UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Inter-dependent Signaling of cGMP and Calcium on Activation of Akt in Murine Osteoblasts

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

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Professor Kathleen French

2011

The Thesis of Trish Phuong Tran is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2011

DEDICATION

I would like to dedicate my Thesis to my loving family. I am completely grateful for the love and encouragement that you have given me. To Mom and Dad, you have always inspired me to do my best and supported my education goals. I have learned my greatest characteristics from the two of you–determination, commitment, and quest for knowledge. To my amazing sister Ly, you are the strongest person I know. You are my role model and your unconditional love makes me the luckiest sibling in the world. Finally, to my brothers who have stood by me and let me know that I always have someone to count on.

EPIGRAPH

“If we knew what it was we were doing, it would not be called *research*, would it?”

-Albert Einstein

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ABSTRACT OF THE THESIS

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Osteoporosis is a common bone disease that is characterized by reduced bone strength. Weight bearing and exercise promotes fluid flow through the bone canalicular canal system resulting in fluid shear stress (FSS) which is believed to stimulate osteoblast proliferation and growth. Unfortunately, individuals with osteoporosis may find exercise unsafe due to the risk of suffering bone fracture. This

advocates for a treatment that can mimic the effects of mechanical loading and induce osteoblast proliferation.

Exposure of osteoblasts to laminar fluid shear stress results in elevation of intracellular calcium leading to increased NO and cGMP synthesis, and activation of cGMP-dependent protein kinases (PKGs). Calcium can exert its anabolic effects in an NO independent signaling pathway. It has been reported that the Akt/mTOR/p70S6K signaling pathway plays a crucial role in regulating the proliferation of osteoblast-like cells in response to oscillatory fluid shear stress. We used pharmacological inhibitors and siRNA-mediated knockdown to analyze the molecular mechanism by which calcium regulates NO/cGMP/PKGII dependent and independent pathways to activate Akt in murine osteoblasts. We found that FSS-induced Akt activation is dependent on both calcium and NO/cGMP/PKGII signaling pathways. Treatment with 8-pCPT-cGMP or a calcium ionophore mimicked the effects of FSS, and worked additively to increase Akt phosphorylation. FSS-induced Akt phosphorylation via the NO/cGMP/PKGII pathways required Src, whereas calcium signaling required both FAK and Src. Our results establish the role of calcium and NO/cGMP/PKGII pathways in regulating osteoblast proliferation and support using PKG-activating drugs as mechano-mimetics for the treatment of osteoporosis.

INTRODUCTION

Osteoporosis: a common bone disease that affects millions

Osteoporosis is a disease that is characterized by a decreased bone mineral density and deteriorated bone matrix due to mineral abnormalities [1]. This common disease leads to an increase risk of fractured bones in the hips, ribs, wrists, and vertebra. Osteoporosis is often associated with weak, brittle bones that make exercise and strenuous activity difficult. An osteoporotic fracture would not ordinarily occur in individuals with normal bone density. Currently, osteoporosis affects approximately 10 million people in the United States, with an additional 34 million people at risk due to low bone density [2]. It is a costly disease with healthcare-associated expenses of approximately 17 billion dollars for the treatment of osteoporosis in 2005 [3].

The dynamic role of the bone in the body

Bone is a living organ that constantly undergoes remodeling in response to signals it receives from the surrounding environment. Cells that play important roles in bone metabolism include osteoblasts, osteocytes, and osteoclasts. Osteoblasts are derived from mesenchymal osteoprogenitor cells and are responsible for secreting collagen and organic compounds into the bone matrix, which it forms. Osteoblasts are also precursors for osteocytes that are functionally important in adult bones. Osteocytes are crucial in bone remodeling because they function as regulators of osteoblast and osteoclast activity [4]. They extend into the canalicular canals and are responsible in phosphate metabolism and calcium availability, making osteocytes necessary for proper bone function [4]. Osteoclasts are derived from circulating

monocytes, resorb the bone matrix, and release minerals into the blood. Some of the important functions of bone include support of soft tissue, energy and mineral storage, and blood cell production. Together, this network of bone cells assists in bone remodeling.

Mechanotransduction

Mechanotransduction is the conversion of a mechanical stimulus to a biochemical signal. Mechanical stimulation has been shown to increase bone growth and strength as a response to stress on the bone [5]. Mechanical loading, such as weight bearing and exercise, increases the interstitial flow of fluid through the canalicular canals and produces shear stress [6]. The resulting fluid shear stress (FSS) induces rapid and transient production of nitric oxide, calcium influx, and prostaglandin E_2 [7]. These secondary messengers act as important mediators of the cell's response to shear stress. FSS initiates numerous signal transduction pathways downstream of these second messengers (such as mitogen activated protein kinase [MAPK] cascade), and promotes anabolic responses in osteocytes/osteoblasts. This leads to increases in the transcription of various genes (e.g. c-fos) and changes in cell proliferation and differentiation [8]. Additionally, previous work by Rangaswami *et al*. proposed a mechanism by which FSS proceeds through the NO/cGMP/PKGII and Src pathways, leading to ERK activation and the upregulation of fos family genes to promote cell growth [9, 10].

We propose to study three protein kinases that are involved in mechanotransduction: Src, FAK, and Akt. Src is a non-receptor tyrosine protein kinase that plays a significant role in cell differentiation, motility, proliferation, and

survival [11]. In a study where c-src was deleted from mice, it lead to osteopetrosis (hardening of the bone) as a result of reduced osteoclast activity [12]. The decreased activity is believed to disrupt the remodeling process of bone formation/resorption and cause osteopetrosis.

Src is regulated by phosphorylation and dephosphorylation. One of the important phoshorylation sites on Src is Tyr 529. When this C-terminal site is phosphorylated, it binds to Src's N-terminal Src- homology 2 (SH2) domain [11]. The intramolecular binding of Tyr 529 to the SH2 domain keeps Src in an inactive state. Dephosphorylation of Tyr 529 disrupts the SH2-phospho-Tyr 529 interaction, allowing autophosphorylation at Tyr 418 and resulting in full activation of its kinase activity [11].

