Published in final edited form as:

Bone. 2010 July ; 47(1): 74–82. doi:10.1016/j.bone.2010.03.014.

Activation of NF- κ B by Fluid Shear Stress, but not TNF- α , Requires Focal Adhesion Kinase in Osteoblasts

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Abstract

When bone is mechanically loaded fluid shear stress (FSS) is generated as a result of the movement of interstitial fluid across the membranes of osteoblasts and osteocytes. This external mechanical loading stimulates changes in the activity of cytoplasmic signaling molecules and alters gene expression in bone cells. This process, referred to as mechanotransduction, is vital for maintaining bone health *in vivo* by regulating the balance between bone formation and bone resorption. This current study focuses on the role of focal adhesions, sites of integrin-mediated cellular attachment to the extracellular matrix, and their proposed function as mechanosensors in bone cells. We examined the role of a key component of focal adhesions and of mechanotransduction, focal adhesion kinase (FAK) in regulation of FSS- and tumor necrosis factor- α (TNF- α)-induced activation of nuclear factor-kappa B (NF- κ B) signaling in osteoblasts. Immortalized FAK^{+/+} and FAK^{-/-} osteoblasts were exposed to periods of oscillatory fluid shear stress (OFF) and NF- κ B activation was analyzed. We determined that FAK is required for OFF-induced nuclear translocation and activation of NF- κ B in osteoblasts. In addition we found that OFF induced phosphorylation of the I κ B kinases (IKK α/β) in both FAK^{+/+} and FAK^{-/-} osteoblasts, but only FAK^{+/+} osteoblasts demonstrated the resulting degradation of NF- κ B inhibitors I κ B α and I κ B β . OFF did not induce the degradation of I κ B ϵ or the processing of p105 in either FAK^{+/+} and FAK^{-/-} osteoblasts. To compare the role of FAK in mediating OFF-induced mechanotransduction to the well characterized activation of NF- κ B by inflammatory cytokines, we exposed FAK^{+/+} and FAK^{-/-} osteoblasts to TNF- α . Interestingly, FAK was not required for TNF- α induced NF- κ B activation in osteoblasts. In addition we determined that TNF- α treatment did not induce the degradation of I κ B β as did OFF. These data indicate a novel relationship between FAK and NF- κ B activation in osteoblast mechanotransduction and demonstrates that the mechanism of FSS-induced NF- κ B activation in osteoblasts differs from the well characterized TNF- α -induced activation.

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Conflict of Interest None.

Keywords

FAK; NF- κ B; Osteoblasts; Mechanotransduction; I κ B

Introduction

Mechanical stimulation plays a critical role in the health of bone tissue by regulating the balance between bone formation and bone resorption. Mechanical loading of bone causes movement of interstitial fluid within bone that generates fluid shear stress (FSS) across the surface of the bone cells [1]. Mechanotransduction is the response of osteoblasts and osteocytes that couples the extracellular stimuli of FSS with cytoplasmic signaling molecules to produce changes in bone metabolism [2]. Focal adhesions, which are composed of integrins, vinculin, α -actinin, actin filaments and several other focal adhesion associated proteins, are proposed as likely mechanosensors in bone cells. Recently, focal adhesion kinase (FAK) was shown to be a key component of mechanotransduction in osteoblasts and was found to be required for FSS induced upregulation of osteopontin and COX-2, key regulators of bone formation and resorption [3]. There is growing evidence that the transcriptional regulator NF- κ B is also important for the regulation of bone health. NF- κ B activity plays a key role in post-natal bone remodeling by inhibiting bone formation in mice [4], and NF- κ B nuclear translocation is activated by FSS in osteoblasts [5]. NF- κ B activity is also involved in tumor necrosis factor – α (TNF- α) mediated stimulation of osteoclastogenesis and inhibition of osteoblast function [6].

