Title: Fluid shear stress suppresses TNF- α -induced apoptosis in MC3T3-E1 cells: Involvement of ERK5-AKT-FoxO3a-Bim/FasL signaling pathways

All authors names

Geng Bin, Zhang Bo, Wang Jing, Jiang Jin, Tan Xiaoyi, Chen Cong, An Liping, Ma Jinglin, Wang Cuifang, Chen Yonggang, Xia Yayi

Geng Bin, Zhang Bo, Wang Jing, Jiang Jin, Tan Xiaoyi, Chen Cong, An Liping, Ma Jinglin, Wang Cuifang

The Second Hospital of Lanzhou University, #82 Cuiyingmen, Lanzhou 730000, Gansu, China

Orthopaedics Key Laboratory of Gansu province, Lanzhou 730000, Gansu, China

E-mail: cxxx@foxmail.com

Xia Yayi (Corresponding author), Chen Yonggang

Department of Orthopaedics, the Second Hospital of Lanzhou University, #82 Cuiyingmen, Lanzhou 730000, Gansu, China

E-mail: xiayayildey@163.com;

Abstract: TNF- α is known to induce osteoblasts apoptosis, whereas mechanical stimulation has been shown to enhance osteoblast survival. In the present study, we found that mechanical stimulation in the form of fluid shear stress (FSS) suppresses TNF- α induced apoptosis in MC3T3-E1 cells. Extracellular signal-regulated kinase 5 (ERK5) is a member of the mitogen-activated protein kinase (MAPK) family that has been implicated in cell survival. We also demonstrated that FSS imposed by flow chamber *in vitro* leads to a markedly activation of ERK5, which was shown to be protective against TNF- α -induced apoptosis, whereas the transfection of siRNA against ERK5 (ERK5-siRNA) reversed the FSS-mediated anti-apoptotic effects. An initial FSS-mediated activation of ERK5 that phosphorylates AKT to increase its activity, and a following forkhead box O 3a (FoxO3a) was phosphorylated by activated AKT. Phosphorylated FoxO3a is sequestered in the cytoplasm, and prevents it from translocating to nucleus where it can increase the expression of FasL and Bim. The inhibition of AKT-FoxO3a signalings by a PI3K (PI3-kinase)/AKT inhibitor (LY294002) or the transfection of ERK5-siRNA lead to the nuclear translocation of non-phosphorylated FoxO3a, and increased the protein expression of FasL and Bim. In addition, the activation of caspase-3 by TNF- α was significantly inhibited by aforementioned FSS-mediated mechanisms. In brief, the activation of ERK5-AKT-FoxO3a signaling pathways by FSS resulted in a decreased expression of FasL and Bim and an inhibition of caspase-3 activation, which exerts a protective effect that prevents osteoblasts from apoptosis.

Key words: Fluid shear stress; Extracellular signal-regulated kinase 5; Forkhead box O 3a; FasL; Bim; Osteoblasts.

1. Introduction

Bone is a living dynamic organ whose internal architecture continuously undergoes remodeling in response to everyday mechanical loading. Exposure to mechanical stimulation is ubiquitous during daily activities, and increased mechanical loading promotes bone formation and suppresses bone resorption [1,2]. Conversely, decreased mechanical stimulation due to bed rest, prolonged immobilization, exposure to a microgravity environment, or muscle paresis causes bone loss [2-4]. Mechanical loading produces fluid flow through the lacunar-canalicular systems, and the fluid flow exerts a fluid shear stress (FSS) at surfaces of the porous spaces of bone [5,6]. It is generally accepted that mechanotransduction, which is the process by which mechanosensitive bone cells convert physical mechanical loading signals into biochemical responses, plays an important role in the maintenance of bone mass and skeletal architectural integrity. Owing to the practical difficulties in investigating if FSS is the primary mediator of mechanical loading *in vivo*, the great majority of the existing studies have observed cellular responses in response to FSS imposed by flow chamber *in vitro* [7-9]. Bone cells translate the mechanical stimulus into biochemical responses, resulting in enhanced proliferation, differentiation, and survival [10-12]. To date, the exact molecular pathway of mechanotransduction whereby FSS regulates these responses in bone still remains unclear.