Focal adhesions form at sites where integrins link the cytoskeleton to the extracellular matrix. Multiple protein-protein interactions occur within focal adhesions, which link numerous structural and signaling networks [13]. Focal adhesion kinase (FAK) is a 125 kDa tyrosine kinase that is important in cell migration, differentiation, and proliferation. It was first identified as a substrate of Src phosphorylation, indicating a potential connection between Src-FAK signaling. In addition, FAK has been suggested to play a role in mechanical sensing by providing a link between the extracellular matrix and the cytoskeleton [13]. FAK has several key tyrosine residues: Tyr 397, Tyr 576, and Tyr 577. Tyr 397 is an autophosphorylation site and Tyr 576/577 is a Src-target site. FAK also has a focal adhesion targeting (FAT) domain that allows it to interact with other focal adhesion proteins (i.e. paxillin). In addition to the FAT domain, FAK contains a FERM domain, located at

its N-terminus, which interacts with the cytoplasmic tail of β1 integrin. When β1 integrin binds its extracellular ligand, FAK autophorylation at Tyr 397 is stimulated [13].

Akt is a serine/threonine protein kinase that occupies a central role in multiple cellular processes such as glucose metabolism, cell growth, and inhibition of apoptosis. Akt lies within the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway. Activated PI3K leads to the translocation of Akt and phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane where PDK1 partially activates Akt by phosphorylation at Threonine 308 [14]. Full activation of Akt requires the mTOR containing complex 2 (mTORC2), which phosphorylates Akt at Serine 473 [14]. Preliminary work in our lab has revealed that FSS leads to Akt phosphorylation at Serine 473. However, at this time, the mechanism by which Akt is activated by FSS and whether Src and FAK are involved is unknown.

Significance of research for the development of therapies for oseoporosis

Since osteoporosis results from reduced bone density and deterioration of the bone matrix, our research focuses on mimicking the effects of mechanical loading to induce bone formation and growth. In this study, we found that both calcium and NO/cGMP/PKGII signaling pathways stimulate Akt phosphorylation, which leads to osteoblast proliferation and increased cell survival. Analysis of the signaling networks revealed the requirement of both Src and FAK in calcium induced Akt phosphorylation. In contrast, only Src was necessary for cGMP induced Akt

phosphorylation. Our findings provide a rational use of pharmaceutical reagents which mimic the effects of FSS to treat osteoporosis.

MATERIALS AND METHODS

Reagents

The following antibodies were used: rabbit polyclonal anti-Src, anti-FAK, phospho-FAK Tyr 397, phospho-FAK Tyr 576/577, anti-Akt, and phospho-Akt Ser 473 (Cell signaling). Rabbit polyclonal anti-ERK-1 and phospho-ERK were from Santa Cruz Biotechnology.

Pharmaceutical inhibitors N-nitro-L-arginine methyl ester (L-NAME) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were from Cayman. cGMP agonist 8-(4-chlorophenylthio)-GMP (8-pCPT-cGMP) (cGMP) and cGMP antagonist 8-(4-chlorophenylthio)-B-phenyl-1, N^2-ethenoguanosine-3',5'-cyclic monophosphorothioate, R_p isomer (R_p)-8-pCPT-PET-cGMPS (RP) were from Biolog. The calcium ionophore A23187 (Calcimycin), the specific Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyramidine (PP2), the inactive analog 4-amino-7-phenylpyrazolo[3,4-*d*]pyramidine (PP3), 1,2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid (acetoxymethyl ester) (BAPTA/AM or simply BAPTA), and ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetic acid (EGTA) were from Calbiochem/EMD.

Cell culture

We obtained murine MC3T3-E1 osteoblast cells (hereafter referred to as MC3T3 cells) with high differentiation ability from the American Tissue Culture Collection. MC3T3 cells used in experiments were at less than 12 passages. Cells were grown in minimal essential medium (MEM) without ascorbic acid, with 10% fetal bovine serum (FBS) at 37ºC in an atmosphere of 5% CO_2.

SiRNA Transfection

Qiagen produced the siRNAs used for transfection, and the targeted sequences are found in the Supplemental Table 1. Cells were plated at 1.0 x 10^6 per well of a 6-well dish or at 0.5 x 10^6 cells per slide. They were transfected 18 hours later at ~40% confluency with 4μl Lipofectamine 2000^{TM} (Invitrogen) and 100ρmol of siRNA in 1ml of MEM+10%FBS. After five hours of transfection, the cells were placed in starvation medium (MEM+0.1%FBS) for 18 hours.

Cell exposure to Fluid Shear Stress

MC3T3 cells were plated on etched glass slides and grown in starvation medium for 24 hours. The cells were transferred to a parallel-plate flow chamber (Cytodyne Inc., San Diego, CA) and subjected to laminar fluid shear of 12 dynes/ cm^2. Sham-treated cells underwent identical manipulations, with the exception of exposure to shear and are labeled "Sham." After FSS, cells were in kept in the flow chamber, without flow, for the indicated time before harvesting.

Western Blot Analysis

The cells were harvested by the addition of hot SDS-sample buffer and lyzed by sonication. Samples were analyzed by SDS-PAGE/immunoblotting. The blots for anti-FAK, phospho-FAK Tyr 397, and phospho-FAK Tyr 576/577 antibodies were blocked at room temperature with 5% bovine serum albumin (BSA) while the other antibodies (anti-Akt, phospho-Akt, anti-Src, etc.) were blocked with 5% milk in TBS-T overnight. The primary antibody (1:1000) was incubated O/N at 4°C and the secondary antibody (1:10,000) was incubated at room temperature for 1 hour. The blots were developed with enhanced chemiluminescence (Pierce).

RESULTS

FSS induces Akt phosphorylation via NO/cGMP/PKGII Pathway

Fluid shear stress (FSS) is a type of mechanical stimulation that has been shown to increase bone density and strength. FSS leads to the activation of eNOS (the most abundant isoform in osteoblasts) and the subsequent activation of the NO/cGMP/PKG pathway. Previous work by Rangaswami *et al.* has shown that FSS-induced ERK phosphorylation is mediated through the NO/cGMP/PKGII pathway [9]. When ERK is activated, it promotes the transcription of fos family transcription factors (c-fos, fra-1, fra-2, and fosB/ΔfosB) leading to osteoblast proliferation. Since Akt is also known to play an important role in cell growth, we reasoned that Akt might be activated by fluid shear stress. It should be noted that Dr. Rangaswami performed the fluid shear stress experiments shown in Fig. 1A-D, Fig. 3A, as well as parallel experiments to those shown in this study.