Proto-typical NF- κ B is composed of a homo- or hetero-dimer of members of the Rel family of DNA binding proteins that in mammals includes: RelA (p65), c-rel, RelB, p105 the precursor of p50, and p100 the precursor of p52. Within the Rel Homology Domain (RHD) that defines this family is a DNA binding region, a dimerization region and a nuclear localization signal. NF- κ B activity is regulated by a second protein family consisting of the I κ Bs (inhibitor of κ B) which includes I κ B α , I κ B β , I κ B γ , I κ B ϵ , Bcl-3, p100 and p105. I κ B family members contain ankyrin repeats that bind to the RHD and facilitate cytoplasmic retention of Rel protein dimers. In most cells, NF- κ B is cytoplasmic in an I κ B-containing complex until it is activated. The majority of studies are consistent with a model in which stimulation of cells activates a preformed complex containing: I κ B kinase- α (IKK α), I κ B kinase- β (IKK β) and IKK γ /NEMO, a non-enzymatic protein that may function as a scaffold. Activated IKK α and IKK β phosphorylate two key serine residues located amino terminal to the ankyrin repeat regions in I κ B α and I κ B β . Phosphorylated I κ B is ubiquitinated on lysine residues near the phosphorylated residues, leading to removal via proteolysis of I κ B from the NF- κ B containing complex. I κ B degradation allows NF- κ B nuclear retention and allows for NF- κ B dependent changes in gene transcription (activation/repression).

To better understand the mechanisms through which mechanical stimulation promotes NF- κ B activation in osteoblasts, we evaluated FSS induced NF- κ B activation in FAK^{+/+} and FAK^{-/-} osteoblasts. We determined that FAK is required for FSS induced nuclear translocation of p65 and NF- κ B transcriptional activation in osteoblasts. We also found that FSS induced the degradation of the NF- κ B inhibitors I κ B α and I κ B β but not I κ B ϵ or p105 in a FAK dependent manner. Re-expression of FAK in FAK^{-/-} osteoblasts rescued FSS induced NF- κ B activation. To compare the role of FAK during FSS induced NF- κ B activation to the well characterized activation of NF- κ B by cytokine, we exposed FAK^{+/+} and FAK^{-/-} osteoblasts to TNF- α . Our analysis determined that FAK is not required for TNF- α induced p65 nuclear translocation or NF- κ B transcriptional activation. Furthermore, we found that in contrast to the effect of FSS on I κ B β degradation TNF- α induced degradation of NF- κ B inhibitor I κ B α .

Materials and methods

Constructs

Full-length green-fluorescent protein (GFP) tagged FAK (FAK-GFP) was a gift of Dr. Lewis Romer (Johns Hopkins University). In order to remove the GFP tag, the FAK cDNA was excised with Bgl II and EcoRI and ligated into pcDNA3.1 at BamHI and EcoRI. The IL-8-LUC reporter construct contained bp-1451-+44 of the IL-8 gene [7] immediately upstream of the firefly luciferase cDNA (pGL3, Promega) [8].

Cell culture

Immortalized FAK^{-/-} and FAK^{+/+} osteoblast clones were established as described in Kim et al. 2007. Briefly, primary cells from mouse calvaria were harvested from *fak^{flxed/flxed}*; *p53^{-/-}* conditional knockout mouse and infected using CMV-Cre adenovirus to knockout the *fak* gene (Cre⁺; FAK^{-/-}; p53^{-/-}) [9]. Single cell cloning was performed using a limiting dilution, and individual clones were established as cell lines. Control calvarial osteoblasts harvested from *fak^{flxed/flxed}* mouse that were not exposed to Cre recombinase (Cre⁻; FAK^{flxed/flxed}; p53^{-/-}) were established in a similar fashion [9]. The mouse clones were cultured in MEM supplemented with 10% FCS and antibiotics. Both FAK^{+/+} and FAK^{-/-} clones express the osteoblast marker osteopontin and alkaline phosphatase and contribute to fetal skeletogenesis *in vivo* [9]. For transient transfection assays, FAK^{-/-} cells were plated at approximately 80% confluent as per manufacturer's instructions. The following day, cells were transfected with FAK cDNA using FuGENE 6 (Roche, Indianapolis, IN) per manufacturer's protocol. Cells were used for experiments on 1-2 days after transfection.

