposed to static or flow conditions were harvested directly into SDS sample buffer and protein concentrations were determined using amino black method (Sheffield et al., 1987). Equal amounts of protein were loaded onto SDS-PAGE gels for separation and transferred to nitrocellulose. The subsequent primary and secondary antibodies were used: phospho-Src (Y418) (Cell Signaling, Boston, MA), total Src (Cell Signaling,

Boston, MA), γ-tubulin (Sigma-Aldrich, St. Louis, MO), lamin B (Santa Cruz, Santa Cruz, CA), HRP conjugated goat anti-rabbit and HRP conjugated goat anti-mouse (Jackson Immunoresearch, West Grove, PA). The secondary antibody signals were detected using a Luminescent Image Analyzer LAS-3000 system (Fujifilm Life Science, Stamford, CT). Densitometry was quantified using Image J software (NIH).

RNA Extraction, cDNA Synthesis and quantitative real-time PCR (qRT-PCR) Analysis. RNA was harvested from cultured MC3T3 osteoblasts and MLO-Y4 osteocytes in Trizol (Invitrogen, Carlsbad, CA). RNA was extracted with chloroform and precipitated with isopropanol. M-MLV reverse transcriptase (Promega, Madison, WI) was used to perform first strand cDNA synthesis. GAPDH (Mm99999915_g1), osteopontin (Mm00436767_m1), and RPLP2 (Mm03059047_gH) real-time PCR primers were obtained (Applied Biosystems, Grand Island, NY). Custom designed primer/probes were prepared for osteocalcin. (forward) 5(-CTGACAAAGCCTTCATGTCCAA-)3 (probe) 5(-AGGAGGGCAATAAGGTAGT-)3 and (reverse) 5(-GGTAGCGCCGGAGTCTGTT-)3. TaqMan Universal PCR Master Mix (Applied Biosystems, Grand Island, NY) was used for amplification in a Mastercycler ep realplex^2 real-time PCR system (Eppendorf, Westbury, NY). The reaction conditions were as follows: 2 minutes at 50°C; 10 minutes at 95°C; 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The ΔΔCT method was used to

evaluate gene expression between samples. RPLP2 and GAPDH were used as a loading control genes.

Immunofluorescence. Images for all experiments were captured using a Nikon inverted immunofluorescence microscope equipped with a CCD camera. MLO-Y4 osteocytes cells were plated onto coverslips and placed into 6 well culture dishes. Cells were serum starved for 24 hrs (0.5% FCS), subjected to static conditions and fixed immediately or fixed after 20 minutes of OFSS (via orbital shaking platform) with 4% paraformaldehyde solution and permeablilized with 0.2% triton after rising with Tris-buffered saline (TBS). Coverslips were treated with normal donkey serum for 30 minutes at 37°C to block non-specific antibody binding. The following primary and secondary antibodies were used: Src and Src Y418 (Cell Signaling, Boston, MA) followed by FITC-conjugated donkey anti-rabbit (Jackson Immunoresearch, West Grove, PA). Texas-Red phalloidin and DAPI (Molecular Probes, Eugene, OR) were used for visualizing F-actin and the nucleus, respectively.

MCOB were plated on glass coverslips, ~2.0 x10^5 cells per slide (~1.1 x10^5 cells/ cm^2), following 24 hrs of serum starvation (0.5% FCS), cells subjected to static culture conditions were fixed immediately (static) or fixed after OFSS (30 minutes, 1 hour, or 1 hour plus 1 hour) (via oscillatory pump) with 4% paraformaldehyde solution and processed for immunofluorescence by permeabilization with 0.2% triton followed by rinsing in Tris-buffered saline (TBS). Slides were then treated with 1% BSA solution in TBS for 30 minutes at 37°C to block non-specific antibody binding. The following primary and secondary

antibodies were used: Pyk2 (BD Biosciences, San Jose, CA), DAPI (Molecular Probes, Eugene, OR), and FITC-conjugated donkey anti-rabbit (Jackson Immunoresearch, West Grove, PA).

DNA Plasmids. The Src biosensor and Src mutant biosensor were generously obtained from Dr. Yingxiao Wang (Wang et al., 2005).

FRET Microscopy. One day prior to imaging, MLO-Y4 cells were electroporated (150V, 9ms) with either the Src biosensor or the Src mutant biosensor (10μg). Cells were plated into 35mm glass bottom dishes (MatTech, Ashland, MA) and maintained in phenol-free, MEM-α media supplemented with 0.5% FCS and antibiotics. The following day, FRET microscopy was performed using an ISS ALBA FastFLIM system (ISS Inc., Champagne, IL) coupled to an Olympus IX71 microscope equipped with a 60 X / 1.2 NA water-immersion objective lens. A 5mW 448 nm diode laser was modulated by the FastFLIM module of the ALBA system at a fundamental frequency of 20 MHz with up to six sinusoidal harmonics. The modulated 5mW 448 nm laser was used to excite the donor fluorophore of either the Src biosensor or Src mutant biosensor. The frequency domain FLIM method was used to obtain fluorescent lifetime(s) based on emission signal changes in the phase and amplitude compared to the excitation source. The phasor plots, lifetime maps and intensity images were analyzed using ISS VistaVision software (ISS Inc., Champagne, IL). Lifetime maps were generated using a two-component fit for the calculation of the donor lifetime. The

phasor plots display the modulation and phase characteristics of the emission signal for every pixel in an image, generating a visual determination of the lifetime (Jameson et al., 1984; Redford and Clegg, 2005). Lifetime data from MLO-Y4 cells were extracted from three different regions of interest (ROI): plasma membrane, cytoplasm and nucleus. Bodipy C12 (Invitrogen Corp., Grand Island, NY) (0.01mg/mL) was used to stain the plasma membrane and outline the nucleus.

Nuclear Fractionation. Cells subjected to either static or OFSS treatment in 6 well dishes were washed with phosphate buffered saline, harvested in ice-cold hypotonic buffer (10mM HEPES, 10mM KCL, 1.5mM $MgCl_2$, 1mM DTT and protease inhibitors), passed through a 22-gauge needle five times and were centrifuged for 10 minutes at 13,000 rpm. The cytosolic fraction was saved while the nuclear fraction pellet was resuspended in buffer C (10mM HEPES, 0.42 M NaCl, 25% Glycerol, 1.5mM $MgCl_2$, 0.5mD EDTA, ddH_2O and protease inhibitors) placed on ice and vortexed for 15 seconds every 10 minutes for an hour to swell nuclear proteins out the nuclei. Finally the samples were centrifuged at 4°C for 15 minutes at 14,000 rpm. The clear supernatant was collected and prepared for western blot analysis.

Immunoprecipitation. For detecting protein-protein interactions *in vivo* co-immunoprecipitation was performed in MC3T3 osteoblasts and MLO-Y4 osteocytes. Immunoprecipitation was performed using Src protein, Src (Y416),

MBD2, normal rabbit serum, or normal mouse serum. Immunoprecipitation buffer contained 1% Triton-X-100, 145 mM NaCl, 10mM Tric-Cl, pH 7.4, 5mM EDTA, 2mM EGTA, and 1mM PMSF. Immune complexes were captured using Protein A sepharose beads (Sigma-Aldrich, Saint Louis, MO) conjugated to either goat-anti rabbit or goat-anti mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PA).

Statistical Analysis. Statistical significance was assessed by either a two-tailed t-test or a two-way analysis of variance (ANOVA) with a p-value of $p<0.05$ or less interpreted as statistically significant.

Chapter III

Nuclear Src Activity Functions to Suppress the Anabolic Response Osteoblasts and Osteocytes to Fluid Shear Stress

ABSTRACT

Global deletion of Src kinase from mice results in increased bone mass. We tested the novel hypothesis that Src plays a previously unrecognized role in bone formation by regulating gene expression in osteoblasts and osteocytes, particularly in response to mechanical loading. Inhibition of Src activity using a pharmacologic inhibitor in MC3T3 osteoblasts and MLO-Y4 osteocytes led to an increase in expression of the anabolic bone gene osteocalcin. Mechanical stimulation of MC3T3 osteoblasts and MLO-Y4 osteocytes by fluid shear stress further enhanced expression of osteocalcin when Src activity was inhibited. Importantly, using a Src biosensor and nuclear fractionation, we report for the first time that Src activity in the nucleus increased in response to fluid shear stress. This study supports the idea that Src plays a nuclear role, suppressing expression of osteocalcin via a previously unrecognized function that limits the anabolic response of osteoblasts and osteocytes to fluid shear stress.

INTRODUCTION

Global disruption of Src, a 60 kDa non-receptor tyrosine kinase, resulted in a mouse with a high bone mass phenotype, demonstrating the importance of Src in bone remodeling (Soriano et al., 1991). The function of both osteoclasts and osteoblasts is altered in Src-/- mice (Marzia et al., 2000; Soriano et al., 1991). Osteoclast numbers are increased at the bone surface, but lack a ruffled border and are inactive (Boyce et al., 1992; Horne et al., 1992; Lowe et al., 1993). Accelerated osteoblastogenesis was observed in the Src-null mice, suggesting Src activity plays a suppressive role in osteoblast differentiation (Amling et al., 2000; Marzia et al., 2000). These findings led to studies focused on producing a Src inhibitor to treat osteoporosis (Hannon et al., 2010; Hannon et al., 2012; Id Boufker et al., 2010; Missbach et al., 1999).

If Src functions to balance bone mass by suppressing anabolic bone genes, it is also likely to affect the response of bone to mechanical loading. In the healthy mammalian skeleton, this process is mediated by osteocytes and osteoblasts that coordinate an appropriate response to mechanical loading resulting in localized net bone gain or loss depending on the type of load experienced at specific sites (Miller et al., 2007; Nicolella et al., 2008; Robling, 2009). Osteocytes and osteoblasts sense and react to mechanical loads generated by the fluid flow through the canalicular system within bone (Buss et al., 1986; Montgomery et al., 1988; Resh, 1994). *In vitro*, osteoblasts and osteocytes respond to mechanical load simulated via the application of oscillatory fluid shear stress (OFSS). Fluid

shear stress causes distortions of the membranes of osteoblasts and osteocytes resulting in the enhanced expression of genes associated with osteoblast activity, up regulating cell proliferation and increasing the release of paracrine factors required for bones to elicit an anabolic response (Knothe Tate et al., 1998; Smalt et al., 1997; Turner and Pavalko, 1998). Src also becomes activated in response to OFSS in osteoblasts and osteocytes (Plotkin et al., 2005; Rangaswami et al., 2010; Rangaswami et al., 2012). The Pilz group recently suggested that Src plays an integral role in relaying mechanical messages in osteoblasts via NO-cGMP-PKG signaling resulting in a proliferative response (Rangaswami et al., 2010). Additionally, this group described the convergence of PKG and FAK on the Src/Akt/β-catenin signaling pathway during osteoblast mechanotransduction (Rangaswami et al., 2012). While Src is activated in osteoblasts and osteocytes in response to OFSS, the impact of Src activation on gene transcription under conditions of OFSS is unclear.

RESULTS

Src kinase represses osteocalcin in static and OFSS conditions

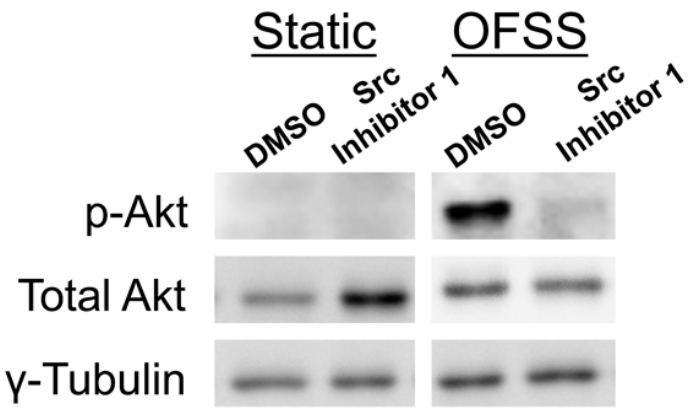
To evaluate the role of Src in basal expression of osteogenic genes in MC3T3 osteoblasts and MLO-Y4 osteocytes we treated cells with a pharmacologic inhibitor of Src activity (Src inhibitor-1, SI1). We first confirmed that SI1 effectively inhibited Src activity by showing that SI1 inhibited fluid flow-induced Akt phosphorylation (Figure 5). To assess OFSS-induced changes in gene expression we utilized real-time quantitative PCR (qRT-PCR) analysis of mRNA (primers listed in Table 2). Osteocalcin expression significantly increased in both MC3T3 osteoblasts and MLO-Y4 osteocytes treated with SI1. (Figure 6). Most importantly, OFSS further enhanced the express of osteocalcin in both MC3T3 osteoblasts and MLO-Y4 osteocytes treated with SI1 compared to static controls treated with carrier (DMSO) only (2.7 and 3.4 fold change, respectively; Figure 7).