To determine whether FSS induces Akt phosphorylation, we subjected MC3T3 cells to FSS and then analyzed the lysates by Western Blot. When subjected to FSS, there was a robust induction of Akt phosphorylation at 5 and 10 minutes (Fig. 1A, lanes 3 and 4). Next, we used pharmacological inhibitors to determine whether FSS-induced Akt phosphorylation is NO/cGMP/PKG dependent. The inhibitors used were: L-NAME for nitric oxide synthase (NOS), ODQ for soluble guanylate cyclase (sGC), and RP for cGMP-dependent protein kinase G (PKG). As seen in Fig. 1B, FSS-induced Akt phosphorylation was drastically reduced in L-NAME, ODQ, or RP treated cells (compare lane 2 with lanes 3, 4, and 5). This result indicates that

inhibiting any of the signaling molecules in the NO/cGMP/PKG pathway blocks FSS-induced Akt phosphorylation.

Mammalian cells contain two different types of PKG, soluble PKGI and membrane bound PKGII. PKGI and PKGII have distinct cellular functions, in part due to differential cellular localization and substrate specificity [9]. To determine whether PKGI or PKGII are involved in mediating Akt phosphorylation, we transfected MC3T3 cells with either PKGI-specific or PKGII-specific siRNA and examined the effect of each knockdown on FSS induced Akt phosphorylation. Control cells were transfected with green fluorescent protein (GFP)-specific siRNA. In PKGI-depleted osteoblasts, FSS induced normal activation of Akt (Fig. 1C, compare lanes 2 and 4). However, in PKGII-depleted cells, there was a significant inhibition of FSS-induced Akt phosphorylation (compare lanes 2 and 6). This result suggests that PKGII is necessary for FSS-induced Akt activation in osteoblasts.

Calcium is crucial in FSS-induced Akt phosphorylation

FSS induces transient calcium influx in osteoblasts [15]. This suggests that calcium signaling may contribute to FSS-induced Akt phosphorylation. To examine the role of calcium in FSS-induced Akt phosphorylation we pretreated MC3T3 cells with calcium chelators, BAPTA and EGTA, one hour prior to laminar fluid flow. As seen in Fig. 1D, there was partial inhibition of FSS-induced Akt phosphorylation when either BAPTA (lane 3) or EGTA (lane 4) was added to the cells. Observable reduction of Akt phosphorylation in the presence of BAPTA or EGTA indicates that FSS-induced calcium influx contributes to Akt phosphorylation.

cGMP mimics the effects of FSS on Akt activation

Since FSS activates Akt through the NO/cGMP/PKGII pathway, we wanted to assess whether cGMP mimics this effect. The addition of 100μM 8-pCPT-cGMP to MC3T3 cells was sufficient to induce Akt phosphorylation, with peaks at 5 and 10 minutes (Fig.2A, lanes 3 and 4). This result mirrors the time course of Akt phosphorylation observed in FSS-induced cells (as seen in Fig 1A). To establish whether cGMP-induced Akt phosphorylation is also mediated through PKGII (as in FSS), we used siRNA transfection to knockdown either PKGI or PKGII. Akt phosphorylation mediated by cGMP was significantly reduced in PKGII knockdown cells, but was unaffected by PKGI knockdown (Fig.2B, compare lanes 2, 4 and 6). From these experiments, we conclude that cGMP can mimic the effects of FSS on Akt phosphorylation in osteoblasts.

Calcium-mediated Akt activation is partially dependent on eNOS

Calcium signaling can lead to eNOS activation and this may occur in response to mechanical stimulation [15]. We have shown that calcium is essential in FSS-induced Akt phosphorylation (using calcium chelators [Fig.1D]), however, it is unknown whether calcium-mediated Akt phosphorylation proceeds through eNOS. To determine if calcium-induced Akt phosphorylation is dependent on the NO/cGMP/PKGII pathway, we performed a time course with the calcium ionophore A23187 (0.3μM) in the presence and absence of L-NAME (4mM). As shown in Fig. 2C, Akt phosphorylation peaked at 5, 10, and 20 minutes in the absence of L-NAME, but a much lower peak was seen at 5 minutes in the presence of L-NAME. Although not yet conclusive, the data suggests that when cells were treated with A23187

(without L-NAME), calcium partially induces Akt phosphorylation through activation of eNOS, but that calcium also signals through an eNOS independent pathway.

Calcium and cGMP Cooperate to Regulate Akt phosphorylation

We found that cGMP mimics the effects of FSS (Fig. 2A) and that calcium can induce Akt phosphorylation independent of cGMP (Fig. 2C, in the presence of L-NAME). From these results, we speculate that both cGMP and calcium signaling pathways could cooperate to increase Akt phosphorylation. To test this hypothesis, we pretreated MC3T3 cells with 4mM L-NAME for one hour. Next, the cells were treated with 0.3μM A23187, 100μM cGMP, or both. We found that activation of both signaling pathways had a greater effect on Akt activation, especially seen in the presence L-NAME (Fig. 2D, compare lanes 2, 3 and 4). These results suggest that NO/cGMP/PKGII dependent and independent pathways activated by calcium work together to regulate Akt phosphorylation.

FSS-induced Akt and ERK phosphorylation requires both Src and FAK

Young *et al*. showed that FSS-induced ERK activation in bone cells proceeded through FAK activation and Rangaswami *et al.* showed that Src is required for FSS and ERK activation [10, 16]. These studies indicate that Src and FAK play important roles in mechanotransduction in the bone. To test the involvement of Src and FAK in mediating Akt phosphorylation, MC3T3 cells were transfected with either Src-specific or FAK-specific siRNA. siRNA-treated osteoblasts were subjected to FSS, and Western Blot data showed a lack of Akt phosphorylation in Src and FAK deficient cells (Fig.3A, lanes 4 and 6 respectively). This result denotes the importance of both Src and FAK for FSS-induced Akt activation.

Calcium-induced Akt activation requires both FAK and Src, but cGMP-induced Akt activation only requires Src

We demonstrated that both cGMP and calcium signaling can mimic the effects of FSS in Akt phosphorylation. As previously done in the FSS experiments, we transfected MC3T3 cells with Src-specific or FAK-specific siRNA. The cells were then treated with cGMP or A23187. As seen in Fig. 3B, cGMP-induced Akt phosphorylation is inhibited in Src deficient, but not FAK deficient cells, as compared to GFP-specific siRNA (compare lanes 2, 4 and 6). Thus, the pathway in which cGMP induces Akt phosphorylation is Src dependent. Next, we examined the role of Src and FAK in calcium-induced Akt activation. When A23187 was added to cells transfected with either Src or FAK siRNA, Akt phosphorylation was drastically reduced in both types of cells. Additionally, we probed the blots with anti-Src and anti-FAK antibodies to check for Src and FAK knockdown in the siRNA-treated samples.

