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Fluid Shear Stress Suppresses Osteoclast Differentiation in RAW264.7 Cells through Extracellular Signal-Regulated Kinase 5 (ERK5) Signaling Pathway

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Although extracellular signal-regulated kinase 5 (ERK5) is known to be critical for osteoclast differentiation, there are few studies on how fluid shear stress (FSS) regulates osteoclast differentiation through the ERK5 signaling pathway. We examined the expression of nuclear factor of activated T cells c1 (NFATc1) in RAW264.7 cells and its downstream factors, including cathepsin K (CTSK), tartrate-resistant acid phosphatase (TRAP), matrix metalloproteinases-9 (MMP-9) and their relationship with ERK5.

Material/Methods: RAW264.7 cells were treated with RANKL, XMD8-92 (ERK5 inhibitor), and then loaded onto 12 dyn/cm^2 FSS for 4 days. Endpoints measured were osteoclast differentiation, bone resorption, and TRAP activity. Cell viability was detected by using the Cell Counting Kit-8 (CCK-8) assay. Western blot was used to analyze protein expression of phosphorylated-ERK5 (p-ERK5), NFATc1, CTSK, TRAP, and MMP-9.

Results: FSS inhibited osteoclast differentiation and expression of NFATc1, CTSK, TRAP, and MMP-9; cell viability was not affected. ERK5 expression increased by FSS but not by RANKL, and it was blocked by XMD8-92. Furthermore, FSS suppressed osteoclast differentiation in RAW264.7 cells through ERK5 pathway.

Conclusions: Our findings demonstrated that FSS inhibited osteoclast differentiation in RAW264.7 cells via the ERK5 pathway through reduced NFATc1 expression and its downstream factors MMP-9, CTSK, and TRAP.

MeSH Keywords: Cell Differentiation • Mitogen-Activated Protein Kinase 7 • NFATC Transcription Factors • Osteoclasts

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Background

Bone is a highly active tissue with 10% remodeling each year and repairs its own micro-damages through continuous reconstruction, maintaining structural, loading and calcium homeostasis [1]. The homeostasis of the skeletal system is maintained by bone remodeling mediated by osteoclasts and osteoblasts. Pathological enhancement of progressive bone resorption by osteoclasts can break this balance leading to many diseases, including osteoporosis, malignant tumors, rheumatoid arthritis and Paget's disease [2]. Osteoclasts are a kind of multinucleated cells derived from the monocyte/macrophage cell lineage [3], and RANKL, which is a tumor necrosis factor (TNF) family member, plays an essential role in osteoclast differentiation [4,5]. Macrophage colony stimulating factor (M-CSF), a survival factor for osteoclast precursor cells, induces the RANKL receptor RANK and activates the RANK/RANKL/OPG signaling pathway-a pivotal regulatory axis in the maturation of osteoclasts [6]. Some studies have shown that stimulation by RANKL alone is sufficient to cause differentiation of RAW264.7 cells into osteoclasts, rendering it an appropriate *in vitro* model of osteoclast differentiation [7–9]. To eliminate the impact of the factors secreted by serum stimulation on the results, we chose RAW264.7 cells and starved cells for 5 hours before studying the effects of fluid shear stress (FSS) and RANKL on protein expression.

Mechanical load is essential in the development of bone tissue, and various mechanical stresses dynamically regulate the balance between osteoblast production and function [10]. Previous studies have shown that FSS can promote osteoblast proliferation [11–15] as well as osteoclast differentiation through multiple pathways [16–19]. Application of FSS promoted osteoclastogenesis from bone marrow cells isolated from the lumbar spine of mice [20]. Oest et al. observed that super physiological oscillatory fluid shear induced upregulation of osteoclast activity and formation of mineral absorption pits without influencing the number of RAW264.7 cells [21]. To explore the effects of different types of FSS on osteoclast differentiation, our laboratory used cyclic FSS to observe its impact on RANKL-induced RAW264.7 cells differentiating into osteoclast.