Activated Src (Y418) accumulates in perinuclear and nuclear regions in response to OFSS

To determine whether Src may affect OFSS-induced transcription via an increase in tyrosine kinase activity in the nucleus, we first examined the total cellular distribution of Src in MLO-Y4 osteocytes in response to OFSS. Immunofluorescence microscopy indicated a shift in the distribution of both total Src and activated Src (as assessed by phosphorylation at tyrosine residue Y418)

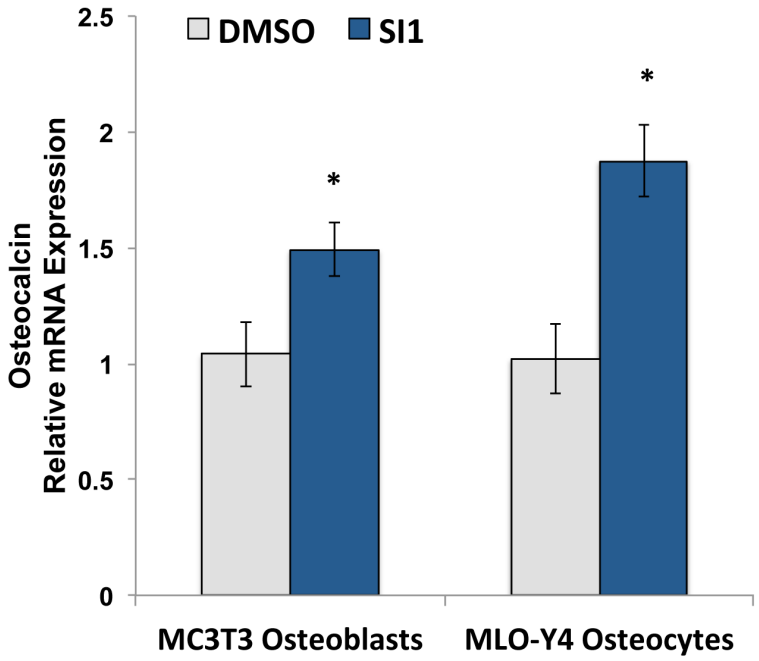
following 20 minutes of exposure to OFSS. Using an antibody that recognizes only activated Src phosphorylated at tyrosine 416 (Y418) we found that activated Src accumulated in the perinuclear/nuclear area of MLO-Y4 osteocytes after exposure to OFSS (Figure 8A, white arrows highlight areas of FA). Total Src protein also increased modestly in the perinuclear/nuclear regions after OFSS, but was not as pronounced as that of the activated (Y418) Src (compare Figures 8A and 8B). This suggests that Src activation by phosphorylation at Y418 may be required for Src to accumulate in this region of the cell.

Figure 5. Treatment with Src inhibitor 1 Prevents Src Activity in Response of OFSS



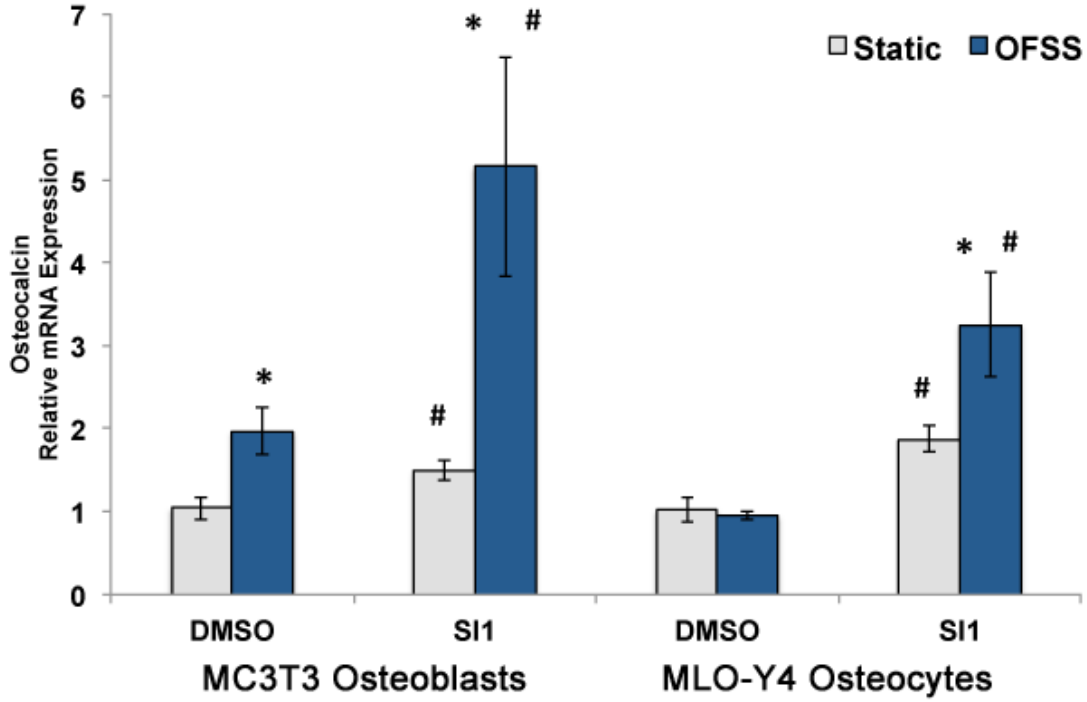
One hour of Src inhibitor 1 (SI1) treatment prevents Src kinase activity. MC3T3 osteoblasts and MLO-Y4 osteocytes were plated at a density of 1.0 x 10^5 into 6 well dishes and serum starved in MEM-α (5% FBS) overnight. Cells were treated with 10μM SI1 for 1 hour prior to 1 hour of OFSS. Western blot analysis of phosphorylated-Akt (Ser308), Total Akt, and γ-tubulin under static and OFSS conditions (1 hour).

Figure 6. Osteocalcin Expression in MC3T3 Osteoblasts and MLO-Y4 Osteocytes Treated with SI1



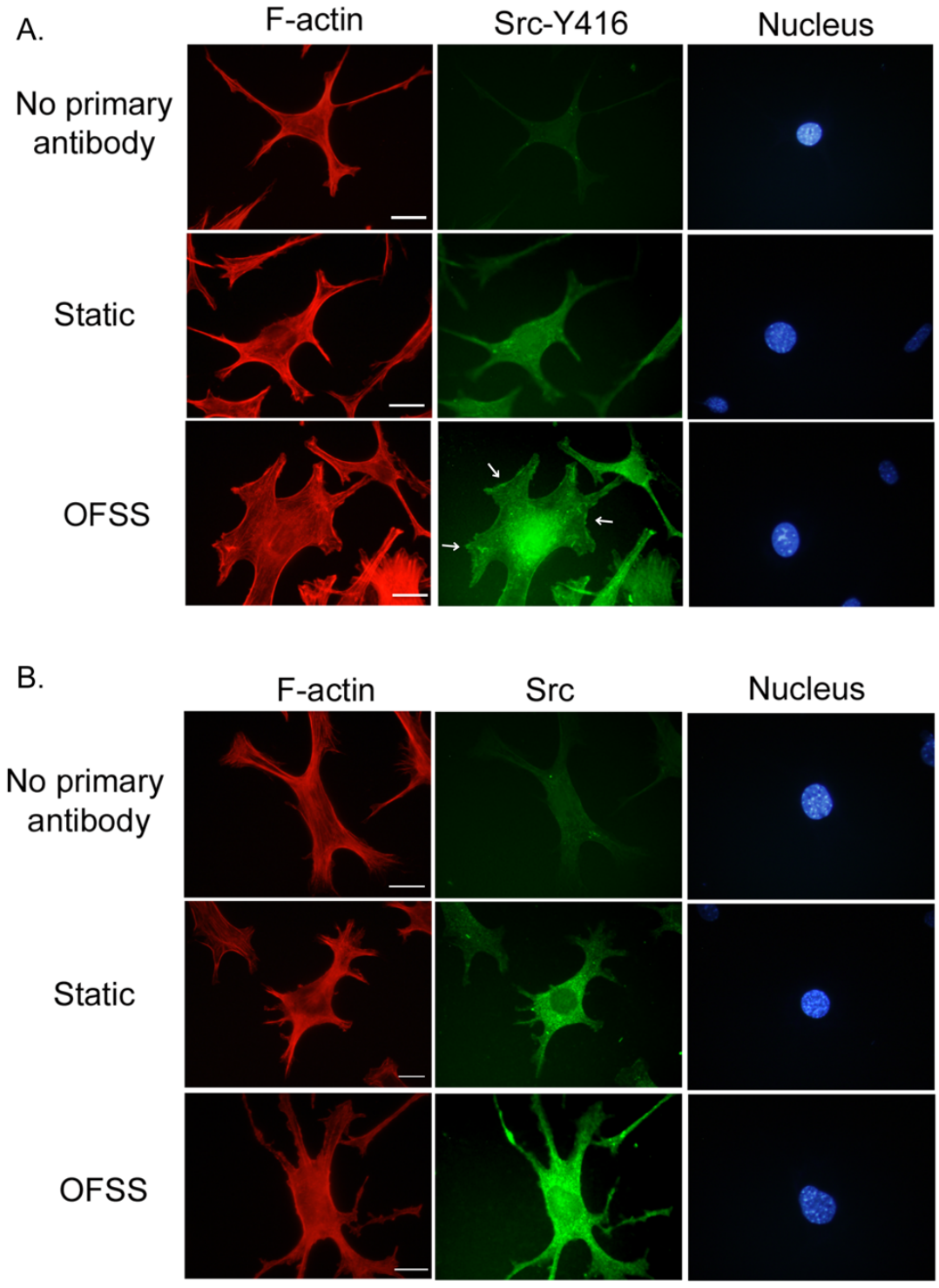
Inhibiting Src kinase activity causes an increase in basal levels of osteocalcin expression. MC3T3 osteoblast and MLO-Y4 osteocytes were plated in 6 well dishes and treated with either control (DMSO) or SI1 (10μM) for one hour. SI1 treatment significantly increases the expression of osteocalcin in MC3T3 osteoblasts and MLO-Y4 osteocytes. *Represents a statically significant increase compared to static control (*$p<0.05$). Error bars represent standard error. An $n \geq 3$ was used and experiments were performed in triplicate.

Figure 7. OFSS-induced Osteocalcin Expression in MC3T3 Osteoblasts and MLO-Y4 Osteocytes Treated with SI1



OFSS further enhanced the expression of osteocalcin in both MC3T3 osteoblasts and MLO-Y4 osteocytes compared to static controls. MC3T3 osteoblasts and MLO-Y4 osteocytes were treated with either control (DMSO) or SI1 (10μM) for 1 hour prior to exposure to either static of OFSS conditions. ($p<0.05$). *Represents a statically significant increase compared to static control (*$p<0.05$). #Represents a statically significant difference between treatment groups (#$p<0.05$) Error bars represent standard error. An $n \geq 3$ was used and experiments were performed in triplicate.