Our previous studies have shown that the application of FSS to osteoblasts results in enhancing MC3T3-E1 cells proliferation and differentiation via extracellular signal-regulated kinase 5 (ERK5,

also known as BMK1) pathway [13-16]. The mitogen-activated protein kinase (MAPK) family includes ERK5, p38, extracellular signal-regulated protein kinase 1/2 (ERK1/2), and c-jun N-terminal kinase (JNK). ERK5 encoded by *MAPK7* gene is the effector kinase of a classical three-tiered MAPK cascades comprising MEK5 (MAPK kinase 5), MEKK2/3 (MAPKK kinase 2/3) and ERK5 [17,18]. In light of protein phosphorylation is one of the crucial mechanisms used to convert external signals into the nucleus, ERK5 is activated by protein phosphorylation and transduce external environmental signals (mechanical loading, oxidative stress, growth factors, and hyperosmolarity) into biological effects (proliferation, differentiation and apoptosis) [19-23]. Recently, our previous results found FSS inhibits TNF- α -mediated signaling events in osteoblast by a mechanism dependent on activation of ERK5 [24], but a detailed role of ERK5 in osteoblast apoptosis has not been well elucidated.

Some studies indicated that the phosphorylation of Forkhead box O (FoxO) family by osmotic stress promotes cell survival in mouse embryonic fibroblasts [25]. The Forkhead box O family includes four members: FoxO1, FoxO3a, FoxO4 and FoxO6 [26]. Among them, FoxO3a is closely related to cells apoptosis [27]. A key event in the FoxO3a cascade is the activation of AKT [28,29], in other words, AKT is the direct upstream mediator of FoxO3a. Activation of FoxO3a provides a mechanism to sequester FoxO3a in the cytoplasm through interaction with 14-3-3 proteins thereby blocking their apoptotic effect [30-32]. However, it is unclear whether ERK5 is involved in FoxO3a activation in the osteoblasts after exposure to FSS. Furthermore, whether ERK5-AKT-FoxO3a signaling pathways is related to the detailed mechanisms that FSS regulated apoptosis in osteoblast remained largely unknown.

This study was undertaken to determine if mechanical stimulus in the form of FSS suppresses TNF- α -induced apoptosis in MC3T3-E1 cells and, if so, to investigate the molecular signaling mechanisms responsible for this anti-apoptotic effect. Here, we postulate that ERK5-AKT-FoxO3a-Bim/FasL signaling pathways play a crucial role in the FSS-mediated anti-apoptotic effect. To identify downstream effect of ERK5 activation in response to FSS in osteoblast, the activation of ERK5 or FoxO3a were inhibited through the transfection of siRNA against ERK5 (ERK5-siRNA) or a PI3K (PI3-kinase)/AKT inhibitor (LY294002), respectively. This study can have important implications for the treatment of osteoporosis and related bone-wasting diseases because the ERK5-AKT-FoxO3a-Bim/FasL signaling pathways used by FSS contain molecular elements that may serve as therapeutic targets.

2. Materials and methods

2.1 Reagents and antibodies

Reagent: α -modified essential medium (α -MEM) and fetal bovine serum (FBS, Life Technologies, USA); Recombinant mouse TNF- α (R&D Systems, USA); ERK5-siRNA (Santa Cruz Biotechnology, USA); Recombinant murine EGF (Peprotech, USA). Antibodies: ERK5 (1:1000), AKT (1:10000), phospho (P)-AKT T308 (1:100), FoxO3a (1:10000), P-FoxO3a S253 (1:5000), FasL (1:1000), and Bim (1:1000, Abcam, USA); P-ERK5 Thr218/tyr220 (1:1000, Cell Signaling Technology, USA); ERK1/2 (1:500) and P-ERK1/2 T202/Y204 (1:500, Bioworld Technology, USA); β -actin (1:800, Sigma, USA).

2.2 Cells culture

The murine osteoblastic MC3T3-E1 cells were obtained from the Chinese Academy of Medical

Sciences (Bei Jing, China), and maintained in a-MEM supplemented with 10% FBS, penicillin, and streptomycin, in a humidified atmosphere containing 95% air and 5% CO₂ at 37°C. To induce osteoblast apoptosis, various concentrations of TNF- α (0, 1, 10, 20, or 40 ng/mL) were added to the cell culture medium.

2.3 Exposure to fluid-induced shear stress

The MC3T3-E1 Cells were exposed to laminar flow (physiological shear stress = 12 dyn/cm²) in a parallel plate flow chamber as described previously [15,33]. Cells were grown to confluence on 20×50mm coverslips. When the cells were nearly 90% confluence, cells were flowed for 1 h in α -MEM after growth-arrest for 6 h in 0% serum. Briefly, this system provides a continuous hydrostatic pressure head to drive culture medium through the channel of the parallel plate flow chamber, producing an accurate FSS of 12 dyn/cm². Control cells were maintained under static conditions with the same medium at 37°C.