Members of the classical mitogen-activated protein kinase (MAPK) family such as extracellular signal-regulated kinase 5 (ERK5), p38, ERK1/2, and JNK1/2/3 [22,23] play crucial roles in osteoclast differentiation [24,25]. Nuclear factor of activated T cells c1 (NFATc1) mainly localized in the cytoplasm and translocates to the nucleus after activation [26,27]. It promotes the transcription of metalloproteinases-9 (MMP-9), tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK), effectors of precursor cell fusion, and osteoclastogenesis and considered as markers of osteoclast differentiation [28]. Genetic ablation of ERK5 in mice increased osteoclast activity in parallel with the expression of osteoclastogenesis markers, including NFATc1 and CTSK [24]. However, the relationship between FSS and ERK5 pathway in osteoclasts remains understudied. We evaluated the effect of FSS on osteoclast differentiation by NFATc1 and its downstream effectors MMP, TRAP, and CTSK via the ERK5 pathway. This study aims to improve the molecular pathway theory of osteoclasts and to combine fluid mechanics with osteoclast differentiation and functional studies. As an emerging research field of osteoclasts, this plays an essential role in understanding the differentiation and function of osteoclasts *in vitro* and *in vivo* and lay the foundation for our future research.

Material and Methods

Antibodies

Antibodies included: ERK5 (1: 1500 for western blot), MMP-9 (1: 1500 for western blot, Abcam, USA); CTSK (1: 1500 for western blot) and TRAP (1: 1500 for western blot) and RANKL (Proteintech, USA); p-ERK5 Thr218/tyr220 (1: 1500 for western blot, Cell Signaling Technology, USA); NFATc1 (1: 1500 for western blot, Santa Cruz, CA, USA); β-actin (1: 1000 for western blot, ZSGB-BIO, China); HRP-conjugated Affinipure goat anti-mouse IgG (1: 3000), peroxidase-conjugated goat anti-rabbit IgG (1: 3000, Proteintech, USA), and XMD8-92 (Selleck, USA).

Osteoclastogenesis

RAW264.7 cells were cultured in alpha-minimum essential media (α-MEM, ThermoScientific, USA) containing 10% fetal bovine serum (FBS; Biological Industries, USA) and 1% penicillin/streptomycin, added to 50 ng/mL RANKL for 4 days. The media refreshed every 2 days.

Mechanical loading for FSS

Cyclic FSS was applied to cells in a parallel plate flow chamber using a closed flow loop [29], as described previously [11–15]. Cells were seeded onto 20×50 mm coverslips (3.0×10^5/cover slip) and placed in the chambers following a 5-hour incubation in serum-free medium and then exposed to 12 dyn/cm^2 FSS laminar flow based on the previous reports [29]. In time gradient related experiments, cells were loaded FSS for 0, 15, 30, 45, 60, or 90 minutes. In addition to the time gradient experiment, cells were loaded FSS for 30 minutes. Unless the cell culture was to be continued, experimentally treated cells were extracted for protein for related analyses immediately (no more than 5 hours).

Cell viability assay

Cell Counting Kit-8 (CCK-8; Dojindo, Japan) was used to measure cell viability according to the manual. Floating cells were