Figure 8. Activated and Total Src Localization in MLO-Y4 Osteocytes Under Static or OFSS Conditions



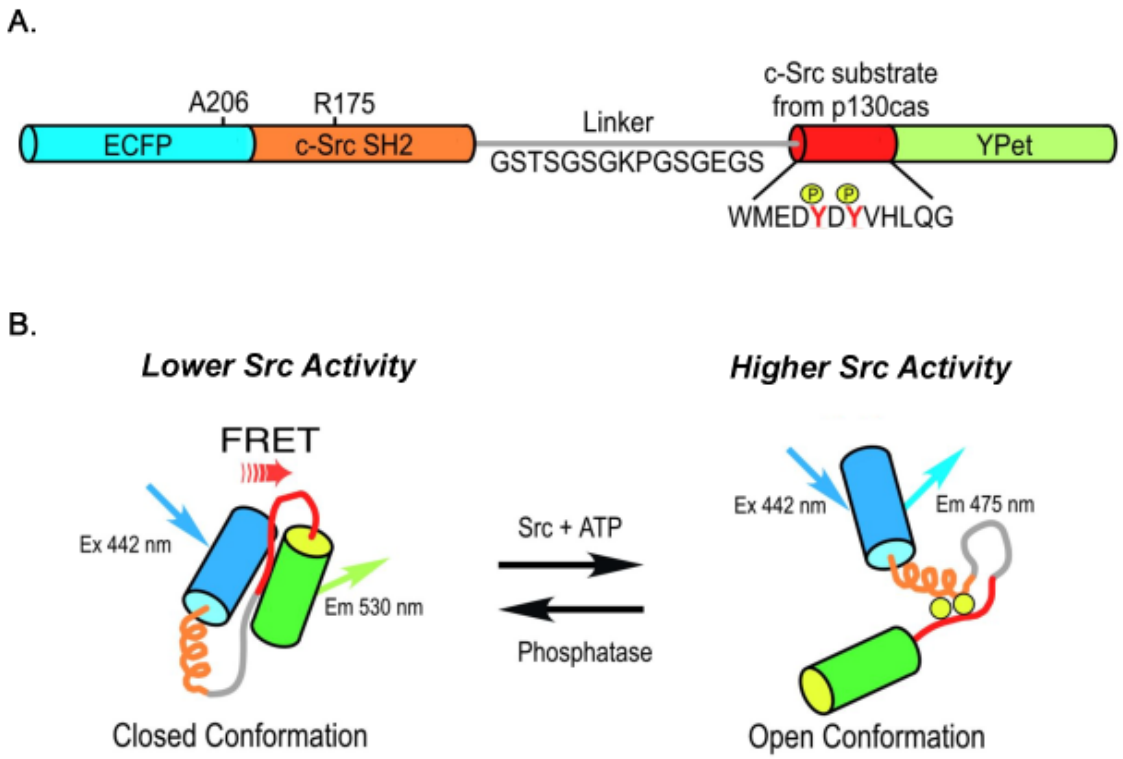
OFSS induces accumulation of Src at perinuclear/nuclear regions in MLO-Y4 osteocytes. (A) Immunofluorescence microscopy of MLO-Y4 osteocytes subjected to static culture conditions or OFSS for 20 minutes. Slides were fixed immediately and processed for immunofluorescence using antibodies against activated Src (Y418 followed by FITC-conjugated secondary antibodies). F-actin was visualized using Texas-Red Phalloidin and the nucleus was visualized using DAPI. White arrows indicate focal adhesions, highlights Src's increased activation at the plasma membrane in response to OFSS. Scale bars=25μm (B) Immunofluorescence microscopy of MLO-Y4 osteocytes subjected to static culture conditions or OFSS for 20 minutes. Slides were fixed immediately and processed for immunofluorescence using antibodies against total Src followed by FITC-conjugated secondary antibodies. F-actin was visualized using Texas-Red Phalloidin and the nucleus was visualized using DAPI. Scale bars=25μm

Nuclear Src activity increases in response to OFSS

To directly examine changes in the sub-cellular distribution of Src tyrosine kinase activity in MLO-Y4 osteocytes in response to OFSS we utilized a Src biosensor to measure Src activity by FRET microscopy. The changing FRET signal from the Src biosensor probe was detected using fluorescent lifetime imaging microscopy (FLIM), which measures the shortened donor lifetime that results from FRET. This approach allows us to map with pixel level resolution the sub-cellular locations of changing Src protein activity. The Src biosensor used here is in a closed conformation under conditions of low endogenous Src activity, resulting in high FRET efficiency and a shortened donor lifetime. Upon phosphorylation of the substrate peptide by endogenous Src, the substrate binds to the phosphopeptide-binding pocket of the SH2 domain, resulting in a more open conformation and diminished FRET leading to an increased donor lifetime (Figure 9). Thus, an increase in Src biosensor lifetime indicates an increase in Src kinase activity.

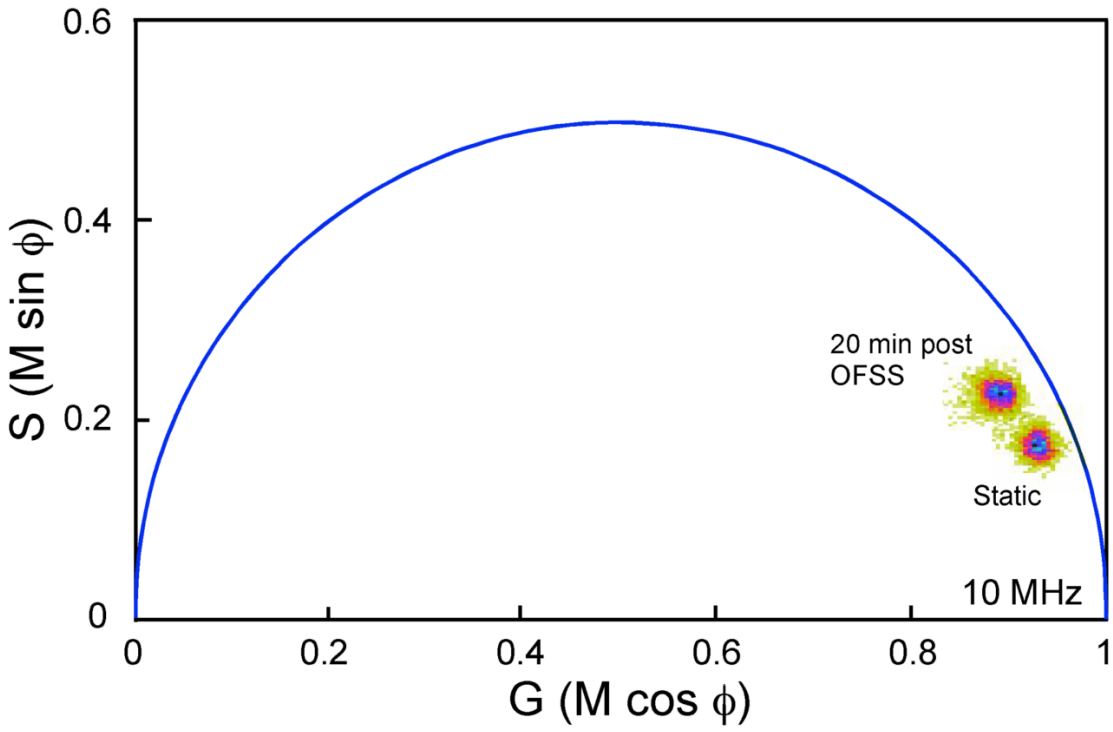
The phasor plot analysis comparing the total population of Src biosensor in MLO-Y4 cells prior to or following exposure to OFSS clearly showed a shift towards longer lifetimes of the Src biosensor, indicating a global increase in Src activity throughout MLO-Y4 cells in response to OFSS (Figure 10). Following exposure to 5 minutes of OFSS, lifetimes of the Src biosensor donor fluorophore were determined for three distinct sub-cellular compartments (regions of interest, ROI) – the membrane, cytoplasm and nucleus - at 10, 15 and 20 minutes post-OFSS using FLIM analysis. Prior to OFSS, Src activity in the nucleus was

Figure 9. Diagram of the Src Biosensor



The Src biosensor is capable of detecting changes in endogenous Src kinase activity. (A) The structure of the Src biosensor. The Src biosensor is made up of a donor fluorophore (ECFP) fused to the Src Homology 2 (SH2) of cytosolic Src (c-Src). A short amino acid linker connects ECFP and SH2 to the acceptor fluorophore (YPet) and c-Src substrate that can be phosphorylated by endogenous Src. Two mutants of the Src biosensor were generated to cause the Src biosensor to remain in a closed confirmation. The R175 site within the SH2 domain was mutated, as well as, the two key phosphorylation sites within the p130Cas substrate. (B) The Src biosensor is in a closed conformation under conditions of low endogenous Src activity, resulting in high FRET efficiency and a shortened donor lifetime. Upon phosphorylation of the substrate peptide by endogenous Src, the substrate binds to the phosphopeptide-binding pocket of the SH2 domain, resulting in a more open conformation and diminished FRET leading to an increased donor lifetime. This is a dynamic and reversible process. Figure was adapted from Wang et al., 2005 (Hum et al., 2012).

Figure 10. Phasor Plot of the Src Biosensor's Donor Lifetime in MLO-Y4 Osteocytes Under Static and OFSS Conditions

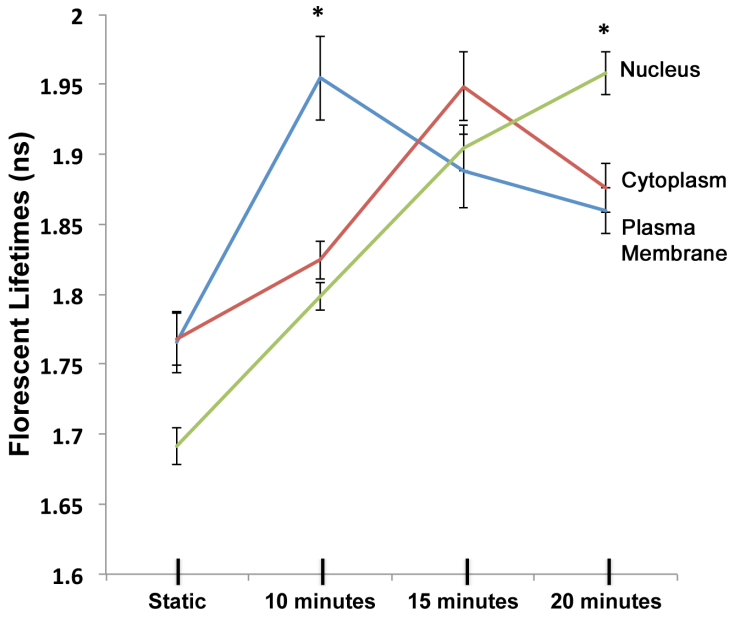


A global increase in Src activity occurs in response to OFSS. Phasor plot overlay of static Src biosensor lifetime and 20 minutes post-OFSS Src biosensor lifetime. The phasor plot analysis displays a shift towards longer lifetimes of the Src biosensor, indicating an increase in Src activity throughout MLO-Y4 cells in response to OFSS.

significantly lower than at either the membrane or in the cytoplasm (Figure 11). Most importantly, significant increase in nuclear Src activity was seen at each time point measured following OFSS and increased steadily during the 20 min post-OFSS period (Figure 12). Prior to OFSS, the average lifetime of the Src biosensor in the nucleus was 1.69 $\pm$ 0.01 nanoseconds. At 10, 15 and 20 minutes post-OFSS nuclear Src biosensor lifetimes significantly increased compared to static (1.80 $\pm$ 0.01, 1.90 $\pm$ 0.02, and 1.95 $\pm$ 0.02 nanoseconds, respectively) (Figure 12). Lifetime maps of the nucleus under static and post-OFSS conditions illustrate the increase in Src activity in response to OFSS (Figure 13). In contrast, Src activity at the membrane and in the cytoplasm peaked at 10 and 15 minutes post-OFSS, respectively, and then decreased at 20 minutes post-OFSS (Figure 11).

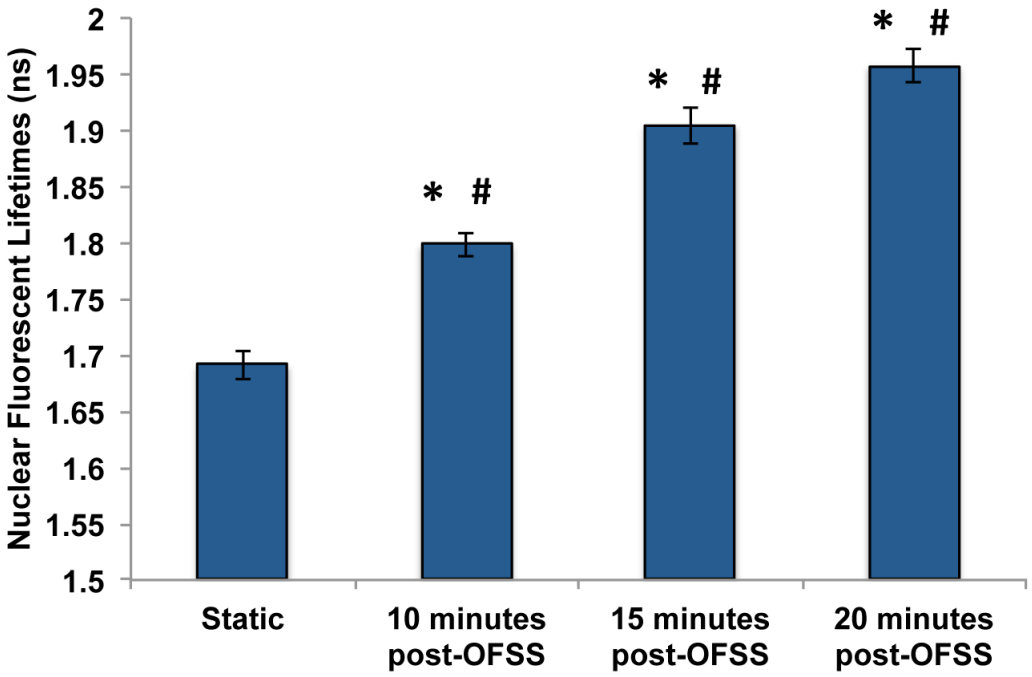
In sharp contrast, there was no change in the fluorescent lifetime of a mutant Src biosensor (Y662F Y664F) following 5 minutes of OFSS (Figure 14). The mutation of the tyrosine residues in the p130Cas substrate blocks Src tyrosine kinase activation of the biosensor, and also changes its confirmation, resulting in higher basal lifetimes compared to the Src biosensor (Figure 15).