Oscillatory fluid flow (OFF) conditions

Osteoblasts were plated on glass slides at 2.0×10^5 cells per slide. Cells were subjected to oscillatory fluid shear stress (10 dynes/cm²) in parallel plate flow chambers at 37°C, 0.25% FCS in MEM using a previously described fluid flow device [10]. Hard-walled tubing was used to connect the pump to the chamber inlet, and a reservoir was attached to the outlet to allow for movement of the fluid and exchange of 5% CO₂. This system subjects cells to oscillating fluid flow at a frequency of 0.5Hz. Static controls were held in cell culture dishes at 37°C in 0.25% FCS in MEM with 5% CO₂.

Immunoblot analysis

Cells subjected to static and oscillatory fluid flow were harvested in SDS sample buffer, and protein concentrations were determined using the amido black method [11]. Equal amounts of protein (15µg) were loaded onto SDS-PAGE gels for separation and transferred to PVDF or nitrocellulose. The following primary and secondary antibodies were used: p65 (Santa Cruz), phospho-IKKα/β (Cell Signaling), IKKα/β (Santa Cruz), IκBα (Cell Signaling), IκBβ (Cell Signaling), IκBε (Cell Signaling), p105, (Cell Signaling), FAK (Upstate), phospho-FAK Y397 (BD), vinculin (Sigma), HRP conjugated goat anti-rabbit and HRP conjugated goat anti-mouse (Jackson Immunoresearch, West Grove, PA). Densitometry was quantitated using Image J software (NIH).

Immunofluorescence

Osteoblasts grown on glass slides were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. Monoclonal antibody against p65 (Santa Cruz) followed by FITC conjugated secondary antibody was used. Images were recorded using an Olympus digital camera attached to a Nikon Diaphot 200 inverted epifluorescent microscope with PlanApo X40 Nikon objectives. Images are representative of at least three independent trials in which at least 50 cells were analyzed per treatment in each trial.

Reporter Assay

Osteoblasts grown to ~80% confluency in 10 cm dishes were transfected with 6 μ g the IL-8 promoter-firefly luciferase construct using FuGENE per manufacture's protocol [7, Vig, 2001 #150]. Transfected cells were incubated over night and the next day were plated for TNF- α treatment or exposure to OFF. Luciferase activities were determined using at Luciferase Assay Kit (Promega, Madison, WI) according to the manufacture's protocol. Data are presented as fold change in activity relative to untreated (TNF- α) or static controls (OFF). Graphical data is representative of at least three independent trials.

TNF- α Treatment

Osteoblasts were plated in 6 well dishes, 1.5×10^5 cells per well, and treated with 10 ng/ml of TNF- α (Calbiochem) in MEM supplemented with 0.25% FCS 37°C, 5% CO₂. Graphical data is representative of at least three independent trials.

Statistical analysis

Statistical significance was assessed by one-tailed *t*-tests with a *p*-value of < 0.05 interpreted as statistically significant. Experiments were performed in triplicate, and $n \geq 3$ for each experimental group in each trial.