2.4 The siRNA transfection

ERK5-siRNA used was purchased from Santa Cruz Biotechnology, USA. The MC3T3-E1 cells were seeded in 35mm diameter dishes or 20×50mm coverslips, and then transfected with ERK5-siRNA or non-silencing siRNA using commercial transfection reagent (Santa Cruz Biotechnology, USA) according to the manufacturer's protocol as previously described [15]. At 48 h post transfection, the protein levels of ERK5 were detected by Western blotting analysis.

2.5 Hoechst 33258 staining

Cell survival was assessed based on nuclear morphological changes that were detected following nuclei staining with Hoechst 33258 (Sigma, USA). Briefly, the MC3T3-E1 cells were seeded in 20×50mm coverslips and incubated for 24 h. Cells were rinsed three times with phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde for 30 min at room temperature (RT). The cells were then stained with Hoechst 33258 in the dark 10 min at RT. After rinsing three times with PBS, the nuclear morphological changes of apoptosis were observed by fluorescence microscope (Olympus, Japan). Apoptotic cells exhibited brightly staining condensed nuclei, nuclear fragmentation, or apoptotic bodies.

2.6 TUNEL assay

TdT-UTP nick end labeling (TUNEL) assay was further used to detect the apoptotic cells by in situ cell death detection kit (Roche, Germany) according to the manufacturer's instruction. The grown cells on coverslips were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton-X100 for 2 min on ice. TUNEL reaction mixture was applied to the MC3T3-E1 cells in a humidified atmosphere for 2 h at 37°C in the dark. Samples were analysed under a fluorescent microscope by using an excitation wavelength in 580 nm. A minimum of 6 fields were randomly selected, and the apoptotic cells were counted in each field. The cells with red fluorescence were expressed as apoptotic cells.

2.7 Measurement of caspase-3 activity

Caspase-3 activity was detected by cleavage of chromogenic caspase substrates, the chromophore DEVD-p-nitroaniline (pNA), which is a caspase-3 substrate, using the capase-3/CPP32 colorimetric

assay kit (BioVision, USA). The pNA light emission can be quantified using a spectrophotometer at 405 nm. The protein samples were prepared by using chilled cell lysis buffer, and were added to the reaction buffer containing DEVD-pNA (200 μ M final concentration). The mixture was incubated at 37°C for 2 h. The absorbance of pNA from apoptotic samples or un-induced control samples were detected with a spectrometer at 405 nm.

2.8 PCR assay

Total RNA was carefully extracted from MC3T3-E1 cells using the TRIzol method (Invitrogen, USA). cDNA was then synthesized using a RT² First Strand kit (QIAGEN, USA) according to the manufacture's instruction. Newly synthesized cDNA was loaded onto RT² Profiler PCR array plates (a 96-well array was used, QIAGEN, USA) and amplified on the Roche LightCycler 480 II Real-Time PCR system. Relative levels of gene expression (fold change) were calculated using the comparative CT ($\Delta\Delta$ CT) relative quantification method of qPCR.

2.9 Immunofluorescence assay

Cells were seeded on sterilized cover glasses placed in a 100 mm dish. After being treated with ERK5-siRNA or LY294002, the cells were exposed to FSS for 1 h. Then, cells were fixed with 4% paraformaldehyde for 1 h, permeabilized with 0.1 % Triton X-100 in PBS for 30 min, blocked with 3% BSA in PBS for 30 min and incubated with primary antibodies overnight at 4°C. Secondary antibody were incubated using goat anti-rabbit antibodies (Invitrogen, UK) for 2 h at RT. All cells were stained with DAPI for nuclear observation. Cells were visualized by immunofluorescence microscopy.

2.10 Western blot analysis

After washing twice with PBS, the cells were lysed in RIPA buffer (Beyotime Biotechnology, China) containing proteinase and phosphatase inhibitors supplemented with 1 mmol/LPMSF. Cell debris was removed by centrifugation at 12,000 $\times$ g for 10 min at 4°C, and the protein concentration of the resulting supernatant was detected using BCA protein assay system (Beyotime Biotechnology, China). The mixture of the cell lysate and loading sample buffer was boiled for 3-5 min. Equal amounts of protein (~50 μ g) were separated by 8-15% SDS-PAGE and transferred to 0.45 μ M PVDF membranes. The membranes were blocked with 5% (W/V) nonfat milk, and then incubated with the appropriate primary antibodies overnight 4°C. Following washing three times in Tris Buffered Saline with Tween (TBST, pH 7.4), the membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000, Invitrogen, UK) for 2 h at RT. Finally, protein expression was detected by the Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., USA) and imaged using a VersaDoc Imaging System (Bio-Rad Laboratories Co., USA).