Figure 11. Subcellular Measurements of the Src Biosensor's Donor Lifetimes in MLO-Y4 Osteocytes in Response to OFSS



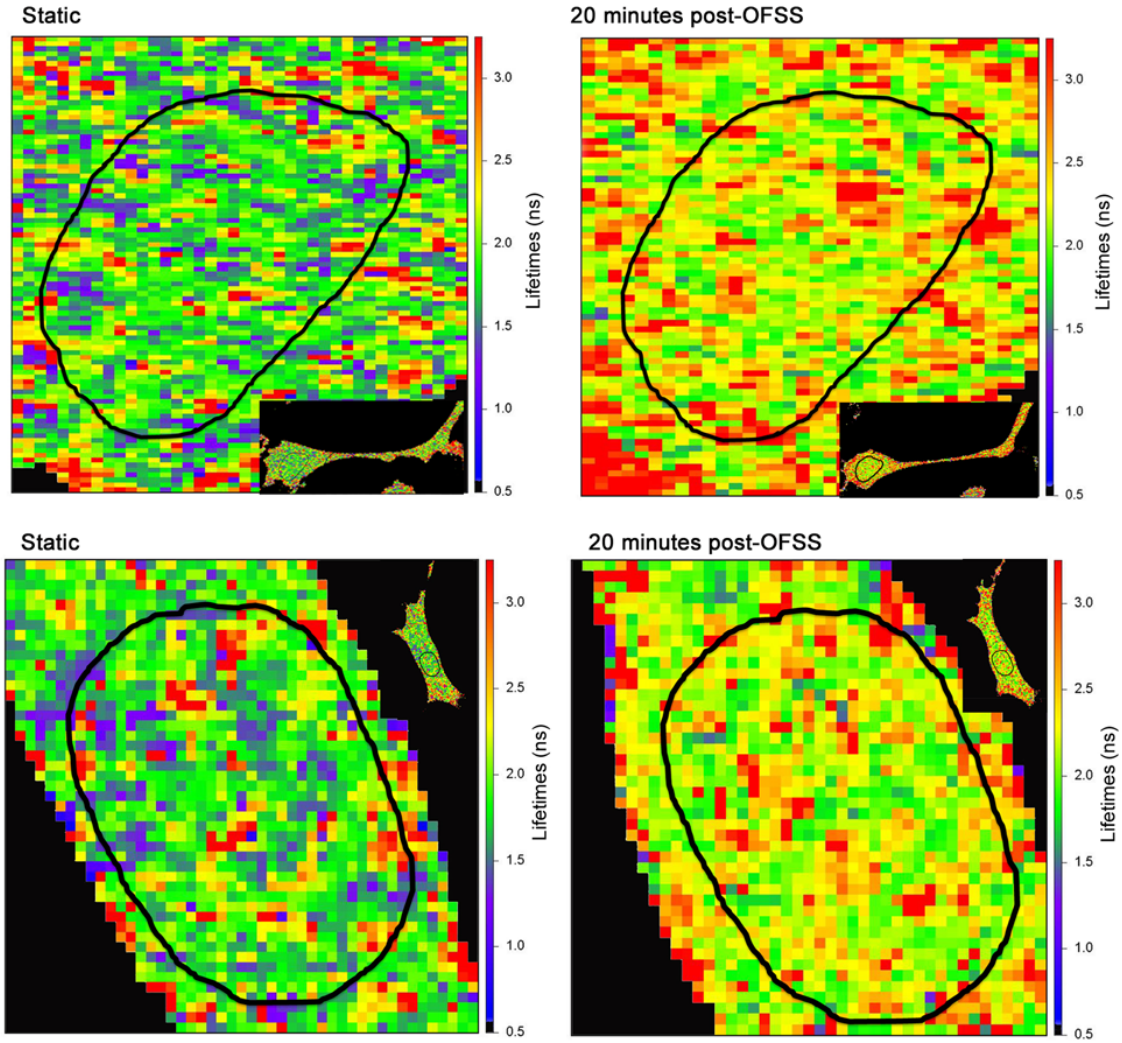
In response to OFSS the subcellular regions of Src activity change. MLO-Y4 osteocytes expressing the Src biosensor were exposed to 5 minutes of OFSS. Images were taken 10, 15, and 20 minutes post-OFSS. A distinct pattern of Src activity develops in the three regions of interest (ROI) examined: plasma membrane, cytoplasm, and nucleus. The plasma membrane displays a significantly longer lifetime 10 minutes after exposure to OFSS compared to the other ROI (*$p<0.05$). Fifteen minutes after OFSS the cytoplasm displays the longest lifetimes. Lifetimes in the nucleus are significantly longer 20 minutes after exposure to OFSS (*$p<0.05$). Graph represents n=3 in which the average lifetime of each ROI (30) at each time point analyzed. Error bars represent standard error.

Figure 12. Lifetimes of Src Biosensor in the Nucleus of MLO-Y4 Osteocytes in Response to OFSS



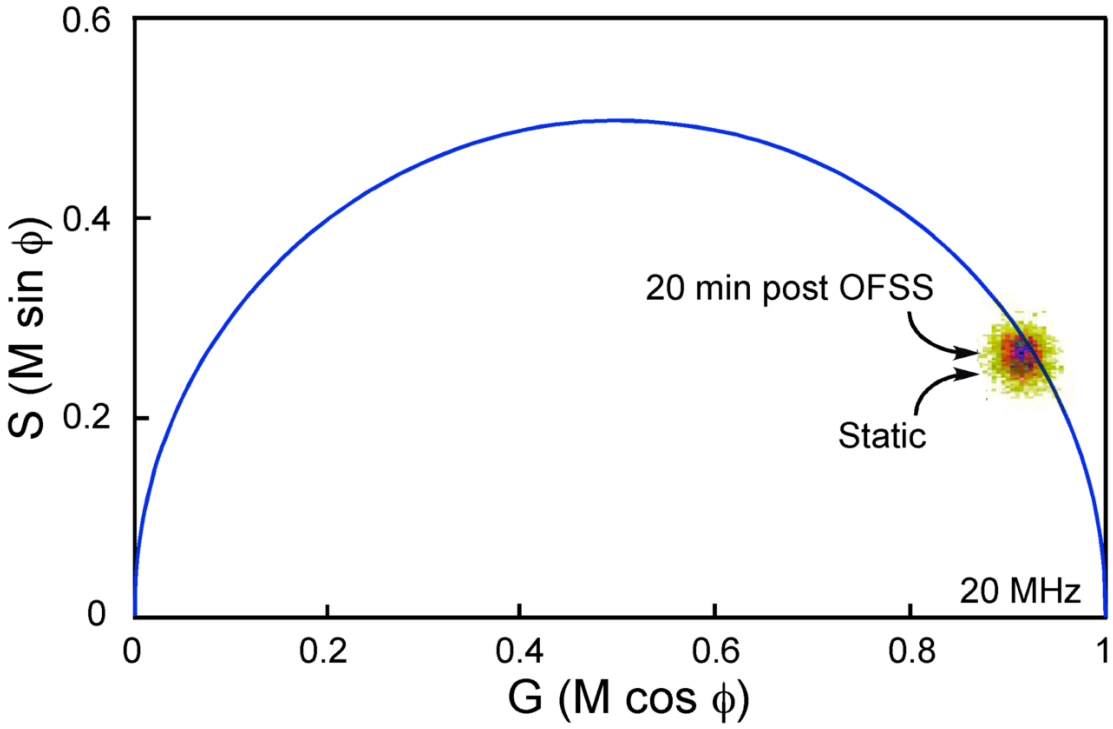
Src activity increases in the nucleus in response to OFSS. A significant increase in nuclear Src activity was seen at each time point measured following OFSS. Graph represents an N=3, with the average of 30 ROI's examined at each time point. Error bars represent standard error. *p<0.05 versus the static control, #p<0.05 versus other OFSS time points examined

Figure 13. Lifetime Maps of the Src Biosensor Under Static or OFSS Conditions



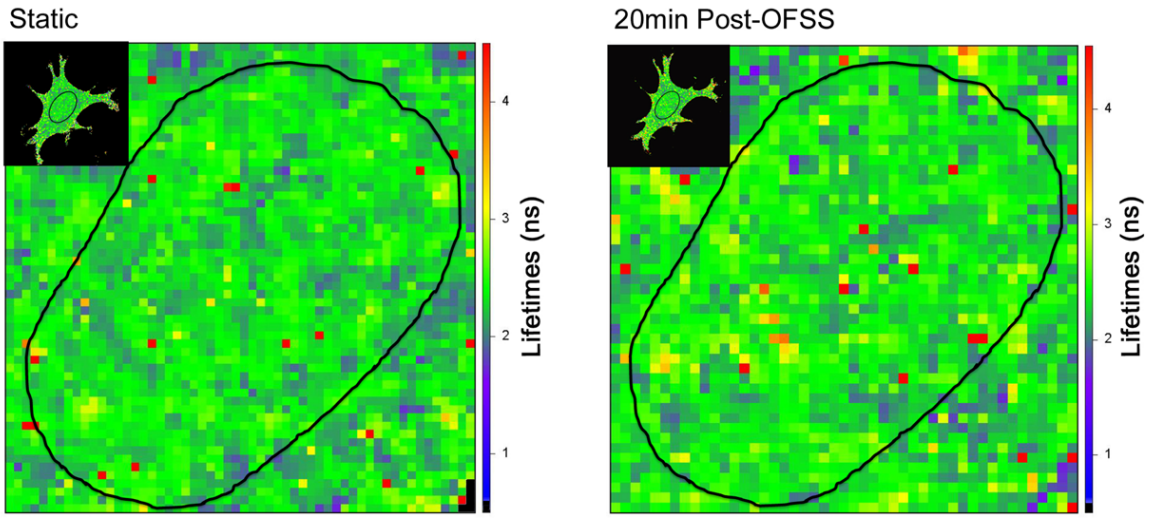
Nuclear Src activity increases in response to OFSS. Lifetime maps of two MLO-Y4 osteocytes under both static and 20 minutes post-OFSS conditions. Areas of short donor lifetimes are indicated by cooler colors, while longer donor lifetimes are indicated by warmer colors.

Figure 14. Phasor Plot of the Mutant Src Biosensor



OFSS does not change the lifetime of the mutant Src biosensor. MLO-Y4 osteocyte cells expressing the mutant Src biosensor we analyzed under static conditions and then exposed to 5 minutes of OFSS. Next, lifetime images were taken at 10, 15, and 20 minutes post-OFSS. The phasor plot above was generated from the overlaid phasor plots of the same static and 20 minutes post-OFSS MLO-Y4 osteocyte.

Figure 15. Lifetime Maps of the Mutant Src Biosensor Under Static or OFSS Conditions

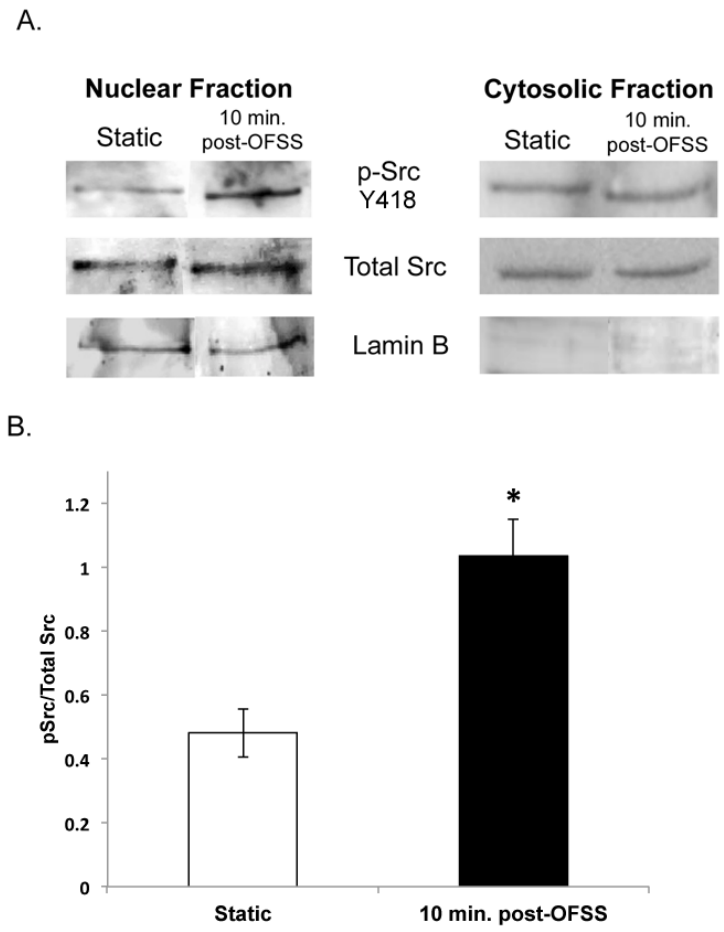


OFSS does not induce a change in the mutant Src biosensor's lifetimes in the nucleus. Mutant Src biosensor lifetimes in MLO-Y4 osteocytes were analyzed under static conditions and after exposure to 5 minutes of OFSS. In nucleus, no change in lifetime occurs in the mutant Src biosensor. Areas of short donor lifetimes are indicated by cooler colors, while longer donor lifetimes are indicated by warmer colors.