Results

Decreased shear-induced p65 nuclear translocation in FAK deficient osteoblasts

Previous work from our group demonstrated that fluid shear stress-induced p65 nuclear translocation in MC3T3 osteoblast-like cells [5]. To examine the role of FAK during fluid shear stress (FSS) induced p65 nuclear translocation, we exposed osteoblasts to 30 minutes, 1 hour and 2 hours of oscillatory fluid flow (OFF) or maintained cells in static culture conditions. We determined that p65 was localized in the cytoplasm in cells maintained under static conditions and following 30 minutes or 1 hour of OFF (data not shown). After exposure to 2 hours of OFF, p65 accumulation in the nucleus was detected in FAK^{+/+} but not in FAK^{-/-} osteoblasts (Fig. 1). Additionally, nuclear accumulation of p65 occurred following exposure to 2 hours of OFF in FAK^{-/-} osteoblasts reconstituted with FAK (FAK^{-/-} + cDNA) (Fig. 1). To determine how long p65 remained localized in the nucleus following OFF, we exposed FAK^{+/+} and FAK^{-/-} osteoblasts to 2 hours of OFF followed by a 1 hour or 4 hour post-flow period in MEM supplemented with 0.25% FCS, 37°C, 5% CO₂. We found that p65 remained in the nucleus for at least 1 hour after stimulation with OFF in FAK^{+/+} osteoblasts, but by 4 hours post-OFF cytoplasmic levels of p65 were similar to those detected in cells maintained under static conditions (Fig. 1). FAK^{-/-} osteoblasts did not exhibit nuclear localization of p65 following a 1 hour or a 4 hour post-OFF period, and re-expression of FAK in FAK^{-/-} osteoblasts rescued nuclear translocation of p65 at 1 hour post-OFF (Fig. 1).

To determine if the requirement for FAK in p65 nuclear accumulation was specific to the OFF response, we evaluated the requirement for FAK in response to TNF- α . Nuclear translocation of p65 was observed in TNF- α treated FAK^{+/+} and FAK^{-/-} osteoblasts (Fig. 1).

FAK is required for FSS-induced but not TNF- α induced NF- κ B activity in osteoblasts

In addition to analyzing FSS induced p65 nuclear translocation, we evaluated whether the nuclear accumulation of p65 lead to activation of NF- κ B dependent gene expression in osteoblasts. FAK^{+/+} and FAK^{-/-} osteoblasts were transfected with a reporter construct composed of the firefly luciferase cDNA under the control of the NF- κ B dependent IL-8 gene promoter (IL-8-Luc). Twenty four hours after transfection, osteoblasts were exposed to 2 hr OFF or maintained in static culture conditions. Following an additional 2 hr post-OFF

incubation period in 10 cm tissue culture dishes in MEM supplemented with 0.25% FCS, cell lysates were collected, and IL-8 promoter activity was analyzed. FAK^{+/+} osteoblasts exhibited a significant increase in IL-8 promoter activity (2.8 ± 0.25 fold increase) in response to OFF. In contrast, there was no significant increase in IL-8 promoter activity in response to OFF in FAK^{-/-} osteoblasts (Fig. 2A). Re-expression of FAK in the FAK^{-/-} osteoblasts rescued IL-8 promoter activation (2.3 ± 0.56 fold increase) in response to OFF (Fig. 2A).

To confirm that FAK^{-/-} osteoblasts were capable of activation the NF- κ B dependent response, we evaluated NF- κ B transcriptional activity in TNF- α treated FAK^{+/+} and FAK^{-/-} osteoblasts. FAK^{+/+} and FAK^{-/-} osteoblasts were transfected with the IL-8-Luc construct and twenty four hours later cultures were treated with TNF- α (or vehicle) for 4 hours. Cell lysates were collected and luciferase activity was analyzed. Both FAK^{+/+} and FAK^{-/-} osteoblasts exhibited a significant fold increase in IL-8 promoter activity in response to TNF- α treatment for 4 hour confirming that NF- κ B signaling was intact in FAK^{-/-} cells of (Fig. 2B).

Activation of p65 in response to OFF and TNF- α in FAK^{+/+} and FAK^{-/-} osteoblasts