2.11 Statistical analysis

All experiments were repeated three times and all tests were run using three independent samples. All data was expressed as the means $\pm$ SD. All statistical analysis was performed with one-way ANOVA. For post hoc test, LSD was used. *P* values < 0.05 were considered statistically significant. SPSS 17.0 was used as a statistical analysis software.

3. Results

3.1 *TNF- α induces MC3T3-E1 cells apoptosis*

The apoptotic role of TNF- α in MC3T3-E1 cells was analyzed using Hoechst staining and TUNEL assay, as delineated in Materials and Methods. The cells were exposed to control (0 ng/ml), 1, 10, 20, and 40 ng/ml for 24 h. The data shows that in relation to the control, TNF- α elevated apoptosis of MC3T3-E1 cells in a dose-dependent manner (Fig. 1 A-D). The percent apoptotic cells became significant at a concentration of 10 ng/ml (7.1%, $p < 0.01$), with a further increase at 20 ng/ml (11.5%, $p < 0.001$) and maximized at 40 ng/ml (22.3%, $p < 0.001$).

3.2 *Effect of ERK5-siRNA in MC3T3-E1 cells*

To silence the expression of ERK5, ERK5-siRNA were stably transfected into MC3T3-E1 cells. Significant down-regulation of ERK5 was detected in ERK5-siRNA transfected cells compared to vector control (Fig. 2 A, B, $p < 0.001$).

3.3 *Fluid shear stress suppresses MC3T3-E1 cells apoptosis induced by TNF- α via a mechanism that requires ERK5 activity*

We investigated the effect of FSS on MC3T3-E1 cells in terms of cell apoptosis. The cells were treated with TNF- α and/or ERK5-siRNA and then exposed to either 12 dyn/cm² or static conditions in a parallel plate flow chamber for 1 h at 37°C. Compared to the cells treated with TNF- α under static conditions, apoptosis was significantly decreased by FSS in TNF- α -treated cells (Fig. 3 A). After steady laminar FSS for 1 h, there was a 3-fold decrease of apoptotic cells (from 24.8% to 7.8%), compared with TNF- α group (Fig. 3 C, $p < 0.001$).

In addition, TNF- α for 24 h triggered 24.8% cells apoptosis detected by nuclear morphology analysis, whereas the transfection of ERK5-siRNA significantly increased TNF- α -induced cells apoptosis (from 24.8% to 38.1%, Fig. 3 C, $p < 0.001$). As shown in figure 3 A, the ability of FSS to suppress TNF- α -induced cells apoptosis was significantly diminished by ERK5 silencing. Compared with FSS+TNF- α group, we found the anti-apoptotic effect of FSS is blocked in the ERK5-siRNA-transfected cells (from 7.8% to 32.1%, Fig. 3 C, $p < 0.001$).

In summary, these data shown FSS protects osteoblast from apoptosis, and ERK5 activity is essential for the anti-apoptotic effect in MC3T3-E1 cells. Similar results were observed with TUNEL analysis, as shown in figure 3B, D.

3.4 *ERK5-mediated repression of akt and activation of foxo3a, fasl, bim, caspase-3, some pro-apoptotic genes*

The pro-apoptotic genes that were most markedly affected by knockout of ERK5 were further identified to define potential requirements for ERK5 in their regulation. The levels of *foxo3a*, *fasl*, *bim*, and *caspase-3* transcripts were significantly increased in TNF- α -treated cells compared to control, whereas *akt* expression was dramatically decreased (Fig. 4 A). The up-regulation of *foxo3a*, *fasl*, *bim*, and *caspase-3* and down-regulation of *akt* in the absence of ERK5 were secondary pro-apoptotic signaling in ERK5-siRNA-transfected cells with TNF- α (Fig. 4 B). Together, these data supported that loss of ERK5 up-regulates TNF- α -induced transcription of *foxo3a*, *fasl*, *bim*, and *caspase-3* gene, and down-regulates expression of *akt* gene. The function of ERK5-related pro-apoptotic genes under various conditions were further detected by western blotting (see below).