Src activation increases in the nucleus in response to OFSS

To validate the observation of increased Src activity in the nucleus of MLO-Y4 cells in response to OFSS; nuclear fractionation followed by Western blot analysis was performed. MLO-Y4 osteocytes were exposed to either static conditions (control) or 5 minutes of OFSS. The control cells or OFSS cells were collected 10 minutes-post-OSS. Src activation, as measured by phosphorylation at Y418, was performed by Western blot. In response to 5 minutes of OFSS activated Src in the nucleus was increased at 10 minutes post-OFSS compared to static conditions (Figure 16).

Figure 16. Nuclear Fractionation of MLO-Y4 Osteocytes



Nuclear Src activation increases in response to OFSS. (A) Western blot analysis of nuclear fractionation blotted for Src activation (Y418), total Src and lamin B in MLO-Y4 osteocytes exposed to 5 minutes of OFSS or static culture conditions. (B) Graph represents quantification of Src activation (Y418)/total Src in nuclear fractions. Error bars represent standard error. Statistically significant difference between static and 10 minutes post-OFSS (*$p<0.05$). An $n \geq 3$ was used, experiments were performed in triplicate.

DISCUSSION

The goal of this study was to investigate a potential role for Src tyrosine kinase in transcriptional regulation of anabolic gene expression in MC3T3 osteoblasts and MLO-Y4 osteocytes, principally in response to OFSS. Expression of osteocalcin, a marker of bone formation in vivo, was increased when Src activity was inhibited in MC3T3 osteoblasts and MLO-Y4 osteocytes. Furthermore, we show for the first time that the increase in osteocalcin expression normally induced by OFSS was further enhanced in MC3T3 osteoblasts and MLO-Y4 osteocytes when Src activity was inhibited by treated with SI1 compared to control cells in which Src activity was not inhibited. We used a Src biosensor to demonstrate an increase in Src activity in the nucleus of MLO-Y4 osteocytes in response to OFSS that was further confirmed using nuclear fractionation. Together these results support the novel concept that Src plays a role in bone remodeling by functioning to curb the anabolic response of MC3T3 osteoblasts and MLO-Y4 osteocytes to OFSS via a mechanism that involves a mechanically-induced increase in nuclear Src activity.

Inhibition of Src caused an increase in osteocalcin mRNA in MC3T3 osteoblasts not subjected to fluid flow, while a flow-induced increase in osteocalcin mRNA normally induced by mechanical stimulation was further enhanced by Src inhibition compared to control cells (Figure 7). Interestingly, in MLO-Y4 osteocytes Src inhibition under static conditions fails to increase osteocalcin expression. Only after exposure to OFSS, does Src inhibition lead to

an increase in osteocalcin expression (Figure 7). Thus, our results suggest Src normally functions to attenuate osteocalcin expression under conditions of both static culture and OFSS. Osteocalcin is an important protein associated with bone formation and its levels in serum directly correlate with measurements of bone mineral density (Delmas et al., 1990; Wolf, 1996). Osteocalcin expression is increased in response mechanical loading in both *in vivo* and *in vitro* models (Kannus et al., 1996; Raab-Cullen et al., 1994). Here we suggest, for the first time, that Src activity affects the expression of osteocalcin in both static and OFSS conditions.

Taken together our immunolocalization, FRET microscopy and nuclear fractionation studies suggest that exposure of MLO-Y4 cells to OFSS results in an increase in the amount of activated Src (Y418 phosphorylation) in the perinuclear/nuclear regions and an increase in Src tyrosine kinase activity in the nucleus in response to OFSS. A subtle increase in total Src in the perinulear/nuclear region was seen in response to OFSS. In contrast, a more pronounced increase in activated Src (Y418) was seen in the perinuclear and/or nuclear regions of MLO-Y4 osteocytes (Figure 8) suggesting that the population of Src that accumulates in this region is activated (as assessed by Y418 phosphorylation). Our nuclear fractionation and FRET data directly demonstrate that Src tyrosine kinase activity increased in the nucleus in response to OFSS. This novel result suggests a previously unrecognized role for Src tyrosine kinase activity in regulating the transcriptional response of MLO-Y4 osteocytes to OFSS. There is not a widely accepted mechanism to explain how Src translocates into

the nucleus, however myrstoylation has been suggested to function in regulating transport of Src to the nucleus (David-Pfeuty et al., 1993). Additionally, myristoylation is one of the ways in which Src maintains its distribution at the plasma membrane where it can participate in integrin-mediated signaling responses to mechanical loading (Resh, 1994). A hydrophobic pocket is predicted to exist in the SH-1 kinase domain of Src, enabling Src to switch between its myristoylated and nonmyristolated forms (Cowan-Jacob et al., 2005). An increase in nuclear Src activation (Y418) was reported in breast cancer tissue samples and correlated with improved patient outcome (Campbell et al., 2008).

We have characterized the formation and function of load-induced multi-protein complexes termed “mechanosomes” that mediate mechanotransduction in bone cells (Bidwell and Pavalko, 2010; Bidwell and Pavalko, 2011; Pavalko et al., 2003b). We propose the existence of mechanosomes that either promote (“GO”) or attenuate (“STOP”) load-induced bone formation. β-catenin/Lef1 is an example of a “GO” mechanosome, while NMP4/p130Cas functions as a “STOP” mechanosome (Childress et al., 2010; Jackson et al., 2005; Robinson et al., 2006; Tamamura et al., 2005; Yang et al., 2010). The Pilz group has described a mechanosome made up of protein kinase G, Src and Src homology 2 domain-containing tyrosine phosphatase 1 and 2 (Rangaswami et al., 2010). Our results extend these findings to suggest that OFSS enhances the activity of a Src-containing “STOP” mechanosome to attenuate the anabolic response of osteoblasts and osteocytes to loading. “STOP” mechanosomes function to prevent the overreaction of “GO” mechanosomes to OFSS, preventing the

overexpression of anabolic bone genes. For instance, OFSS-induces an increase in osteocalcin expression in both MC3T3 osteoblasts and MLO-Y4 osteocytes, which we propose is limited by the activity of a Src-containing "STOP" mechanosome. When Src activity is inhibited under OFSS conditions, the expression of osteocalcin is further enhanced due to the absence of activity from a Src-containing "STOP" mechanosome that functions to attenuate the expression of anabolic bone genes. Under static conditions inhibiting the activity of Src causes a modest increase in osteocalcin expression. This suggests that a Src-containing "STOP" mechanosome may have a role in attenuating the transcription of anabolic bone genes under static conditions as well.

We suggest that increased Src tyrosine kinase activity in the nucleus in response to mechanical loading may serve as transient "off switch" to attenuate the anabolic response of bone to mechanical loading. Once activated by mechanical stimulation, Src may further increase its activity in the nucleus and participate in a mechanism to prevent an over-reaction to physical stimulation. Clinical trials are underway testing the effectiveness of Src inhibitors on suppressing bone resorption by osteoclasts. Our results suggest the possibility that load-bearing exercise could enhance the efficacy of Src inhibitors in patients treated with Src inhibitor. Further studies will be needed to determine the detailed molecular mechanism(s) by which Src activation and activity in the nucleus regulates gene expression. It is noteworthy however that a previous report suggested that Src might be capable of regulating methyl-CpG-binding domain protein 2 (MBD2) mediated expression of the myogen promoter during

skeletal muscle differentiation (Luo et al., 2009). Src may also function as a "STOP" mechanosome by targeting the activity of activator protein 1 (AP1) transcription factor to suppress anabolic bone gene transcription. The C-terminal region of Src can negatively regulate AP1 activity. AP1 is a heterodimer comprised of two transcription factors, *c-fos* and *c-jun*. Src can bind and phosphorylate c-Jun at Y26 and Y170 resulting in the ubiquitination of c-Jun and decreased AP1 activity (Zhu et al., 2006). Since osteocalcin contains an AP1 binding site in its promoter region, this may serve as a mechanism by which Src activity could regulate anabolic bone gene transcription (Lian et al., 1989).

Using FRET microscopy with FLIM analysis to investigate endogenous Src tyrosine kinase activity permits the mapping the sub-cellular locations of changing Src activity in living cells. FLIM analysis does not require corrections for spectral-bleed through, which is necessary for other FRET-based imaging approaches. The Src biosensor used here is in a closed conformation under conditions of low endogenous Src activity, resulting in high FRET and shortened donor lifetimes. Upon phosphorylation of the substrate peptide by endogenous Src, the substrate binds to the phosphopeptide-binding pocket of the SH2 domain, resulting in an open conformation and diminished FRET leading to an increased donor lifetime. Through the use of a Src biosensor we observed that mechanical stimulation by fluid flow increases Src activity in the nucleus of MLO-Y4 osteocytes 20 minutes after exposure to a brief (5 min) bout of OFSS. This approach confirmed that OFSS initially (10 min post-OFSS) has the greatest impact on activity of Src that is localized at the membrane (where integrins detect

OFSS stimulation). Subsequently, OFSS-induced Src activity was highest in the cytoplasm (15 min) and by 20 min the greatest increase in Src activity was seen in the nucleus. There is some concern when examining a substrate phosphorylated by Src that related kinases such as Yes, Abl, Jak2 or the Ser/Thr kinase ERK1 might also produce a change in lifetime of the Src biosensor. A previous reported on the specificity of this Src biosensor found a change of less than 2% in emission ratio in the Src biosensor by Yes, Abl, Jak2 or Ser/Thr kinase ERK1 (Wang et al., 2005). Additionally, the mutant Src biosensor does not respond to endogenous Src activity and remains in a closed conformation. Recently, using Src and FAK biosensors, Src activity was reported to decrease and FAK activity to increase during differentiation from human mesenchymal stem cells to osteoblasts (Liao et al., 2012). Our study is the first to report spatial and temporal changes, via FLIM analysis, in Src activity in MLO-Y4 osteocytes in response to OFSS.

In conclusion we suggest Src may play a significant functional role in attenuating the transcription of anabolic bone genes, such as osteocalcin, in response to OFSS. This effect is evident *in vitro* under basal (static) conditions, as well as, following exposure to mechanical loading (OFSS). *In vivo*, load-bearing exercise promotes skeletal health by adjusting bone remodeling and bone mass. An underappreciated aspect of load-induced bone formation may be the existence of negative feedback signals mediated by Src within osteocyte directed skeletal mechanotransduction pathways that may limit the beneficial bone forming effects of exercise. Pharmacological interventions that inhibit Src

activity could disable those negative feedback signals and dynamically enhance skeletal health. Among the questions raised by this study is the precise molecular mechanism(s) through which Src activity is increased in the nucleus in response to OFSS and how it aids in repressing transcription. Future studies will need to define how Src participates in attenuating anabolic bone gene expression by identifying Src binding partners in the nucleus that have the capacity to directly alter transcription and the epigenome.

Chapter IV

Pyk2 May Function as a "STOP" Mechanosome By Interacting with MBD2 in Osteoblasts and Osteocytes

ABSTRACT

Pyk2 plays an important role in bone remodeling. Pyk2 null mice exhibit increased bone mass, due to compromised osteoclast function. Undefined is Pyk2's role in mediating mechanotransduction in bone cells. We tested the hypothesis that Pyk2 suppresses anabolic targets of OFSS-induced mechanotransduction in osteoblasts and osteocytes. In this study we sought to determine Pyk2's localization, effect on the abundance of proteins and transcripts associated with anabolic signaling, and association with other signaling molecules under static and OFSS conditions. We found Pyk2 suppressed OFSS-induced Cox-2 protein expression and osteopontin gene expression, and displayed nucleocytoplasmic shuttling. These observations of Pyk2's effect on protein and gene expression and localization are consistent with the phenomena we have proposed of mechanosomes. To determine whether Pyk2 may form a complex similar to a mechanosome, co-immunoprecipitation experiments examined Pyk2's association with MBD2 under static and OFSS conditions. A constitutive interaction between Pyk2 and MBD2 was observed and was not OFSS dependent. However, when examining the activated form of Pyk2 (Y402), OFSS induced an increase in the association of Pyk2 and MBD2. Additionally,

the association between activated Src (Y418) and Pyk2 (Y402) increases in response to OFSS. In summary, we found that in response to OFSS Pyk2 is capable of nucleocytoplasmic shuttling, increased association with MBD2 and suppression of Cox-2 protein expression and osteopontin expression, thus meeting the criteria of functioning as a “STOP” mechanosome.