Because FAK^{-/-} osteoblasts did not exhibit a significant increase in NF- κ B activation in response to OFF, we next examined the phosphorylation status of p65 since phosphorylation of p65 is important for p65 transcriptional activity [12]. In comparison to osteoblasts maintained in static culture conditions, neither the FAK^{+/+} nor FAK^{-/-} osteoblasts demonstrated changes in phosphorylation of p65 at serine 536 following 30, 60 or 120 minutes of OFF (Fig. 3A). This data contrasts with previous reports indicating that p65 is phosphorylated in response to unidirectional fluid shear stress in endothelial cells [13]. Oscillatory fluid flow and unidirectional fluid flow can have differential effects on osteoblasts [14]. Therefore, we also examined phosphorylation of serine 536 in p65 in response to unidirectional fluid flow (10 dynes/cm²) in FAK^{+/+} osteoblasts. Interestingly, FAK^{+/+} osteoblasts did show a significant increase (1.5-2 fold) in serine 536 phosphorylation in response to 30 minutes of unidirectional fluid flow as compared to static controls (data not shown) suggesting that p65 phosphorylation may be differentially induced depending on the type of fluid flow profile that is experience by cells.

To confirm that the FAK^{-/-} osteoblasts were capable of phosphorylation of p65 on serine 536, we analyzed the phosphorylation status of serine 536 in TNF- α -treated FAK^{+/+} and FAK^{-/-} osteoblasts. We found that FAK^{+/+} and FAK^{-/-} osteoblasts exhibited significant increases in phosphorylation of p65 at serine 536 in response to 30 and 60 minutes of TNF- α treatment as compared to untreated samples (Fig. 3A, B).

FSS- induced IKK α / β activation and I κ B degradation is impaired in FAK^{-/-} osteoblasts

To further evaluate the role of FAK in FSS-induced activation of NF- κ B, we examined the phosphorylation of IKK α / β at serine 180/181, respectively. We observed a significant increase (2-3 fold) in IKK α / β phosphorylation in FAK^{+/+} and FAK^{-/-} osteoblasts in response to 30-120 minutes of OFF (Fig. 4A, B). We also analyzed the steady-state levels of several I κ B family members, I κ B α , I κ B β , I κ B ϵ and p105, in response to OFF. FAK^{+/+} osteoblasts exhibited a significant reduction (~80%) in I κ B α and I κ B β (~60%) protein following 1 hour of OFF (Fig. 4A, C, D). In contrast, FAK^{-/-} osteoblasts did not exhibit a decrease in the steady-state levels of I κ B α or I κ B β protein in response to 1 hour of OFF. Interestingly, neither I κ B ϵ nor p105 protein levels changed in response to OFF in either FAK^{+/+} or FAK^{-/-} osteoblasts (Fig. 4A). Re-constitution of FAK^{-/-} osteoblasts with FAK rescued the FSS induced degradation of I κ B α and I κ B β proteins in response to 1 hour of OFF (Fig. 4A, C, D).

TNF- α induced IKK α/β phosphorylation and I κ B degradation in FAK $^{-/-}$ osteoblasts

To further compare the requirement for FAK in OFF versus TNF- α activation of NF- κ B, we treated FAK $^{+/+}$ and FAK $^{-/-}$ osteoblasts with TNF- α for 0, 15, 30 or 60 minutes. Cellular proteins were harvested, and western blot analysis was performed to determine if FAK was required for TNF- α induced IKK phosphorylation and degradation of NF- κ B inhibitors I κ B α , I κ B β , I κ B ϵ and p105. TNF- α treatment induced a significant increase (2-3 fold) IKK α and IKK β phosphorylation in FAK $^{+/+}$ and FAK $^{-/-}$ osteoblasts (Fig. 5A, B). This resulted in a significant reduction in I κ B α protein level (~80%) after 15 and 30 minutes of exposure to TNF- α (Fig. 5A, C). Both FAK $^{+/+}$ and FAK $^{-/-}$ osteoblasts exhibited a significant reduction in p105 protein levels, ~40-60%, after 30 and 60 minutes of TNF- α treatment in (Fig. 5A, D). TNF- α treatment had no effect upon I κ B β or I κ B ϵ protein levels in either FAK $^{+/+}$ or FAK $^{-/-}$ osteoblasts (Fig. 5A).