Differential phosphorylation of FAK at Tyr 397 and Tyr 576/577

We observed that cGMP-induced Akt phosphorylation was dependent on Src, but calcium-induced Akt phosphorylation was dependent on both Src and FAK (Fig. 3B, C). As mentioned previously, FAK has key phosphorylation sites at Tyr 397 (autophosphorylation site) and Tyr 576/577 (Src-target site), therefore we wanted to examine whether theses sites were differentially phosphorylated when cells were treated with cGMP or A23187. We found that treatment with the calcium ionophore induced phosphorylation at both Tyr 397 and Tyr 576/577, indicating FAK and Src activation (Fig. 4B). However, cGMP only induced phosphorylation at Tyr 576/577, presumably due to Src activation (Fig. 4A).

Src inhibition does not affect calcium-induced FAK autophosphorylation at Y397, but does affect phosphorylation at Tyr 576/577

In many cells, Src and FAK are found in a complex, with Src phosphorylating FAK and contributing to full FAK activation. To determine whether Src inhibition affected calcium-induced phosphorylation of FAK, M3C3T3 cells were pretreated with PP2 (10µM), a Src family kinase inhibitor, or its inactive analog PP3(10µM) for one hour. Treatment for 5 minutes with A23187 showed that Src inhibition selectively blocked FAK phosphorylation at Tyr 576/577, but not Tyr 397 (Fig.5A). Similar results were obtained using siRNA to knockdown Src (Fig.5B). Our results show that Src inhibition does not affect calcium-induced FAK autophosphorylation at Tyr 397, suggesting that calcium can activate FAK independently of Src. On the other hand, phosphorylation of Tyr 576/577, which is known to be a Src-target site, is inhibited in the presence of the pharmacological inhibitor PP2 or treatment with a Src-specific siRNA, thus Tyr 576/577 is phosphorylated by Src in these cells.

cGMP-induced Akt activation is calcium independent

To address whether cGMP-induced Akt phosphorylation is dependent on calcium, MC3T3 cells were pretreated with BAPTA or EGTA, followed by the addition of cGMP. We saw that pretreatment with BAPTA or EGTA did not affect cGMP-induced Akt phosphorylation (Fig. 6, lanes 2, 3, and 4). These results are consistent with our proposed mechanism that calcium and cGMP can activate Akt independent of each other.

DISCUSSION

The additive effects of cGMP and calcium mimic FSS

Osteoporosis is a disease that is most often a result of deteriorated bone matrix. The cells that produce the matrix are of osteoblast lineage, which suggests that if mechanical loading can induce osteoblast proliferation, it would help in the treatment of osteoporosis. The ability to reproduce the anabolic effects of mechanical stimulation such as fluid shear stress may be helpful for the treatment of osteoporosis. In this study we found that both cGMP and calcium mimic the effects of FSS to induce Akt activation. Although it is known that FSS stimulates the production of calcium and NO, the downstream signaling events have not been fully characterized. The novel finding that calcium induces Akt phosphorylation by both NO/cGMP/PKGII dependent and independent pathways unravels a mechanism by which Src and FAK modulate osteoblast proliferation.

The use of a cGMP analog and calcium ionophore to study FSS signaling pathways is advantageous for a number of reasons. First, MC3T3 cells are very sensitive to shear forces and moving the cells from the incubator to the flow chamber can easily cause background signals. Second, the flow has to be of a certain rate and pressure to produce fluid shear stress analogous to that found *in vivo*. This requires careful calibration of the flow apparatus. Thus, the use of pharmacological reagents simplifies the task of analyzing signaling pathways downstream of FSS.

A proposed mechanism of Akt phosphorylation induced by cGMP and calcium signaling

A proposed mechanism leading to Akt phosphorylation mediated by inter-dependent signaling of cGMP and calcium is shown in Fig. 7. Calcium signaling

independent of the NO/cGMP/PKGII pathway induces the autophosphorylation of FAK Tyr 397. Autophosphorylation of FAK Tyr 397 recruits Src (by binding to Src's SH2 domain), and may induce Src activation resulting by autophosphorylation at Tyr 418 [17]. Src may be activated by calcium through other mechanisms. Once Src is activated, it phosphorylates FAK at Tyr 576/577, further stimulating FAK activation (now phosphorylated at Tyr 397 and Tyr 576/577). FAK then signals downstream to Akt by an undetermined pathway, inducing phosphorylation at Akt Ser 473.

Calcium signaling via NO/cGMP/PKGII dependent pathway also leads to Akt phosphorylation. First, intracellular calcium induces eNOS activation and the subsequent production of NO/cGMP/PKGII. PKGII may signal to Shp1/2 (as seen in FSS-induced ERK activation) and leads to Src activation with phosphorylation of Tyr 418 [10]. The activation of Src induces Akt phosphorylation in an unknown mechanism. Since Akt is known to phosphorylate eNOS and increase production of NO and cGMP, this signaling pathway may lead to a positive feedback cycle on Akt activation [18]. The activation of Akt leads to osteoblast proliferation and increased cell survival.

FSS initiates mechanotransduction and activates both Akt and ERK

From this study, and previous work in the lab, we found that Akt and ERK are activated in response to FSS by mechanotransduction. While we have not reported the data, Western blot analysis showed that phospho-ERK showed a similar response to cGMP and calcium stimulation as seen in Akt. Akt and ERK are in distinct signaling pathways, but the activation of both Akt and ERK are crucial for bone growth and strength. Previous work has shown that ERK activation leads to increased

transcription of growth promoting transcription factors. PI3K is known to be upstream of Akt and can lead to its activation. Akt signals to numerous downstream targets, including the anti-apoptotic proteins BAD and GSK3, the growth promoting TSC1/TSC2 complex, and leading to the activation of S6K [19]. A study found that the Akt/mTOR/p70S6K pathway plays a crucial role in regulating the proliferation of osteoblasts like cells in response to oscillatory fluid shear stress [20]. Together with the findings that FSS induces Akt phosphorylation and cGMP and calcium mimic these effects, the next step would be to study whether PI3K plays a role in cGMP and/or calcium induced Akt activation. While previous work has shed light on FSS signaling through ERK, understanding the contribution of the PI3K/Akt/mTOR pathway provides additional information on how FSS increases bone cell growth, proliferation, and inhibition of apoptosis.