3.5 *ERK5 is activated by fluid shear stress, and the activation of AKT and FoxO3a by FSS are blocked*

in ERK5-siRNA-transfected MC3T3-E1 cells

In view of the effects of FSS and/or ERK5-siRNA with regard to decreasing or increasing TNF- α -induced apoptosis, whether ERK5 is activated by FSS was detected in the subsequent experiment. Our previous studies have shown that ERK5 is phosphorylated by FSS in MC3T3-E1 cells, and the phosphorylation of ERK5 was markedly increased at 15min and reached a peak at 45 min [13-15]. As shown in Fig. 5 A, B, the results also indicated that ERK5 is phosphorylated and activated by steady FSS for 1h ($p<0.001$). Compared to FSS group, phosphorylation of ERK5 was strongly reduced in ERK5-siRNA+FSS group ($p<0.001$).

We further tested the phosphorylation levels of AKT and FoxO3a by the exposure to FSS or transfection with ERK5-siRNA in MC3T3-E1 cells. As shown in Figure 5 A, D, E, FSS promoted AKT activation that up-regulates FoxO3a phosphorylation ($p<0.001$).

To examine the role of AKT and FoxO3a in this signaling cascade, cells were treated with the antagonist of AKT (LY294002) and/or exposed to FSS (Fig. 6). The transfection with ERK5-siRNA prevented the FSS-mediated phosphorylation of AKT and FoxO3a (Fig. 5 D, E, $p<0.001$). Similarly, LY294002 was also sufficient to attenuate the phosphorylation of AKT and FoxO3a induced by FSS ($p<0.001$), but no significant change was observed for the phosphorylation level of ERK5 (Fig. 6 A-D).

In brief, these data suggested that FSS enhances ERK5 activity, leading to the up-regulation of AKT activity and, ultimately, to increased phosphorylation of FoxO3a. In the other words, AKT-FoxO3a are the downstream targets of ERK5 signaling.

3.6 FSS increased nuclear translocation of P-ERK5, but decreased nuclear translocation of FoxO3a

Because the activities of ERK5 and FoxO3a are known to be highly influenced by their subcellular localization, we found FSS increased nuclear translocation of P-ERK5, but decreased nuclear accumulation of FoxO3a (Fig. 7). Pre-treatment with ERK5-siRNA attenuated the FSS-induced nuclear translocation of P-ERK5 and FSS-decreased nuclear translocation of FoxO3a in osteoblast (Fig. 7). However, Pre-treatment with LY294002 only inhibited FSS-decreased nuclear translocation of FoxO3a (Fig. 7). Briefly, activated ERK5 by FSS shuttles from the cytosol to the nucleus. Because FoxO3a has been identified as direct target of AKT (Fig. 5 and 6), FoxO3a is phosphorylated through an ERK5-AKT-dependent mechanism. Phosphorylated FoxO3a is sequestered in the cytoplasm (Fig. 7).

3.7 ERK5 silencing increases the expression of FasL and Bim induced by TNF- α in MC3T3-E1 cells

As shown in figure 8 A-C, we found that the treatment with TNF- α for 24 h significantly up-regulates the expression of FasL and Bim in MC3T3-E1 cells ($p<0.001$). To show that deletion of ERK5 contributes to the up-regulation of FasL and Bim, we treated cells with small interfering RNA against ERK5. These results indicated the transfection of ERK5-siRNA significantly increases FasL ($p<0.05$) and Bim ($p<0.01$) protein expression compared with control (Fig. 8 b, c). As expected, MC3T3-E1 cells transfected with ERK5-siRNA treated with TNF- α for 24 h caused a significant increase in the expression of FasL ($p<0.01$, Fig. 8 B) and Bim ($p<0.05$, Fig. 8 C). These data suggested that ERK5 plays a crucial role in the apoptotic effects of TNF- α via the regulation of FasL and Bim protein expression.

3.8 Fluid shear stress down-regulates the expression of FasL and Bim induced by TNF- α in MC3T3-E1 cells

To further determine if ERK5 signaling is necessary for down-regulation of FasL and Bim by FSS, the ERK5-siRNA-transfected MC3T3-E1 cells treated with TNF- α were exposed to FSS for 1 h. Western blotting analysis demonstrated a decrease of FasL and Bim protein levels in the presence of FSS for 1 h (Fig. 9 A-C, $p < 0.01$). In contrast, after the treatment of TNF- α , the transfection of ERK5-siRNA blocked the down-regulation of FasL and Bim by FSS (Fig. 9 A-C, $p < 0.01$). Taken together, these results suggested that the expression of FasL and Bim induced by TNF- α are down-regulated by FSS via ERK5 signaling in MC3T3-E1 cells.