Discussion

In this study we sought to understand the mechanisms through which FSS induced activation of NF- κ B in osteoblasts. Previous work by our group demonstrated that p65 nuclear translocation is induced by FSS in osteoblasts [5]. We have now extended this work by examining the signaling pathway involved in p65 nuclear localization and determined that FAK is required for FSS induced p65 nuclear translocation and NF- κ B trans-activation activity in osteoblasts. We also found that FSS did not result in an increase in p65 phosphorylation on serine 536 in either FAK $^{+/+}$ and FAK $^{-/-}$ osteoblasts.

To better understand the molecular mechanisms operating in response to mechanical stimulation, we examined upstream signaling events known to lead to p65 nuclear translocation and NF- κ B transactivation activity. We determined that FAK is not required for OFF-induced serine phosphorylation of IKK α/β . Although the time course of IKK α/β phosphorylation was slightly different between FAK $^{+/+}$ and FAK $^{-/-}$ osteoblasts, FSS induced significant increases in IKK α/β serine phosphorylation in both FAK $^{+/+}$ and FAK $^{-/-}$ osteoblasts. We also examined the steady-state levels of the I κ B family members in response to FSS in FAK $^{+/+}$ and FAK $^{-/-}$ osteoblasts. FAK is needed for FSS induced degradation of I κ B α and I κ B β proteins, which significantly decrease following 1 hour of OFF in FAK $^{+/+}$ osteoblasts but not in FAK $^{-/-}$ osteoblasts. Furthermore, we found that FSS does not affect the steady-state levels of I κ B ϵ or p105 in osteoblasts. While several reports in a variety of cell types indicate that mechanical stimulation can induce NF- κ B activity, IKK activation, I κ B family protein degradation our study is the first to examine these event in osteoblasts in response FSS [13,15-39].

To better characterize the role of FAK in OFF-induced NF- κ B activation, we compared the effects of OFF to TNF- α induced responses in the FAK $^{+/+}$ and FAK $^{-/-}$ osteoblasts. FAK was not necessary for TNF- α induced p65 activation (as measured by phosphorylation of serine 536), p65 nuclear translocation or NF- κ B trans-activation activity as measured in IL-8 firefly luciferase reporter assays in osteoblasts. FAK was also not required for TNF- α induced activation of IKK α/β as measured by phosphorylation of serines 180 and 181, respectively or for degradation of I κ B α or p105. Interestingly, TNF- α did not cause degradation of I κ B β or I κ B ϵ in osteoblasts. Our data are consistent with the recent report that TNF- α induced NF- κ B activation is FAK independent in endothelial cells [13]. However, Funakoshi-Tago et al. demonstrated that fibroblasts require FAK for TNF- α induced NF- κ B activation, which implies that this phenomenon may be cell type specific [40].

We have previously demonstrated that FAK is a key signaling component of FSS-induced mechanotransduction in osteoblasts [3], and previous reports indicate that FAK is important for bone healing and remodeling in mice [9]. This present study extends these findings by providing a mechanistic basis through which FAK regulates FSS gene expression in osteoblasts

and demonstrates for the first time that FAK is necessary for the regulation of FSS induced NF- κ B activity. Our study is the first to examine the relationship between FAK and NF- κ B activity in response to mechanical stimulation in osteoblasts. While our group reported that FSS induced p65 nuclear translocation is dependent on intracellular calcium release, we were not able at that time to examine the role of FAK during FSS induced Ca^{2+} release [5]. While our data indicate that the degradation of I κ B α and I κ B β are important for the FSS induced p65 nuclear translocation and NF- κ B transcriptional activity, it is important to acknowledge that calcium signaling may also be contributing. Rezzonico et al reported that FAK interacts with the large conductance calcium-activated hSlo potassium channel in human osteoblasts further implying that FAK^{-/-} osteoblasts may have altered calcium signaling [41]. Further investigation will be required to determine the role of FAK during FSS induced intracellular Ca^{2+} release in osteoblasts.