FIGURES

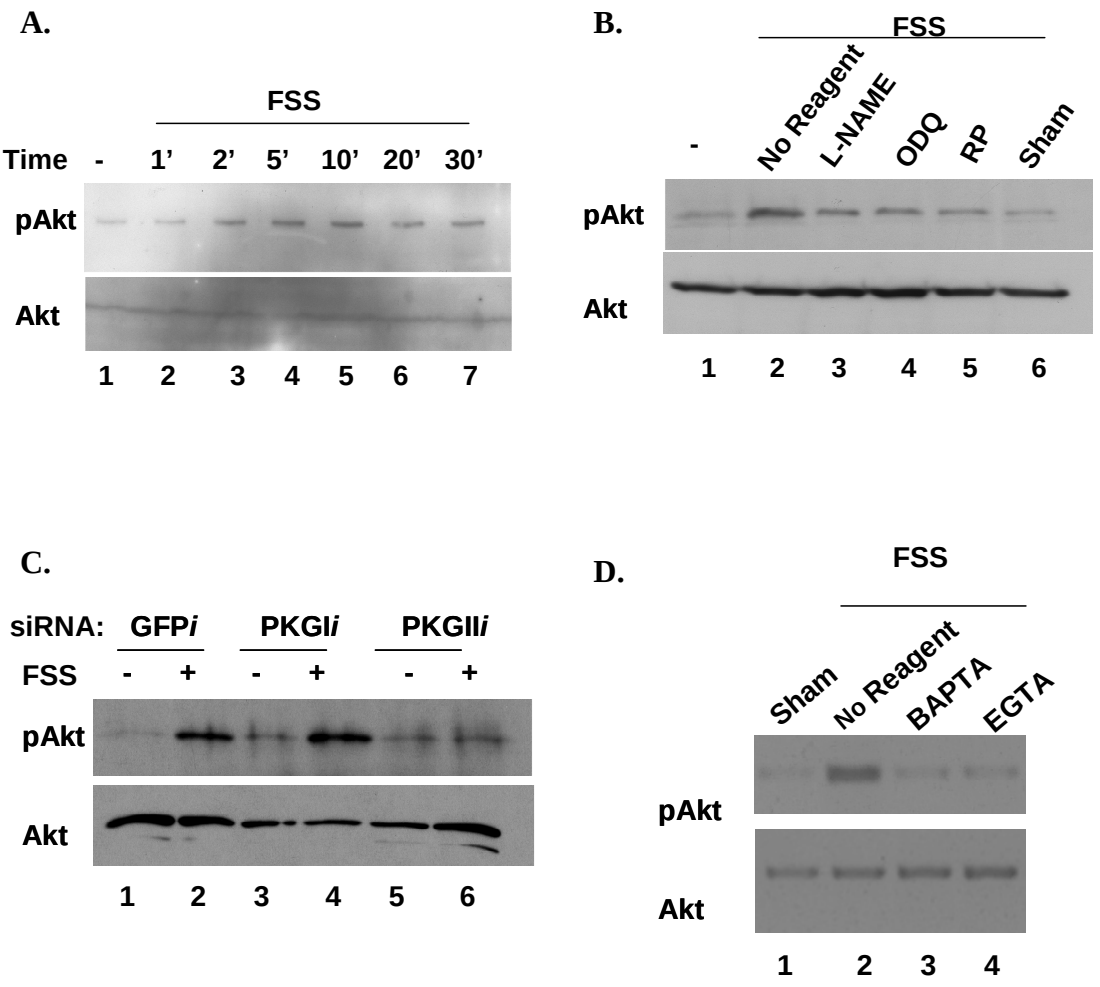
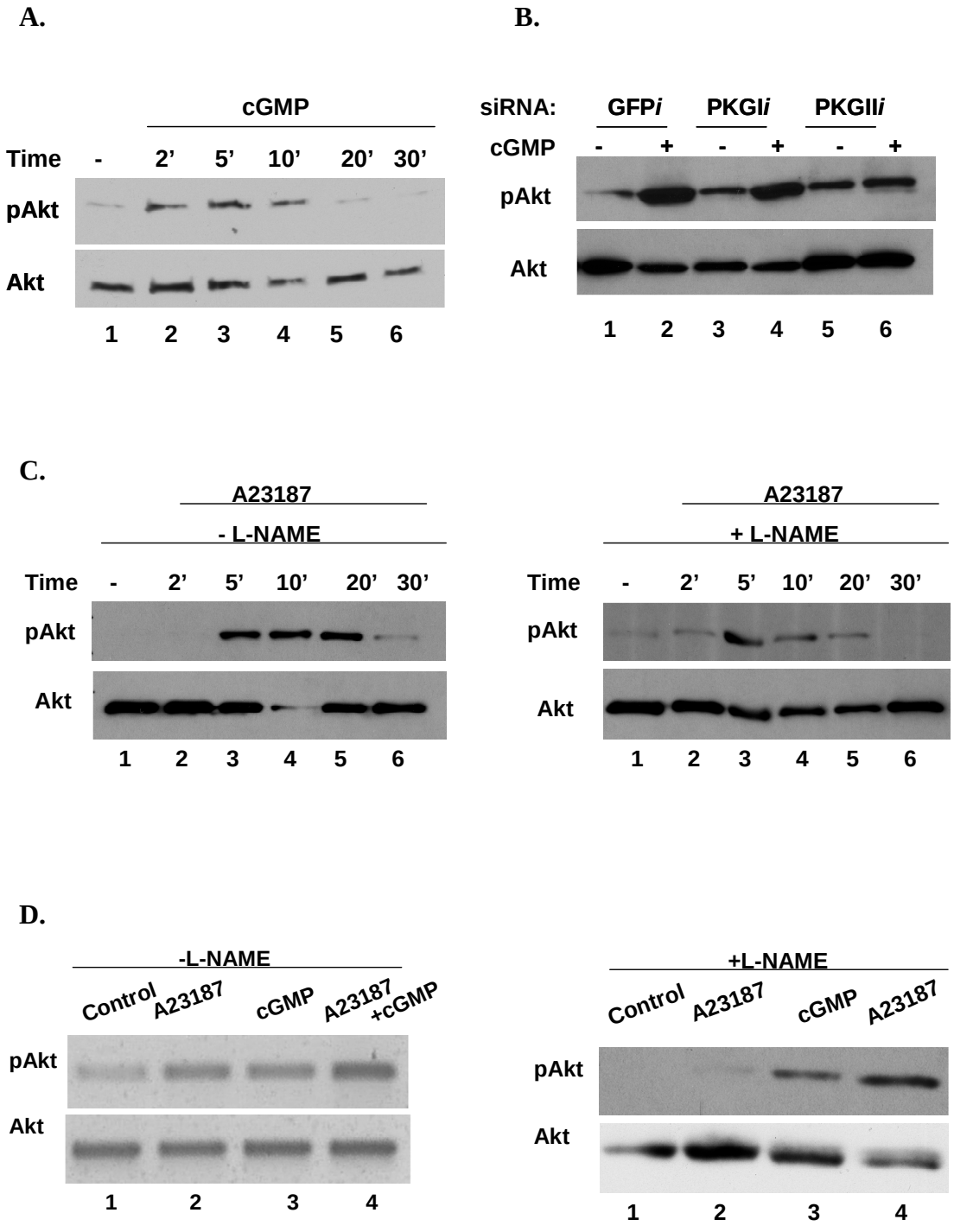