3.9 ERK5-AKT-FoxO3a signaling pathway is essential for regulation of FasL and Bim by FSS

As shown in Fig. 10 A, C-E, FSS enhanced phosphorylation of ERK5, AKT, and FoxO3a compared to TNF- α group ($p < 0.01$). The inhibition of FasL and Bim by FSS was blocked by transfection of ERK5-siRNA or treatment with LY294002 (Fig. 10 B, F, $p < 0.01$). These results further indicated ERK5-AKT-FoxO3a signaling is necessary for regulation of FasL and Bim in MC3T3-E1 cells.

3.10 Fluid shear stress inhibits TNF- α -induced activation of caspase-3 via ERK5-AKT-FoxO3a-Bim/FasL signaling pathways

The protease family of caspases is the final common signal transduction pathway of cells apoptosis. To investigate the involvement of caspase in TNF- α -induced MC3T3-E1 cells apoptosis, the activity of caspase-3 was also detected (Fig. 4 and 11). To test the relation between caspase-3 and ERK5 signaling, caspase-3 activity was estimated after ERK5 knockdown. As could be expected, the treatment with TNF- α for 24 h in MC3T3-E1 cells activated caspase-3 $\times 14.6$ -fold (Fig. 11 A, $p < 0.001$). Compared to TNF- α group, MC3T3-E1 cells transfected with ERK5-siRNA and treated with TNF- α for 24 h displayed a 1.4-fold activation of caspase-3 (Fig. 11 A, $p < 0.001$). Activation of ERK5 by FSS significantly decreased caspase-3 activity (Fig. 11 A, $p < 0.01$). In contrast, the inhibition of activation of caspase-3 by FSS was blocked via the transfection of ERK5-siRNA in MC3T3-E1 cells (Fig. 11 A, $p < 0.001$).

Similar results were observed by treatment with LY294002, exposure to FSS for 1 h and following treatment with TNF- α for 24 h in MC3T3-E1 cells (Fig. 11 B). Because AKT-FoxO3a are the downstream targets of ERK5 signaling (see Fig. 4 and 5), the inhibition of AKT-FoxO3a signaling also increases activity of caspase-3. These results indicated that FSS inhibits TNF- α -induced activation of caspase-3 via ERK5-AKT-FoxO3a-Bim/FasL signaling.

In summary, these all data indicated that FSS protects osteoblast from apoptosis, and the activation of ERK5 by FSS inhibits TNF- α -induced osteoblast apoptosis. Furthermore, the phosphorylation of ERK5 is required to phosphorylate AKT and FoxO3a in osteoblast. After the exposure to FSS, AKT-FoxO3a were activated by phosphorylated ERK5 inhibited the expression of FasL and Bim and the activity of caspase-3. Briefly, ERK5-AKT-FoxO3a-Bim/FasL signaling pathways is necessary for fluid shear stress protects osteoblast from apoptosis.

4. Discussion

Fluid shear stress, a complex physiological response to flow stimuli, is associated with osteoporosis and related bone-wasting diseases, while it is also required for the maintenance of normal

bone mass [34]. It is widely accepted that osteoblast apoptosis plays a key role in the maintaining of bone mass and skeletal architectural integrity [35-38]. However, detailed molecular mechanism explaining how FSS degrades osteoblast apoptosis remained to be investigated. Here, our results, for the first time, shown an essential correlation between FSS-mediated anti-apoptotic effects with ERK5-AKT-FoxO3a-Bim/FasL signaling pathways.

In this study, we have made several novel observations and determined multiple mechanisms through which mechanical stimulation of the MC3T3-E1 cells by FSS may suppress cells apoptosis. These mechanisms appear to be involved: an initial FSS-mediated activation of ERK5 that phosphorylates AKT to increase its activity, and a following phosphorylation of FoxO3a by activated AKT ultimately decrease the protein expression of FasL and Bim. In addition, the activation of caspase-3 by TNF- α was significantly inhibited by aforementioned mechanisms. Among several novel observations, the major findings of this study are the protective effect of FSS against TNF- α -induced apoptosis via ERK5-AKT-FoxO3a-Bim/FasL signaling pathways, and we think very importantly, these conclusions are strongly supported by the following findings that (1) FSS markedly phosphorylated ERK5 and subsequently enhanced activation of AKT-FoxO3a signaling (Fig. 5); (2) blocking ERK5 signaling by transfecting the siRNA against ERK5 increased MC3T3-E1 cells apoptosis, and inhibited FSS-mediated anti-apoptotic effect (Fig. 3); (3) inhibiting AKT-FoxO3a by the transfection of ERK5-siRNA or a chemical inhibitor (LY294002) abolished the anti-apoptotic effect of FSS (Fig. 6, 10, 11); and (4) activating ERK5-AKT-FoxO3a signaling pathways down-regulated the expression of FasL and Bim and reduced the activity of caspase-3 (Fig. 8-11).