INTRODUCTION

Bone tissue adapts to changes in mechanical loading from the external environment by modeling and remodeling. This is an example of mechanotransduction, a process by which a mechanical signal is detected and converted into biochemical and transcriptional responses inside the cell (French, 1992). On the cellular level in bone mechanical loading causes changes in interstitial fluid flow that is detected by osteoblasts and osteocytes. Focal adhesions (FA) play an important role in mechanotransduction and are thought to serve as mechanosensors of osteoblasts and osteocytes (Geiger and Bershadsky, 2001; Thompson et al., 2012). FA are mainly composed of structural proteins such as integrins, vinculin, α-actinin, and actin filaments and adhesion-associated signaling proteins like FAK, Pyk2, and Src (Geiger and Bershadsky, 2001). Focal adhesions are ideal launching sites for either “GO” or “STOP” mechanosomes, in response to changes in fluid shear stress. Mechanosomes are made up of an adhesion-associated protein and a transcription factor and either serve to promote the anabolic response of bone to mechanical loading (“GO”) or suppress its response to mechanical loading (“STOP”) (Bidwell and Pavalko, 2010; Bidwell and Pavalko, 2011; Pavalko et al., 2003b). Much work has been done to describe protein complexes like β-catenin/Lef1 that function as a “GO” mechanosome, however few complexes have been described to function as a “STOP” mechanosome. Pharmacological

manipulation of “STOP” mechanosomes may prove to be a novel therapeutical target.

FAK is a non-receptor tyrosine kinase found at sites of focal adhesions. By associating with the integrins of FA, FAK becomes activated by autophosphorylation at tyrosine 397 (Calalb et al., 1995). We have previously reported FAK to be important in mediating the mechanotransduction signal in osteoblasts exposed to OFSS (Young et al., 2009). In response to OFSS osteoblasts lacking FAK fail to appropriately increase protein levels of Cox-2, c-Fos, and osteopontin. Furthermore, OFSS-induced IkB-β and IkB-α degradation and NF-kB nuclear translocation was impaired in FAK -/- osteoblasts (Young et al., 2010).

Pyk2 is another member of the FAK family of non-receptor tyrosine kinases and is found to associate with focal adhesions. FAK and Pyk2 are closely related in structure, sharing ~45% homology (Herzog et al., 1996; Inazawa et al., 1996). Pyk2 is highly expressed in osteoclasts and its role within osteoclast function is more clearly defined than in osteoblasts and osteocytes. Mice lacking Pyk2 exhibit mild osteopetrosis and impairment of osteoclast function (Gil-Henn et al., 2007; Okigaki et al., 2003). More specifically, osteoclasts lacking Pyk2 fail to form a functional sealing zone causing impaired bone resorption. Pyk2’s signaling capacity in osteoblasts or osteocytes, particularly in response to OFSS, has not been described.

RESULTS

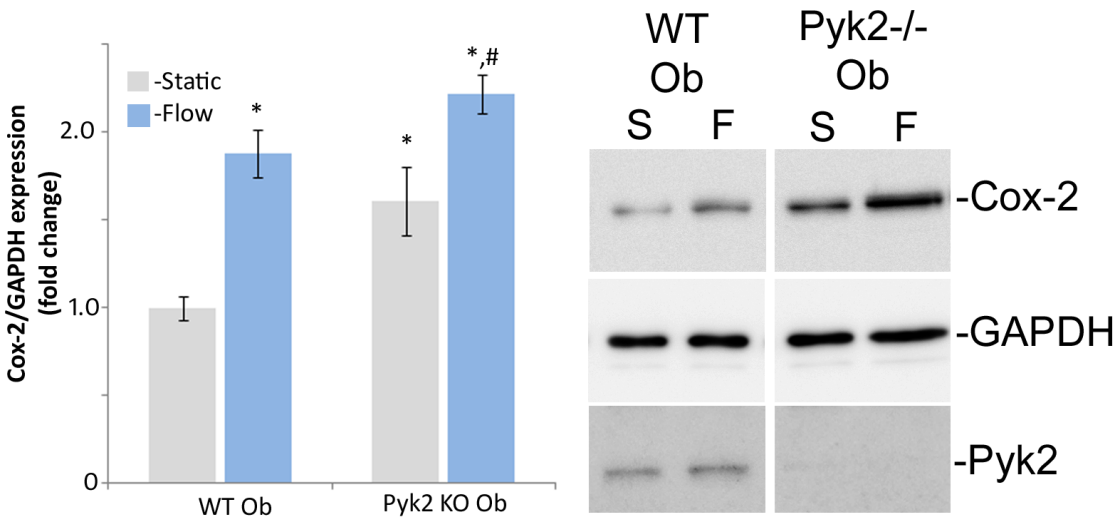
OFSS-induced Cox-2 protein expression is enhanced in Pyk2 -/- osteoblasts

Wild-type MCOB and Pyk2 -/- osteoblasts were subjected to either static or 1 hour of OFSS. OFSS induces a significant 1.8 fold increase in Cox-2 expression in wild-type MCOB's (Figure 17). In the absence of Pyk2, the OFSS induced increase of Cox-2 is significantly higher than static Pyk2 -/- osteoblasts and elevated compared to the Cox-2 protein levels of wild-type MCOB in exposed to OFSS (Figure 17). OFSS does not induce any changes in the protein levels Pyk2 in either the wild-type MCOB or Pyk2 -/- osteoblasts (Figure 17).

Basal osteopontin expression is elevated in Pyk2 -/- osteoblasts and further enhanced in response to OFSS in Pyk2 -/- osteoblasts

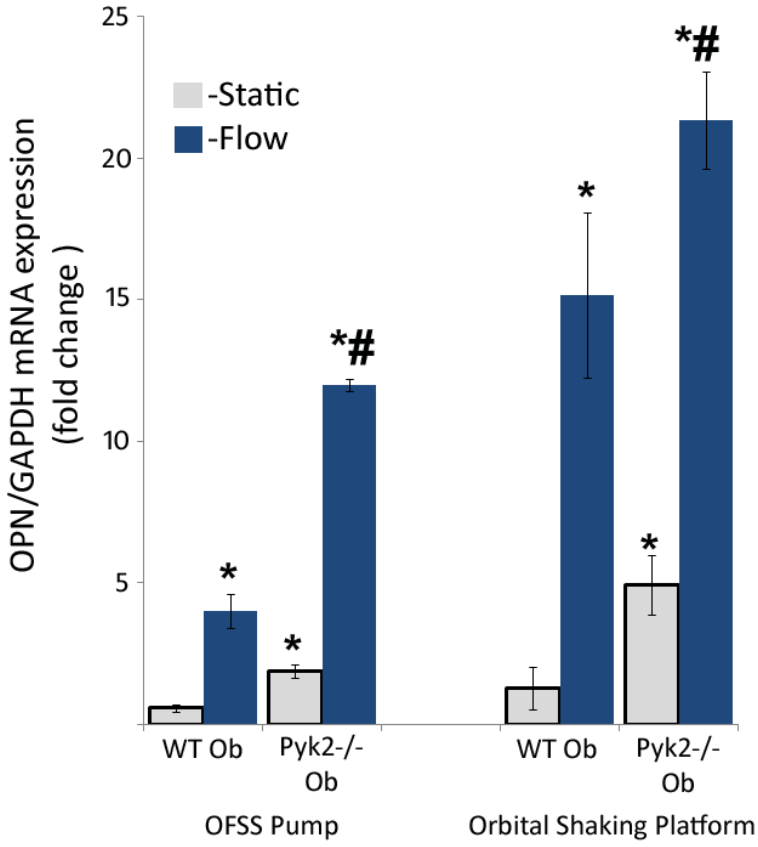
To determine if Pyk2 -/- might play a role in regulating changes of gene transcription in response to OFSS, relative expression of mRNA was examined. Under static conditions, Pyk2 -/- osteoblasts expressed significantly higher levels of osteopontin (Figure 18). Both wild-type MCOB and Pyk2 -/- osteoblasts were exposed to either static or OFSS conditions. Using either method of inducing OFSS (OFSS pump or orbital shaking platform), Pyk2 -/- osteoblasts expressed enhanced levels of osteopontin expression compared to wild-type MCOB's in response to OFSS (Figure 18).

Figure 17. Cox-2 Protein Expression in Wild-type MCOB and Pyk2 -/- Osteoblasts Under Static and OFSS Conditions



Cox-2 protein expression is enhanced in Pyk2 -/- osteoblasts. Wild-type MCOB and Pyk2 -/- osteoblasts were exposed to either static of one hour of OFSS. Representative of immunoblots for Cox-2, GAPDH, and Pyk2 show a OFSS-induced increase in Cox-2 protein expression. OFSS does not cause a change in the loading control, GAPDH. Confirmation of Pyk2 knockout is seen in bottom immunoblot. Graph represents the densitometry units of Cox-2, normalized to GAPDH. In wild-type MCOB, OFSS results in a 1.8 fold change in Cox-2 protein expression. Pyk2 -/- osteoblasts have elevated levels of Cox-2 protein under static conditions, and after OFSS the level of Cox-2 protein expression is highest compared to all other groups (static control, wild-type MCOB static control, and wild-type MCOB OFSS). Error bars represent standard error. *$p<0.05$ vs. static control; #$p<0.05$ vs. wild-type MCOB. N=3 in three separate trials.

Figure 18. Osteopontin Expression in Wild-type MCOB and Pyk2 -/- Osteoblasts Under Static and OFSS Conditions



Osteopontin expression is elevated in Pyk2 -/- osteoblasts under static conditions and further enhanced after exposed to OFSS. Wild-type MCOB and Pyk2 -/- osteoblasts were exposed to either static or 1 hour of OFSS conditions, using either an OFSS pump or orbital shaking platform. Static Pyk2 -/- osteoblasts expressed significantly higher levels of osteopontin compared to static wild-type MCOBs. In response to OFSS, using either method, the absence of Pyk2 further enhances the OFSS-induced osteopontin expression. Error bars represent standard error. *$p<0.05$ vs. static control, #$p<0.05$ vs wild-type MCOB. N=3 in three separate trials.

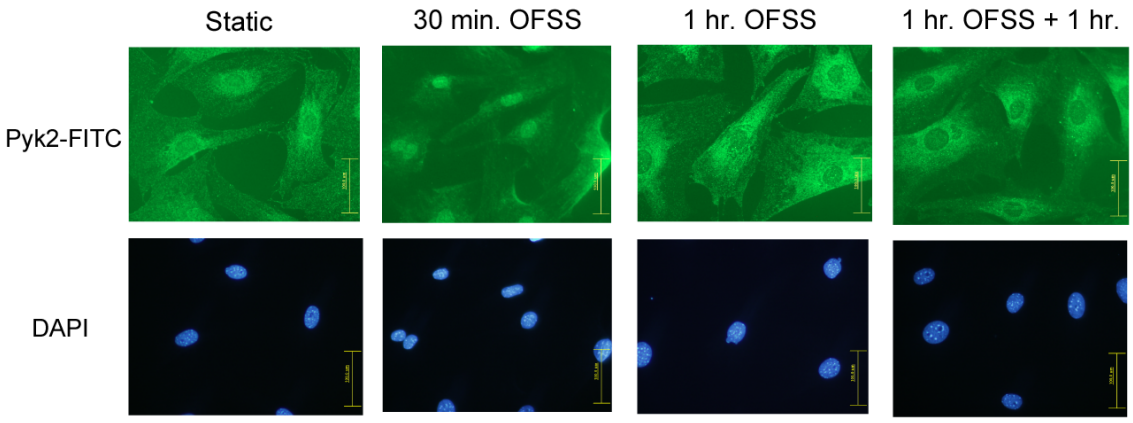
Pyk2 accumulates in the nucleus in response to OFSS

After observing OFSS-induced changes in anabolic gene transcription in the absence of Pyk2 we next monitored the distribution of Pyk2 after periods of OFSS. Immunofluorescent microscopy was used to examine the cellular distribution of Pyk2 in MCOB's that were exposed to either static conditions or OFSS (30 minutes, 1 hour, or 1 hour + 1 hour of rest). Static MCOBs displayed a relatively even distribution of Pyk2 throughout the cells, with a subtle concentration of Pyk2 in the perinuclear/nuclear region (Figure 19). In response to 30 minutes of OFSS, Pyk2 preferentially accumulates in the nucleus of MCOB's (Figure 19). After exposure to an hour of OFSS, the accumulation of Pyk2 in the nucleus is less robust than at the 30 minute OFSS time point (Figure 19). In response to 1 hour of OFSS and 1 hour of rest, Pyk2's absence in the nucleus is even further enhanced compared to static. This suggests that Pyk2 is capable of shuttling between the cytoplasm and the nucleus in response to OFSS (Figure 19).