Figure 1: Fluid shear stress induces Akt phosphorylation via NO/cGMP/PKGII and Calcium. Akt phosphorylation was determined by Western blot using a phospho-Ser 473 specific antibody (pAkt). Blots were reprobed with a total Akt antibody as a loading control. (A) MC3T3 cells were exposed to laminar flow at 12dynes/cm^2 in a time course of 1, 2, 5, 10 minutes. For the 20 and 30 min. time points, cells were exposed to 10 min. laminar flow followed by a 10 or 20 min. static rest before harvesting (B) Cells were treated as a static control (Lane 1), no reagent added (lane 2), sham (Lane 6), or pre-treated with pharmacological inhibitors (lanes 3, 4, 5). The cells were pre-treated with media and 4mM L-NAME (Lane3), 10μM ODQ (Lane 4), or 100μM RP (Lane 5) for one hour. (C) MC3T3 cells were transfected with 100ρmol Green Fluorescent Protein (GFP) siRNA, 100ρmol PKGI specific-siRNA, or 100ρmol PKGII specific-siRNA. Lanes 2, 4, and 6 were subjected to 10 minutes of laminar flow. (D) MC3T3 cells were pre-treated with calcium chelators, BAPTA (10μM) or EGTA (5mM), for one hour before exposure to laminar flow.

Figure 2: cGMP and Calcium mimics the effects of FSS on Akt phosphorylation. (A) MC3T3 cells were treated with 100µM 8-pCPT-cGMP (cGMP) in a time course of 2, 5, 10, 20, and 30 minutes and Akt phosphorylation was determined by Western blotting using a phospho-Ser 473 specific antibody (pAkt) as in Figure 1. (B) MC3T3 cells were transfected with 100µmol GFP siRNA (lanes 1 and 2), 100ρmol PKGI-specific (lanes 3 and 4), or 100ρmol PKGII-specific siRNA (lanes 5 and 6). After transfection, cells were treated with 100µM cGMP for 5 minutes (lanes 2, 4, and 6). (C) Calcium ionophore A23187 (0.3 µM) was added to MC3T3 cells at 2, 5, 10, 20, and 30 minutes under conditions with and without 4mM L-NAME. (D) Treatment of cells with 0.3 µM A23187 (lane 2), 100µM cGMP (lane 3), then with both 0.3µM A23187 and 100µM cGMP (lane 4) with and without 4mM L-NAME.

Figure 2


A.
cGMP
Time - 2' 5' 10' 20' 30'
pAkt
Akt
1 2 3 4 5 6
B.
siRNA: GFPi PKGIi PKGIIi
cGMP - + - + - +
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Akt
1 2 3 4 5 6
C.
A23187
- L-NAME
Time - 2' 5' 10' 20' 30'
pAkt
Akt
1 2 3 4 5 6
A23187
+ L-NAME
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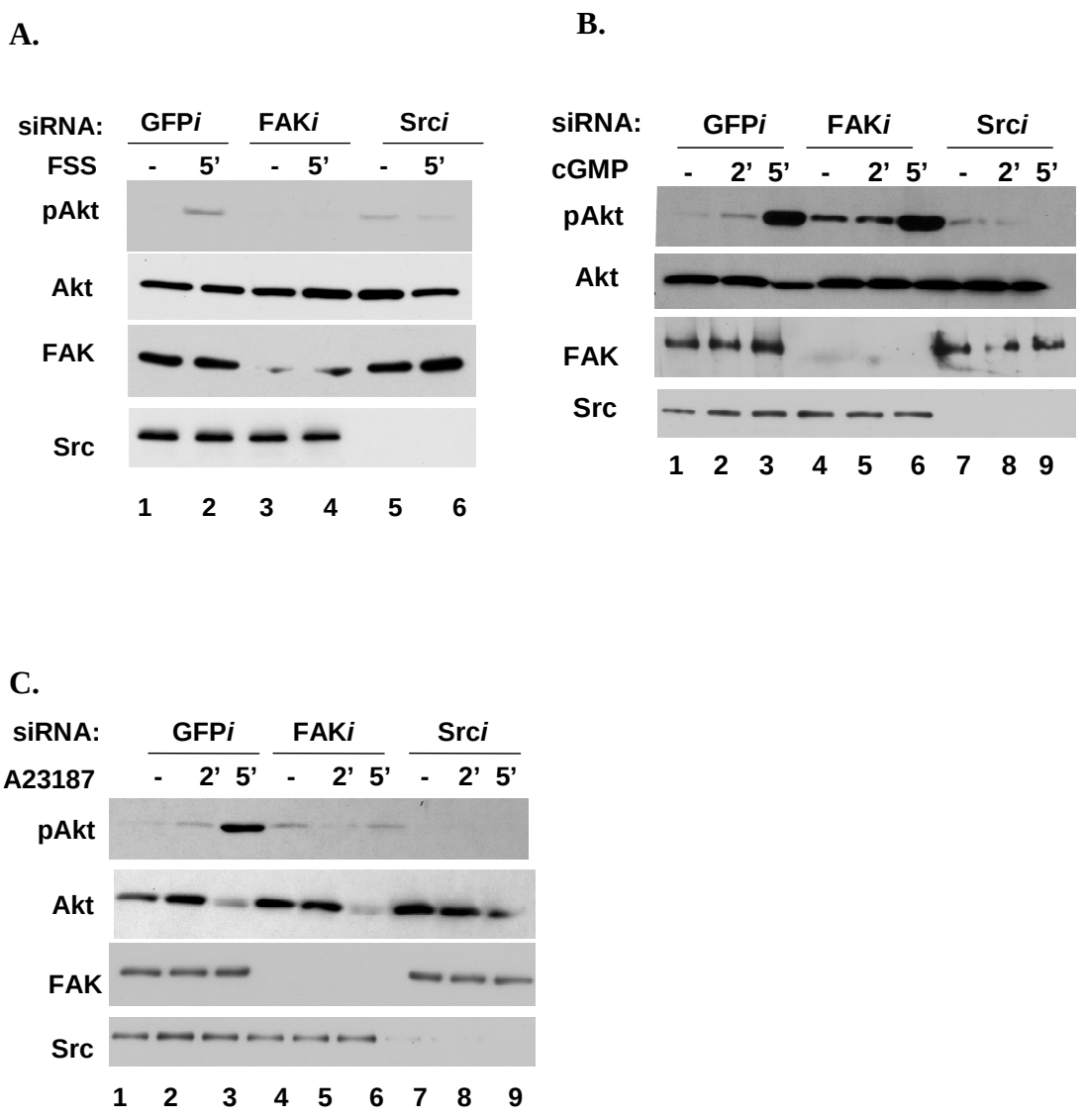
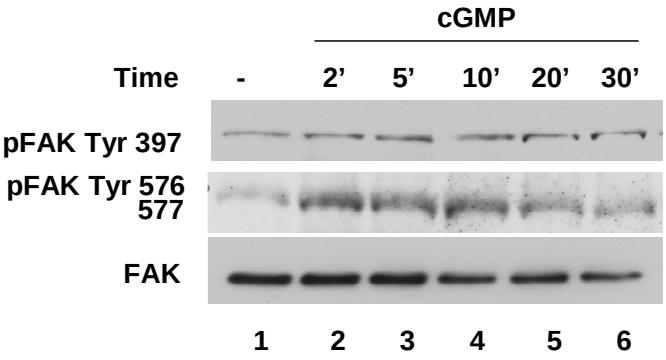