The role of ERK5 signaling in osteoblast is poorly studied. Our lab previously demonstrated that the inhibition of ERK5 activation by transfection of siRNA against ERK5 or pharmacological inhibitors (BIX02188 and BIX02189 compounds) significantly suppresses osteoblast proliferation and differentiation [13-16]. Our results indicated ERK5 silencing significantly decreases AKT phosphorylation, in other words, activation of ERK5 is required for AKT phosphorylation by FSS (Fig. 5). In this study, we found that ERK5 contributes to inducing FoxO3a activation by phosphorylated AKT (Fig. 5, 6). P-FoxO3a is sequestered in the cytoplasm via its association with 14-3-3 as previously described [39]. Under the exposure to FSS, the phosphorylated FoxO3a translocates to the nucleus where it decreases FasL and Bim protein expression (Fig. 8, 9, 11). FasL and Bim-mediated caspase-3 activation enhances TNF- α -induced osteoblast apoptosis.

The studies on FSS-mediated anti-apoptotic effect in osteoblasts remain poor and require further investigation. Oscillatory fluid shear stress (OFSS) suppressed TNF- α -induced osteoblast apoptosis which is explained by down-regulation of TNFR1 and blockade of TNFR1 downstream signaling by OFSS [40]. Triplett et al. found PKC ζ is essential for FSS-enhanced insulin-like growth factor-I-activated AKT and ERK phosphorylation and FSS-mediated anti-apoptotic effect in osteoblasts [41]. Pavalko et al. demonstrated that application of FSS to osteoblasts results in suppression of TNF- α -induced apoptosis via a mechanism involving PI3K/AKT activation and inhibition of caspase-3 activity [42]. In addition, some studies found mechanical stimulus blocked cells apoptosis through prostaglandin E₂ (PGE₂), Connexin 43, or Bcl-2/Bax expression in MLO-Y4 cells [43-45]. Our results are consistent with those reports. Therefore, our findings also act in a complementary mechanism to FSS-induced anti-apoptotic effect, that is, the multiple pro-survival signaling pathways, including ERK5, AKT, FoxO3a, FasL, Bim, and caspase-3, are obviously involved in mediating the protective effect of FSS in osteoblasts.

In conclusion, FSS activates MEKK2/MEKK3, and then MEK5 is phosphorylated.

Phosphorylated MEK5 is the sole upstream ERK5 and directly activate ERK5. Activated ERK5 shuttles from the cytosol to the nucleus and activates AKT. FoxO3a is phosphorylated through an ERK5-AKT-dependent mechanism. Phosphorylated FoxO3a is sequestered by the 14-3-3 protein in the cytoplasm, and prevents it from translocating to nucleus where it can increase the protein expression FasL and Bim. The decrease of FasL and Bim expression reduces the activity of caspase-3, and ultimately exerts a protective effect that prevents osteoblasts from apoptosis.

Author disclosure statement

The authors declare that there are no conflicts of interest related to this work.

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Figure.1

Fig. 1. The effects of TNF- α on MC3T3-E1 cells apoptosis. Cells were treated with control (0 ng/mL), 1, 10, 20, or 40 ng/mL TNF- α for 24 h. DNA fragmentation and condensed nuclei were visualized using Hoechst 33258 and TUBEL assay (A, B). Scale bar = 25 μ m. Quantitative analysis of the effects of TNF- α -induced osteoblast apoptosis by Hoechst abnormal nucleic acid content and TUNEL positive cells count(C, D). n=3, * p <0.05; ** p <0.01; *** p <0.001 relative to control , or to the 1 or 40 ng/ml groups.

Figure.2

Fig. 2. The transfection of ERK5-siRNA limits ERK5 expression in MC3T3-E1 cells. The cells were transfected with ERK5-siRNA or vector. The silencing of ERK5 was confirmed with western blotting analysis, and blotting with β -actin was the control for equal loading (A). Quantitative analysis of the ratio of ERK5/ β -actin (B). n=3, * p <0.05; ** p <0.01; *** p <0.001.

Figure.3

Fig. 3. The effects of ERK5-siRNA on anti-apoptotic effect of fluid shear stress. The cells were transfected with ERK5-siRNA, exposed to FSS (12 dyn/cm²) or left static for 1 h, and then stimulated with TNF- α for 24 h. DNA fragmentation analysis with Hoechst staining and TUNEL assay (A, B). Scale bar = 25 μ m. Quantitative analysis of effects of FSS, TNF- α , and/or ERK5-siRNA in MC3T3-E1 cells (C, D). n=3, * p <0.05; ** p <0.01; *** p <0.001.