Pyk2 and MBD2 complex under basal conditions and increase their association in response to OFSS

To further determine if Pyk2 plays a nuclear role in the regulation of anabolic bone genes, we examined its association with MBD2. We performed a co-immunoprecipation assay to examine the possibility of a Pyk2 complex with MBD2. Western blot antibodies to either Pyk2 or MBD2 were used to detect immunoprecipitated proteins. In static osteoblast lysates, endogenous Pyk2

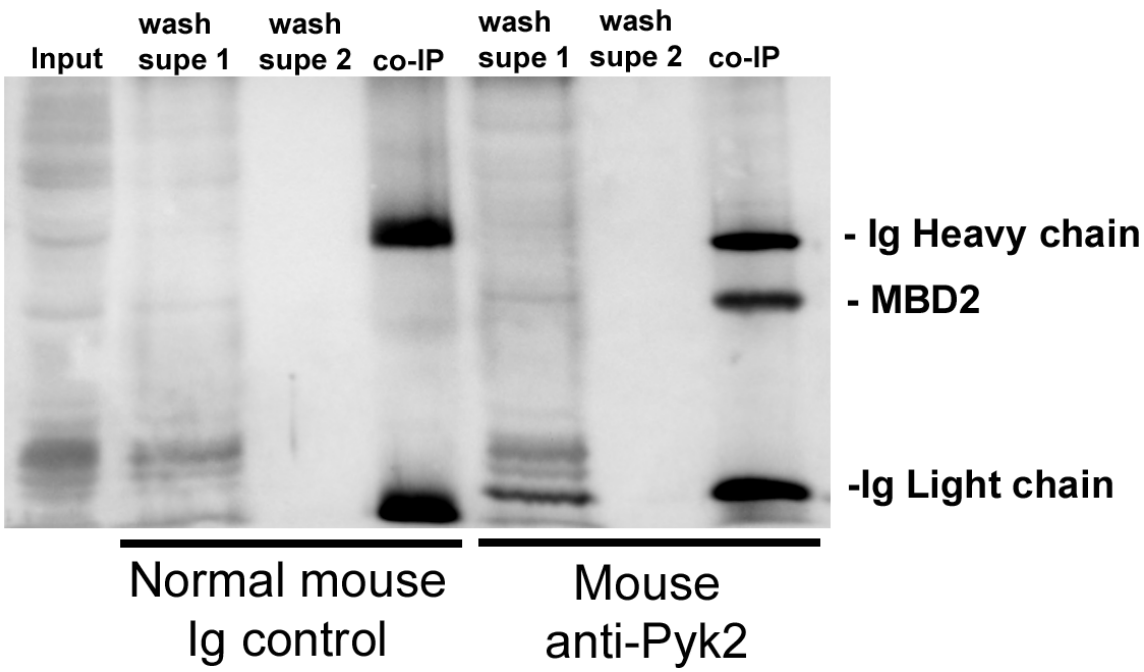
Figure 19. Immunofluorescence of Pyk2's Localization in MCOB Under Static and OFSS Conditions



OFSS-induces Pyk2 nucelocytoplasmic shuttling. Immunofluorescence microscopy of MCOB's subjected to either static culture conditions or OFSS (30 minutes, 1 hour, or 1 hour + 1 hour of rest). Slides were fixed immediately and processed for immunofluorescence using antibodies against Pyk2, followed by FITC-conjugated secondary antibodies. The nucleus was visualized using DAPI. Scale bars = 100μm

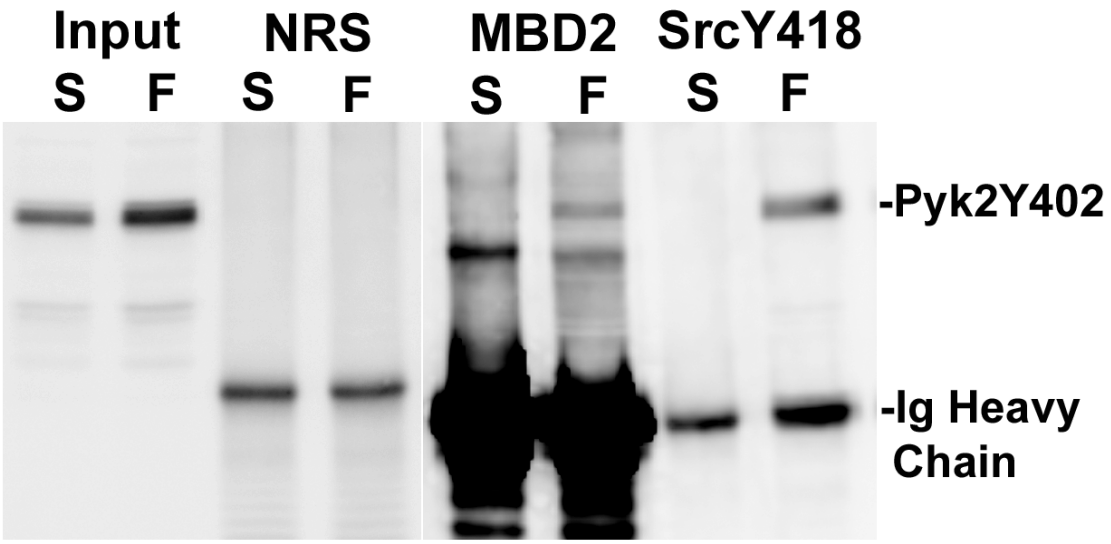
forms a complex with MBD2 (Figure 20). A complex of Pyk2/MBD2 was detected using antibodies to either Pyk2 or MBD2. Next, we compared the association of Pyk2/MBD2 under static and OFSS conditions using antibodies for the activated form of Pyk2 (Y402). In response to 20 minutes of OFSS MBD increases its interaction with the activated Pyk2 (Y402) (Figure 21). Additionally, OFSS-induced an association of Pyk2 (Y402) and Src (Y418) (Figure 21).

Figure 20. Co-immunoprecipitation Between MBD2 and Pyk2 in MLO-Y4 Osteocytes



A complex between MBD2 and Pyk2 forms under static conditions in MLO-Y4 osteocytes. Co-immunoprecipitation between MBD2 and Pyk2 was performed from MLO-Y4 osteocytes harvested under static conditions. MBD2 was not associated with Pyk2 when control normal mouse Ig was used in the immunoprecipitation. Anti-MBD2 antibody was used to probe the blot.

Figure 21. Co-immunoprecipitation Between MBD2 and Pyk2 (Y402) and Src (Y418) and Pyk2 (Y402) in MLO-Y4 osteocytes.



OFSS-induces the association of MBD2 and Pyk2 (Y402) and Src (Y418) and Pyk2 (Y402) in MLO-Y4 osteocytes. Co-immunoprecipitation was performed in MLO-Y4 osteocytes harvested under static (S) or OFSS (F) conditions. Normal rat serum (NRS), MBD2, and Src (Y418) were the antibodies used for the immunoprecipitation. Anti-Pyk2 (Y402) specific antibody was used to probe the blot.

DISCUSSION

In this study the potential for Pyk2 to function as a “STOP” mechanosome was examined. In the absence of Pyk2, Cox-2 and osteopontin expression are modestly increased. OFSS, induced by either oscillatory pump or orbital shaking platform, further enhanced Cox-2 and osteopontin expression in Pyk2 null osteoblasts. Therefore, Pyk2 functions to suppress the expression of the anabolic bone gene osteopontin and protein expression of Cox-2 under static and OFSS conditions. To further evaluate the potential “STOP” mechanosome properties of Pyk2, immunofluorescence was used to visualize the localization of Pyk2 under static and OFSS conditions. MCOB under static conditions displayed a fairly even distribution of Pyk2, with a subtle concentration of Pyk2 in the perinuclear/nuclear region (Figure 19). Pyk2 appeared to be capable of shuttling in and out of the nucleus in response to OFSS, as seen by an accumulation of Pyk2 in the nucleus (after 30 minutes of OFSS) and later an absence in the nucleus (1 hour of OFSS and 1 hour of rest). An OFSS-induced nucleocytoplasmic shuttling mechanism of Pyk2 has yet to be described, but other studies in fibroblasts have observed Pyk2’s nucleocytoplasmic shuttling behavior in response to membrane depolarization (Faure et al., 2007; Faure et al., 2013). The nucleocytoplasmic shuttling of Pyk2 is possible through the nuclear localization sequence (NLS) and the nuclear export sequence (NES) that are both located in the FERM domain (Figure 2) (Lim et al., 2008a; Lim et al., 2010; Ossovskaya et al., 2008). While more work will need to be done to define

the molecular mechanism(s) that cause OFSS-induced nucleocytoplasmic shuttling of Pyk2, this behavior is a hallmark characteristic of a mechanosome.

If Pyk2 is to function as a mechanosome it must also complex with a transcription factor and bind to target gene(s) to alter transcription. Pyk2 null osteoblasts exhibit increased levels of the protein Cox-2, as well as increased expression of osteopontin under basal conditions. OFSS-induced increases in Cox-2 and osteopontin were further enhanced in the absence of Pyk2. These changes in Cox-2 protein expression and osteopontin transcription might be mediated by the OFSS-induced increased in the association of Pyk2 and MBD2. In MLO-Y4 osteocytes a basal interaction between Pyk2 and MBD2 was observed. When examining the activated form of Pyk2 (Y402), an increase in its association with MDB occurs in response to 20 minutes of OFSS. While not a transcription factor, MBD2 is a member of the methyl CpG-binding protein family and functions to repress transcription (Boeke et al., 2000; Hendrich and Bird, 1998; Ng et al., 1999). Specifically, MBD2 and methyl CpG binding protein 2 (MeCP2) bind heterochromatin through their interaction with methylated DNA at CpG islands. The complex then translates the DNA methylation signal into transcriptional repression by recruiting histone deacetylases and other silencing complexes to sustain a heterochromatic state (Bird and Wolffe, 1999; Leonhardt and Cardoso, 2000). In muscles cells the interaction of FAK and MBD2 in the nucleus has been observed during differentiation, leading to the disruption of the repression complex and increased expression of myogenin (Luo et al., 2009). Similarly, in response to membrane depolarization Pyk2 binds MBD2 in the

nucleus of nerve cells, but the functional outcome of this observation has yet to be explained (Faure et al., 2007). Alternatively, in fibroblasts, a nuclear accumulation of Pyk2 was accompanied by an accumulation of Hic-5 (Aoto et al., 2002). Pyk2 and Hic-5 are both found at FA sites and in the nucleus. Hic-5 can bind the FAT domain of Pyk2 (Figure 2). Hic-5 is both an adhesion-associated protein and co-activator of nuclear receptors. Finally, Hic-5, along with Pyk2, shuttle away from sites of FA in response to cyclic strain in osteoblasts (Guignandon et al., 2006).

In summary, this study supports the hypothesis that Pyk2 may function as part of a “STOP” mechanosome. Pyk2 represses the expression of anabolic protein and gene expression under basal conditions, as well as, in response to OFSS. Typical of proposed mechanosome behavior, OFSS-induced nucleocytoplsmic shuttling of Pyk2. While a complex of Pyk2 and MBD2 exists under static conditions, OFSS enhances their association. A Pyk2/MBD2 “STOP” mechanosome may repress the transcription of anabolic bone genes through MBD2’s transcriptional repression capabilities. Future studies will need to examine the specificity of a Pyk2/MBD2 “STOP” mechanosome gene target(s) and explain mechanistically how transcription is repressed.