Figure 3: Akt activation is dependent on both Src and FAK. (A) MC3T3 cells were transfected with 100ρmol GFP siRNA, 100ρmol Src-specific siRNA, or 100ρmol FAK-specific siRNA. After transfection and starvation, the cells were placed in the flow chamber and subjected to laminar flow. The proteins were probed with antibodies specific for phospho-Akt Ser 473 (pAkt), total Akt, FAK, and Src. (B) Cells were tranfected with 100ρmol GFP siRNA, 100ρmol Src-specific siRNA, or 100ρmol FAK-specific siRNA prior to the addition of 100μM 8-pCPT-cGMP for 2 and 5 minutes. Lanes 1, 4, and 9 are controls. (C) Cells were transfected with siRNAs as in (B) and treated with 0.3μM calcium ionophore A23187.

A.



B.

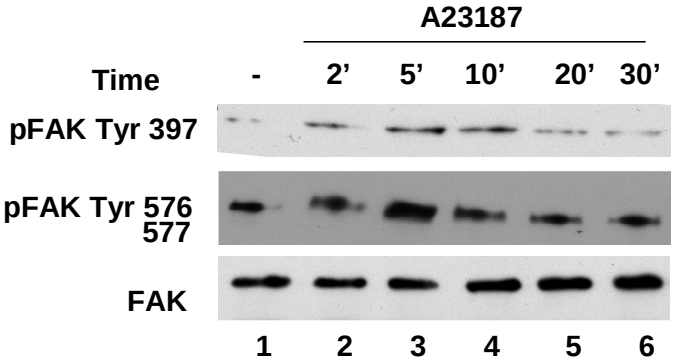


Figure 4: cGMP and Calcium treatments lead to differential phosphorylation of FAK at two key sites. (A) Cells were treated with 100µM 8-pCPT-cGMP for the indicated time intervals. (B) Cells were treated with 0.3µM calcium ionophore A23187 instead of cGMP. In both (A) and (B), the cells were harvested and the lysates were analyzed by Western Blot with phospho- FAK Tyr 397 (pFAK Tyr 397), phospho-FAK Tyr 576/577 (pFAK Tyr 576/577).