p65 expression and activation via phosphorylation at serine 536 were not affected by exposure to 30-120 minutes of OFF. Recently, Petzold et al reported that FAK regulates activation of p65 via phosphorylation of serine 536 and increased NF- κ B transcriptional activity in response to unidirectional fluid flow in endothelial cells [13]. To better understand the differences in these reports, we exposed FAK^{+/+} osteoblasts to 30 minutes of unidirectional fluid flow and found a significant 2-fold increase in p65 phosphorylation at serine 536. Previously we demonstrated that OFF and unidirectional fluid flow have different affects on osteoblasts [14]. Silacci et al. reported that oscillatory fluid flow and unidirectional fluid flow differentially regulate the NOS III gene in endothelial cells [29]. Our study re-enforces this concept by demonstrating that osteoblasts respond differently to varying types of mechanical stimulation. It is important to note that there may be significant differences in chemotransport, supply of nutrients and waste removal, during OFF versus unidirectional flow [42,43]. These potential effects may be contributing to the differences observed when osteoblasts are exposed to OFF versus unidirectional flow. Our lab routinely uses OFF when stimulating osteoblasts because *in vivo* mechanical loading of bone is thought to be dynamic and oscillatory [10]. While OFF did not change the steady state levels of p65 phosphorylation at serine 536 at the time points examined in this study, the timing of the phosphorylation events may be different in response to differing mechanical stimulation. Further examination of different time points should be examined to determine this.

Petzold et al. also reported that shear stress induced p65 nuclear translocation was FAK independent in endothelial cells. This is in contrast with the results presented herein demonstrating that OFF-induced p65 nuclear translocation is FAK dependent and can be rescued by the re-expression of FAK in FAK^{-/-} osteoblasts. Several differences between the two studies may account for these different results including cell type and the method of mechanical stimulation. In addition, our osteoblasts were exposed to 10 dynes/cm² on uncoated glass slides while the endothelial cells were exposed to greater fluid shear stress (24 dynes/cm²) on fibronectin coated slides [13]. Any or all of these differences in experimental methods could contribute to the differing results and could also reflect differences in endothelial cell versus the bone cell niche.

While this study and our previous reports indicate that FAK is a key regulator of mechanotransduction in osteoblasts, appropriate regulation of NF- κ B activity is important for bone homeostasis *in vivo*. The deletion of NF- κ B family members p50/p52 in mice resulted in osteopetrosis, impaired tooth eruption and excessive long bone calcification [44,45]. Chang et al. reported that inhibition of NF- κ B activity in a time and stage specific manner resulted in increased trabecular bone formation and increased bone mineral density in mice [4]. In addition, the role of NF- κ B activity during osteoclastogenesis has been extensively studied (reviewed in [46-48]). However, the effects of FSS on NF- κ B activity are not well understood. Jacobs et al. reported that OFF inhibits TNF- α induced NF- κ B activation in osteoblast-like

cells [49], and our present results demonstrate the requirement of FAK for FSS-induced p65 nuclear localization and NF- κ B trans-activation activity. These studies indicate the importance investigating the FSS-induced mechanisms that regulate NF- κ B activity in order to better understand how NF- κ B activity regulates bone homeostasis.

While examining the role of FAK in FSS-induced activation of NF- κ B activity, we found several differences between TNF- α and FSS on activation of NF- κ B in osteoblasts. TNF- α treatment did not cause degradation of I κ B β , but did result in a reduction in steady-state levels of I κ B α and p105. In contrast, FSS resulted in a reduction of steady-state levels of I κ B α and I κ B β , but not p105. TNF- α treatment resulted in a rapid and robust increase in p65 phosphorylation on serine 536, but FSS did not elicit a change in the phosphorylation of p65 on serine 536. These data indicate distinct and potentially significant differences between cytokine and mechanically induced signaling in osteoblasts in addition to the aforementioned differences in response to unidirectional fluid flow versus oscillatory fluid flow. Together, these differences in response highlight the importance of considering various types of mechanical stimulation that are likely experienced by bone cells *in vivo*.