Figure.4

Fig. 4. Relative expression (fold change) of select genes by PCR array in MC3T3-E1 cells. To identify pro-apoptotic genes that strictly correlate with ERK5 expression, gene expression profiles were compared between control cells and TNF- α -treated cells (A), and further investigation of gene expression was conducted in ERK5-siRNA-transfected cells with/without treatment of TNF- α (B).

Figure.5

Fig. 5. Fluid shear stress up-regulated ERK5 phosphorylation in MC3T3-E1 cells. After cells were exposed to FSS (12 dyn/cm²) or static conditions for 1 h, the protein levels of P-ERK5, ERK5, P-AKT, AKT, FoxO3a, P-FoxO3a, and β-actin was detected by immunoblotting (A). Quantitative analysis of the ratio of P-ERK5/β-actin, ERK5/β-actin, P-AKT/AKT, and P-FoxO3a/FoxO3a (B-E). n=3, **p*<0.05; ***p*<0.01; ****p*<0.001.

Figure.6

Fig. 6. LY294002 inhibits AKT-FoxO3a signalling, but has no effect on the activation of ERK5 by FSS in MC3T3-E1 cells. The cells treated with LY294002 were exposed to FSS (12 dyn/cm²) or static conditions for 1 h. Cell lysates were analyzed by western blotting (A). Quantitative analysis of the ratio of P-ERK5/ERK5, P-AKT/AKT, and P-FoxO3a/FoxO3a (B-D). n=3, **p*<0.05; ***p*<0.01; ****p*<0.001.

Figure.7

Fig.7. The nucleus translocation of P-ERK5 and FoxO3a. Cells were seeded on cover glasses in a 100 mm sterilized dish, and then were exposed to FSS for 1 h with or without the treatment of ERK5-siRNA or LY294002. P-ERK5 and FoxO3a nuclear translocation (green fluorescence) and nuclei location (blue fluorescence) were visualized using immunofluorescence microscopy. Scale bar = 10 μ m.

Figure.8

Fig. 8. ERK5-siRNA increases MC3T3-E1 cells apoptosis via up-regulation of FasL and Bim. Western blotting for FasL, Bim, and β -actin levels after the treatment with TNF- α for 24 h in cells transfected with ERK5-siRNA or vector control (A). Quantitative analysis of the expression of FasL and Bim after treatment with TNF- α and/or ERK5-siRNA in MC3T3-E1 cells (B, C). $n=3$, * p <0.05; ** p <0.01; *** p <0.001.

Figure.9

Fig. 9. ERK5 activity is necessary for FSS-mediated down-regulation of FasL and Bim in MC3T3-E1 cells. The cells were transfected with ERK5-siRNA, exposed to FSS (12 dyn/cm²) or left static for 1 h, and then stimulated with TNF- α for 24 h. Cell lysates were prepared and SDS-PAGE was performed followed by western blotting (A). Quantitative analysis of the expression of FasL and Bim after treatment with TNF- α , FSS, and/or ERK5-siRNA in MC3T3-E1 cells (B, C). n=3, * p <0.05; ** p <0.01; *** p <0.001.

Figure.10

Fig. 10. The role of ERK5-AKT-FoxO3a-Bim/FasL signaling pathways on TNF- α -induced cell apoptosis. MC3T3-E1 cells were exposed to FSS for 1 h, or treated with TNF- α for 24 h. The protein levels of P-ERK5, ERK5, P-AKT, AKT, FoxO3a, P-FoxO3a, and β -actin were detected by western blotting (A, C-E). The effect of transfection of ERK5-siRNA or treatment with LY294002 in MC3T3-E1 cells. The expression of FasL, Bim, and β -actin was determined (B, F). $n=3$, $*p<0.05$; $**p<0.01$; $***p<0.001$.

Figure.11

Fig. 11. Fluid shear stress inhibits activity of caspase-3 induced by TNF- α via ERK5-AKT-FoxO3a-Bim/FasL signaling pathways. Effect of ERK5 silencing, TNF- α , and FSS on the activity of caspase-3 (A). Effect of ERK5-siRNA, LY294002, TNF- α , and FSS on the activity of caspase-3 (B). MC3T3-E1 cells were transfected with ERK5-siRNA or treated with LY294002, exposed to FSS for 1 h, and then treated with TNF- α for 24 h. The absorbance of caspase-3 was detected by a spectrometer. n=3, * p <0.05; ** p <0.01; *** p <0.001.