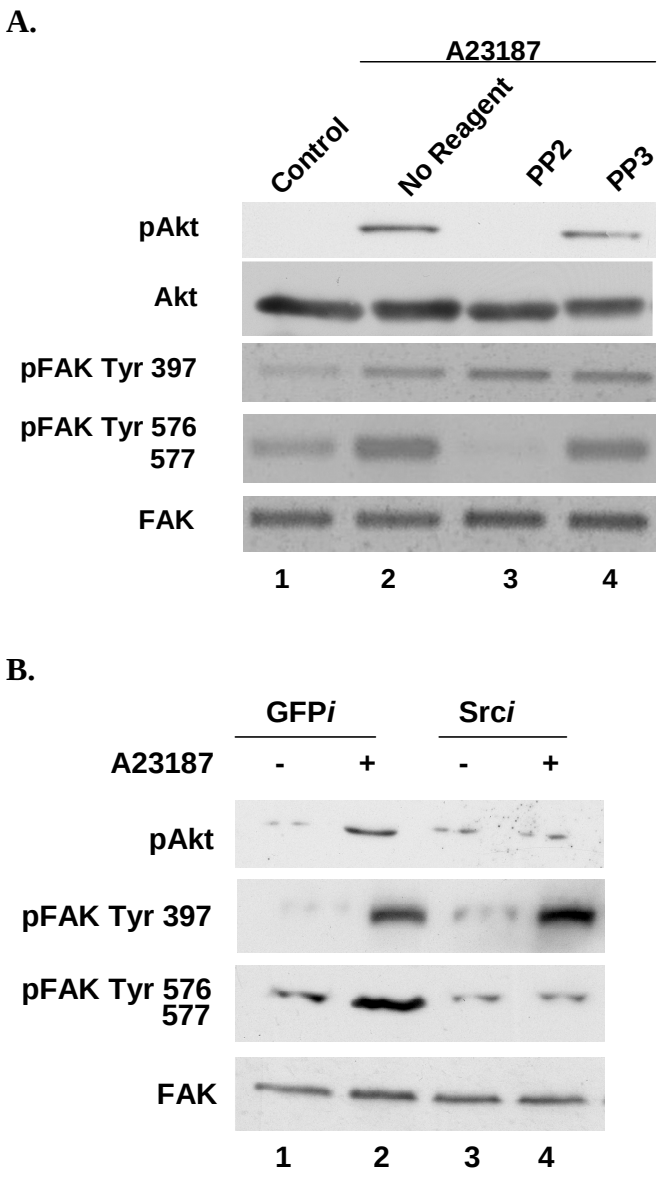


Figure 5: FAK is downstream of Src. (A) MC3T3 cells were treated with either 10µM PP2 or 10µM PP3 for one hour, and then 0.3µM A23187 was added for 5 minutes. (B) Cells were transfected with Src- specific siRNA and then treated with 0.3µM A23187 for 5 minutes. In both (A) and (B), the cells were harvested and analyzed by Western Blot with phospho-specific antibodies for phospho-FAK Tyr 397 (pFAK Tyr 397), phospho-FAK Tyr 576/577 (pFAK Tyr 576/577), and phospho-Akt Ser 473 (pAkt).

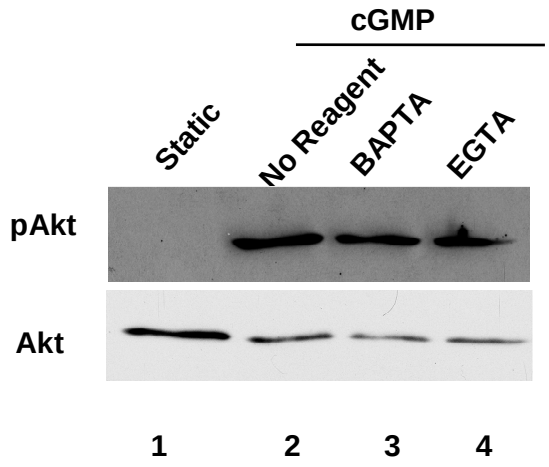


Figure 6: cGMP-induced Akt phosphorylation is calcium-independent. MC3T3 cells were pretreated with calcium chelators 10µM BAPTA (lane 3) or 5mM EGTA (lane 4) for one hour, then 100µM 8-pCPT-cGMP was added to lanes 2, 3, and 4 for 5 minutes.

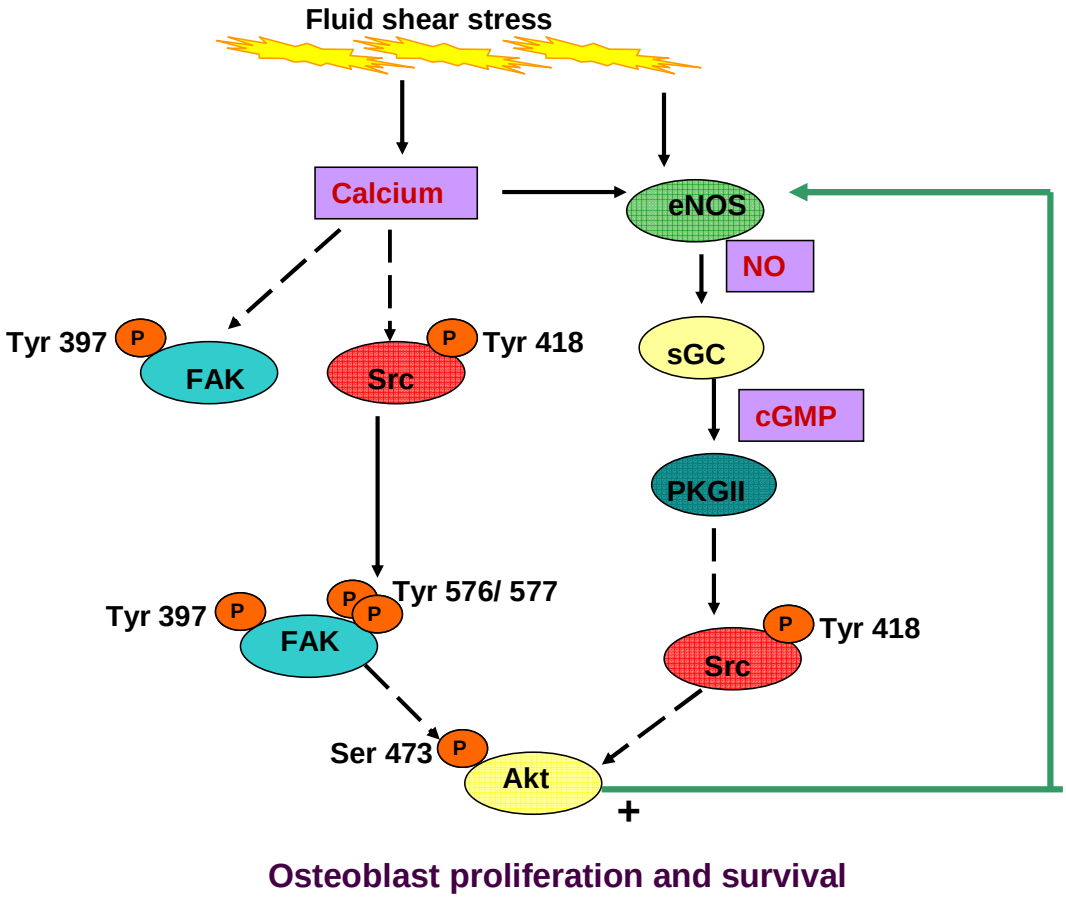


Figure 7: A proposed model mechanism of cGMP and calcium signaling to Akt. When osteoblasts are exposed to FSS, Akt phosphorylation can occur through two independent pathways. There is a possible positive feedback cycle once Akt is activated that acts on endothelial nitric oxide synthase (eNOS). The solid arrows indicate direct activation. The broken arrows show interaction through an indirect or unknown pathway.

SUPPLEMENTAL FIGURES AND TABLES

Supplemental Table 1: siRNA Sequences

SiRNA	**Target Sequence**
PKGIα/β	5’ -CCGGACAUUUAAAGACAGCAA-3’
PKGII #4	5’ -CCGGGTTTCTTGGGTAGTCAA-3’
Src	5’-CAGCAACAAGAGCAAGCCCAA-3’
FAK	5’-TGCAATGGAACGAGTATTAAA-3’
GFP	5’-AAGCTGACCCTGAAGTTCATC-3’

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