CONCLUSIONS AND PERSPECTIVES

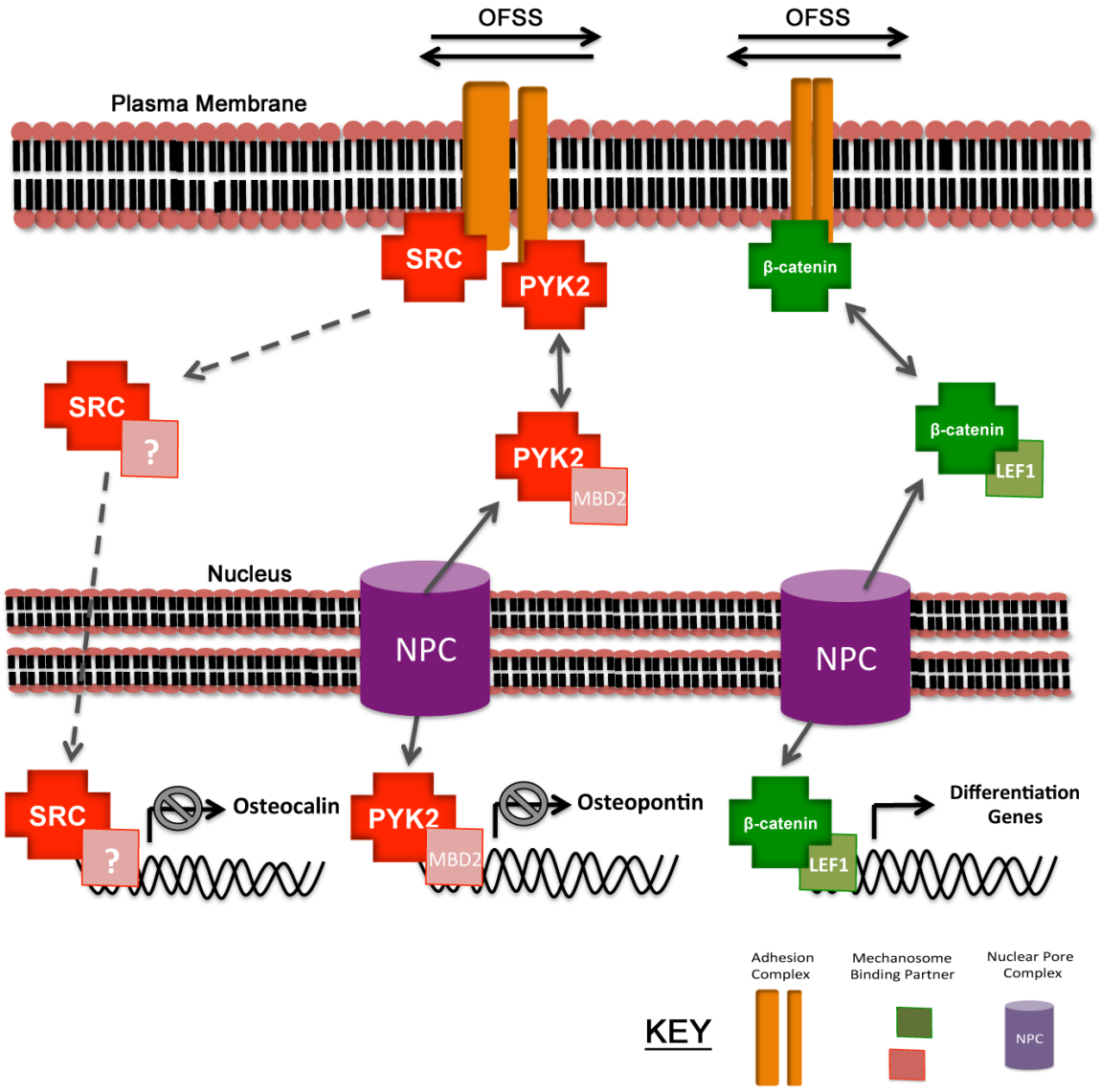
The aim of this thesis study was to better understand the signaling mechanisms that osteoblasts and osteocytes use to suppress the anabolic response of bone to mechanical loading. The roles of Src and Pyk2 were examined due to their shared knockout phenotype, increased bone mass. I investigated the capacity of Src and Pyk2, two adhesion-associated proteins, to function as “STOP” mechanosomes in osteoblasts and osteocytes. After initially finding that inhibiting Src activity caused an increase in osteocalcin expression, which was further enhanced in response to OFSS, Src’s pattern of activity in response to OFSS was examined. OFSS-induced a pattern of increased Src activity that started at the plasma membrane and propagated to the nucleus. A novel observation in bone cells; Src has not previously been reported to function in the nucleus in response to OFSS. In further experimentation, it was confirmed by nuclear fractionation that OFSS-induced an increase in Src activation. Prior to the initiation of this project, it was thought that Src’s role in response to OFSS was limited to its location at sites of FA. Previously, it was shown that OFSS-induces an increase in Src activation, as observed from whole cell lysates, and serves to propagate signaling pathways. Through the use of a pharmacological inhibitor of Src activity, a biosensor for Src, and traditional molecular biology techniques I discovered that in response to OFSS Src’s role extends beyond its functions at the plasma membrane. These data suggest, for the first time, Src may participate in a “STOP” mechanosome aiding in the attenuation of

expression of osteocalcin, an anabolic bone gene (Figure 22). Further studies will examine the mechanisms by which Src suppresses gene transcription in response to OFSS.

Next, the potential of Pyk2 to function as part of a “STOP” mechanosome was examined. The absence of Pyk2 caused modest increases in Cox-2 protein expression and osteopontin expression. The first indication that Pyk2 might function as a “STOP” mechanosome was demonstrated when OFSS-induced enhanced expression of Cox-2 and osteopontin expression in Pyk2 null osteoblasts. OFSS-induces Pyk2 nucleocytoplasmic shuttling, suggesting Pyk2 localizes like a “STOP” mechanosome. Further, these data indicate MBD2 associates with Pyk2 under static conditions, but more importantly OFSS-induces an increase in Pyk2 and MBD2 association. MBD2 could serve as the mechanism by which a Pyk2-contining “STOP” mechanosome functions to suppress gene transcription (Figure 22). Since activated Src increases its association with activated Pyk2 in response to OFSS, future studies will need to examine the possibility of a Src/Pyk2/MBD2 “STOP” mechanosome.

In conclusion, this work supports the hypothesis that “STOP” mechanosomes exist to suppress the anabolic response of osteoblasts and osteocytes to fluid shear stress. OFSS-induces Src and Pyk2 activation and subsequent suppression of anabolic protein and gene expression. In response to OFSS, Src may increase its activity in the nucleus and suppress gene transcription by association with a repression complex. OFSS-induces the nucleocytoplasmic shuttling which leads to an increased association with MBD2

Figure 22. Summary Figure



"GO" and "STOP" Mechanosomes Relay OFSS-induced Signals That Result in Changes in Gene Transcription. The findings in this dissertation support the proposed "STOP" mechanosome model. Specifically, OFSS-induced the nuclear activity of Src and Pyk2, resulting in the suppression of anabolic bone genes. The discovery of two new "STOP" mechanosomes complements findings of the "GO" mechanosome, β-catenin and Lef1. A dotted line was used for the Src mechanosome because it is unknown whether it translocates to the nucleus or if a nuclear form of Src carries out gene suppression.

and repression of the anabolic bone gene, osteocalcin. Taken together these data support the mechanosome hypothesis, and indicate that “STOP” mechansomes are triggered for activation in response to OFSS and actively suppress anabolic bone genes.

Future studies will be necessary to further describe how “STOP” mechanosomes function to balance the response of “GO” mechanosomes to mechanical loading. Specifically, it will be important to describe the mechanism(s) by which “STOP” mechanosomes participate in repressing anabolic bone gene transcription. Therapeutically manipulating “STOP” mechanosomes to inhibit negative feedback signals could enhance bone mass. Pharmacologically targeting “STOP” mechanosomes could provide increased sensitivity and magnitude of the anabolic response of bone to loading, which would be particularly important for patients with decreased mobility and/or muscle strength.

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CURRICULUM VITAE

Julia M. Hum

EDUCATION

Indiana University, Indianapolis, Indiana
August 2008-September 2013
Ph.D., Cellular and Integrative Physiology
Thesis: Signaling Mechanisms that Suppress the Anabolic Response of Osteoblasts and Osteocytes to Fluid Shear Stress

Saint Mary's College, Notre Dame, Indiana
August 2003-May 2007
B.S., Biology
Senor Comprehensive Project: Bioavailability and Remineralization Potential of Different Topical Fluoride Gels

FELLOWSHIPS

National Science Foundation
G-K12 Urban Educators Program
May 2012-May 2013

PROFESSIONAL AFFILIATIONS

American Society for Bone and Mineral Research, Student Member
2011-2013

Indiana Physiological Society, Student Member
2011-2013

The American Physiological Society, Student Member
2010-2013

PUBLICATIONS

Scientific Manuscripts

Hum, J.M., Day, R.N., Bidwell, J.P., Pavalko, F.M. Nuclear Src activity functions to suppress the anabolic response of osteoblasts and osteocytes to fluid shear stress (*Under Review, Journal of Cellular Biochemistry*)

Hum, J.M., Rodenberg, E.J., Young, S.R.L., Pavalko, F.M. Pyk2 may function as a "STOP" mechanosome through its association with MBD2 *(In Preparation)*

Hum, J.M., Siegel, A.P., Pavalko, F.M., Day, R.N. Monitoring biosensor activity in living cells with fluorescence lifetime imaging microscopy Int J Mol Sci 2012 Nov 7: 13(11): 14385-400

Young S.R., **Hum J.M.,** Rodenberg E.J., Turner C., Pavalko, F.M. Non-overlapping functions for Pyk2 and FAK in osteoblasts during fluid shear stress-induced mechanotransduction PLoS ONE 2011 Jan 25: 6(1): e16026

Wang H., Young S.R., Gerard-O'Riley R., **Hum J.M.,** Yang Z., Bidwell J.P., Pavalko F.M. Blockade of TNFR1 signaling: a role of oscillatory fluid shear stress in osteoblasts J Cell Physiol 2011 Apr 4: 1044-51

Educational Publications

Hum, J.M., Judd, M., Finkhouse, C., Marrs, K.A. Down to the Bare Bones: Teaching bone remodeling in the high school classroom *(In Preparation)*

Hum, J.M., Judd, M., Finkhouse, C., Marrs, K.A. The Integumentary Challenge: A problem-based learning skin activity for the high school classroom *(In Preparation)*

PRESENTATIONS

Oral Presentations

1) **Hum, J.M.,** Young, S.R.L., Day, R.N., Pavalko, F.M. Spatial patterns of Src activity differ in response to mechanical loading or EGF in osteocytes. American Society for Bone and Mineral Research Annual Meeting, Minneapolis, MN. (2012)

2) **Hum, J.M.,** Young, S.R.L., Day, R.N., Pavalko, F.M. Live imaging of Src activity in osteocytes in response to mechanotransduction. International Bone and Mineral Society Sun Valley Workshop, Sun Valley, ID. (2012)

3) **Hum, J.M.,** Young, S.R.L., Day, R.N., Pavalko, F.M. Live imaging of Src activity in osteocytes in response to mechanotransduction. Indiana Physiological Society Annual Meeting, Muncie, IN. (2012)

4) **Hum, J.M.,** Young, S.R.L., Day, R.N., Pavalko, F.M. Live imaging of Src activity in osteocytes in response to mechanotransduction. Sigma Xi Society Research Competition, Indianapolis, IN. (2011)

Poster Presentations

5) **Hum, J.M.,** Young, S.R.L., Day, R.N., Pavalko, F.M. Nuclear Src activity increases in MLO-Y4 osteocytes in response to mechanical loading. Biomedical Engineering Society Annual Meeting, Hawaii, HI. (2013)

6) Wang H., Young, S.R., Gerard-O'Riley R., **Hum J.M.,** Yang Z., Bidwell J.P., Pavalko F.M. Blockade of TNFR1 signaling: a role of oscillatory fluid shear stress in osteoblasts. Indiana Physiological Society, Indianapolis, IN. (2011)

7) **Hum, J.M.,** Young, S.R.L., Norvel, S., Gerard-O'Riley,R., Pavalko, F.M. Mechanical Regulation of Bone via Wnt/B-catenin Signaling. Society for Physical Regulation in Bone and Medicine Annual Meeting, Tucson, AZ. (2010)

AWARDS

American Society for Bone and Mineral Research
Young Investigator Award
2012

Indiana University School of Medicine
Charles H. Turner Young Investigator Bone Research Award
2012

Indiana Physiological Society
Peter Lauf and Norma Adragna Research award
2012

Indiana Physiological Society
Abstract Award
2011

Society for Physical Regulation in Biology and Medicine
Travel Award
2010

Saint Mary's College
Lumen Christi Award
2007

TEACHING EXPERIENCE

Indiana University School of Medicine, Indianapolis, Indiana
G717: Cellular Basis for Systems Biology
August 2011-December 2011
August 2012-December 2012
Teaching Assistant

Marine Biological Laboratory, Woods Hole, Massachusetts
Optical Microscopy Workshop
September 27th, 2012-October 7th, 2012
Teaching Assistant

Butler University, Indianapolis, Indiana
BI303: Principles of Physiology
October 2011, October 2012
Guest Lecturer

Southport High School, Indianapolis, Indiana
Biology II
August 2012-May 2013
Teaching Fellow

PROFESSIONAL AND UNIVERSITY SERVICE

Indiana University School of Medicine
Graduate Office, Student Mentor
2009-2012

Indiana University School of Medicine
Graduate Office, Student Ambassador
2009-2012

Indiana University School of Medicine
Invited Orientation Speaker
“Talk, Think, and Study Like a Graduate Student”
2012, 2013

Indiana University School of Medicine
Invited Panel Participant
Graduate Student Panel
2011