In summary, we demonstrated novel differences between TNF- α and FSS induced NF- κ B activation and uncovered additional mechanistic differences in the response of osteoblasts to unidirectional fluid flow and oscillatory fluid flow. We also found that FAK is not required for TNF- α induced NF- κ B activation. Importantly, our study demonstrated a novel function for FAK during FSS induced mechanotransduction in osteoblasts. We demonstrated that FAK is required for NF- κ B transcriptional activation, p65 nuclear translocation and the FSS-induced decreased expression of I κ B α and I κ B β .

Acknowledgments

This work was supported by NIH R01AR052682 to FMP and by a post-doctoral fellowship AR056188 to SRLY.

Citations

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Figure 1. FSS induced p65 nuclear translocation is FAK dependent

Immunostaining for the p65 subunit of NF-κB was performed on FAK^{+/+} and FAK^{-/-} osteoblasts maintained in static culture conditions (Static), exposed to 2 hours of OFF, exposed to 2 hours OFF followed by a 1 hour post-flow incubation or exposed to 2 hours of OFF followed by a 4hr post-flow incubation. Re-expression of FAK in FAK^{-/-} osteoblasts via transfection of FAK cDNA (FAK^{-/-} + cDNA) rescued FSS induced p65 nuclear translocation. Immunostaining for the p65 subunit of NF-κB was also performed on FAK^{+/+} and FAK^{-/-} osteoblasts treated with TNF-α for 60 minutes (TNF-α).

Figure 2. OFF induced activation of NF-κB-dependent promoter requires FAK

(A) Quantification of IL-8 promoter reporter assay expressed in Fold Activation (OFF/static). IL-8 promoter-firefly luciferase construct transfected into FAK^{+/+} and FAK^{-/-} osteoblasts were maintained in static culture conditions or exposed to 2 hours of OFF followed by a 2 hour post-flow incubation. (B) Quantification of IL-8 promoter reporter assay. Experiments were performed in triplicate, and results are expressed in Fold Activation (TNF-α treated/untreated). Transfected FAK^{+/+} and FAK^{-/-} osteoblasts were untreated or treated with TNF-α for 4 hours. ^aStatistically significant difference between static and OFF or untreated and TNF-α treated ($p < 0.05$). Error bars represent SE.

Figure 3. Analysis of p65 phosphorylation in response to OFF and TNF- α treatment

(A) Western blot analysis of the expression and activation of p65, measured by phosphorylation of serine 536, in FAK^{+/+} and FAK^{-/-} osteoblasts maintained in static culture conditions (0 minutes) or exposed to 30, 60 or 120 minutes of OFF or treated with TNF- α for 0, 30 or 60 minutes. Vinculin was analyzed as a loading control. (B) Quantification of TNF- α induced p65 activation measured by phosphorylation of serine 536 in FAK^{+/+} and FAK^{-/-} osteoblasts. Experiments were performed in triplicate, and results are expressed in raw densitometry units. ^aStatistically significant difference between static and OFF or untreated and TNF- α treated ($p < 0.05$). Error bars represent SE.

Figure 5. TNF- α induced degradation of I κ B α and p105 does not require FAK

(A) Western blot analysis of IKK α/β expression and IKK α/β activation measured by phosphorylation of ser180/181, I κ B α , I κ B β , I κ B ϵ and p105 in FAK $^{+/+}$ and FAK $^{-/-}$ osteoblasts untreated or treated with TNF- α for 0, 30 and 60 minutes. FAK $^{-/-}$ osteoblasts re-expressing FAK via transfection of FAK cDNA (FAK $^{-/-}$ + cDNA) were also analyzed. Vinculin was analyzed as a loading control. (B-D) Quantification of (B) activation of IKK α/β (phosphorylation of ser180/181), (C) I κ B α , (D) p105. Experiments were performed in triplicate, and results are expressed in fold change (treated/untreated). ^aStatistically significant difference between untreated and TNF- α treated ($p < 0.05$). Error bars represent SE.