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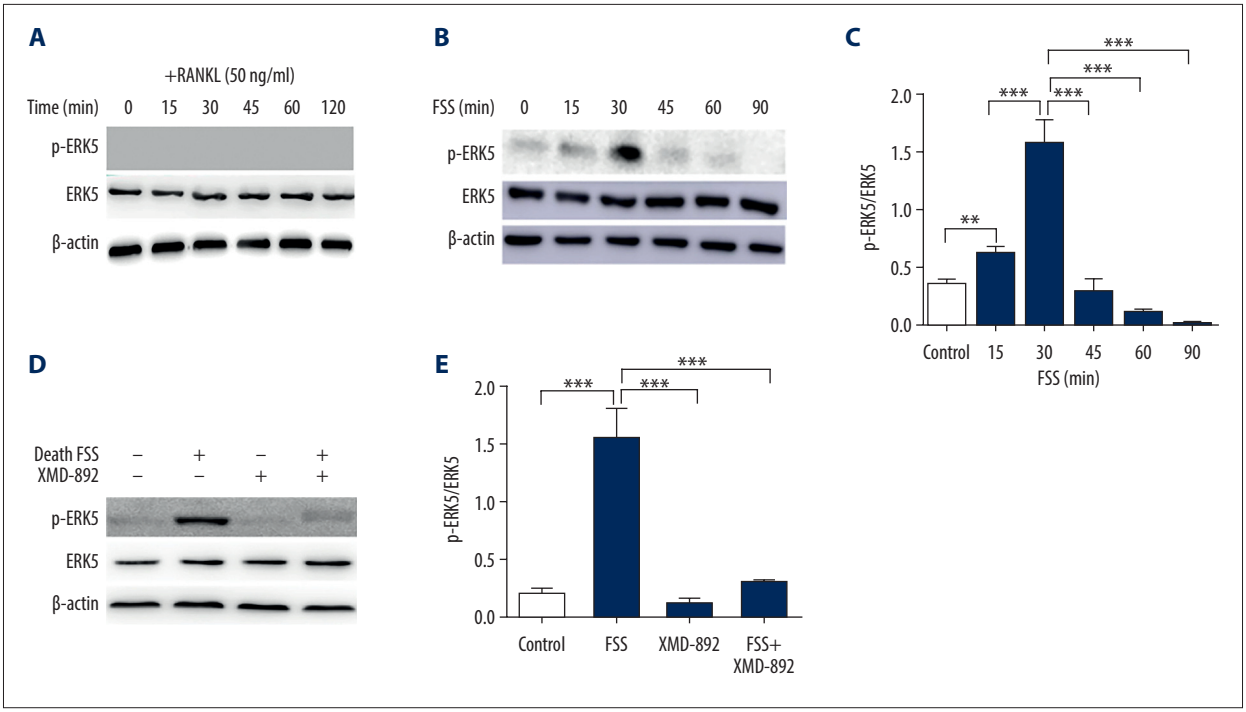

Figure 1. ERK5 expression is increased by FSS but not by RANKL. (**A**) RANKL cannot promote ERK5 phosphorylation. After incubation in serum free medium for 5 hours, cells were added in RANKL (50 ng/mL) for different time. Subsequently, extracted protein and detected by western blot, then measured the protein expression. (**B–E**) FSS promotes ERK5 activation, and XMD8-92 can inhibit it. Before loading FSS or adding XMD8-92 then loading FSS, RAW264.7 cells were cultured in serum free medium for 5 hours, then extracted protein and detected by western blot and measured the protein expression. Data are shown as mean±standard deviations; n=3, * P<0.05, ** P<0.01, *** P<0.001. ERK5 – extracellular signal-regulated kinase 5; FSS – fluid shear stress.

removed at the specific time points, and then we added 100 uL medium containing 1/10 CCK-8 reagent each well and incubated for 4 hours. Viability was assessed after measuring absorbance at 450 nm.

Osteoclastogenesis assay, TRAP staining and TRAP activity assay

To detect the formation of mature osteoclasts (nuclei ≥3) after 4 days of induction, cells were stained by TRAP (Sigma-Aldrich, USA) according to the instruction manual. TRAP-positive cells were counted under an optical microscope. The activity of TRAP is not inhibited by tartaric acid, while other acid phosphatases (ACPs) are inhibited by tartaric acid. We purchased this kit in Nanjing Jiancheng Bioengineering Institute, using this principle and according to instructions, the activity of TRAP can be detected. TRAP enzyme catalyzes the hydrolysis of the substrate to produce free phenol; the phenol reacts with the diazonium salt to synthesize an azo compound, and the activity of the enzyme is calculated by a colorimetric method at 530 nm.

Resorption pit assay

Bovine bone slices were procured from the Lan Zhou 940 Hospital, Gansu, China, and plated in 6-well plates. RAW264.7 cells were inoculated onto bovine bone slices (1.0×10^4/slices) and loaded FSS for 30 minutes every 2 days with or without the addition of RANKL. After 7 days of induction, bone slices were washed thrice in phosphate-buffered saline (PBS), fixed in 10% paraformaldehyde for 15 minutes, dehydrated in a gradient of ethanol, and dyed with toluidine blue for 15 minutes. Finally, slices were washed and allowed to dry naturally. The area of resorbed pits in each slice was measured and analyzed using ImageJ software.

Western blots

After loaded FSS with or without XMD8-92 and RANKL pre-incubation, the cells washed in PBS 3 times, and lysed in radioimmunoprecipitation assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMSF) (100: 1) on ice. Samples were centrifuged at 4°C at 14 000 g for 15 minutes, mixed with loading buffer and boiled for 5 minutes. Proteins were separated on 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel

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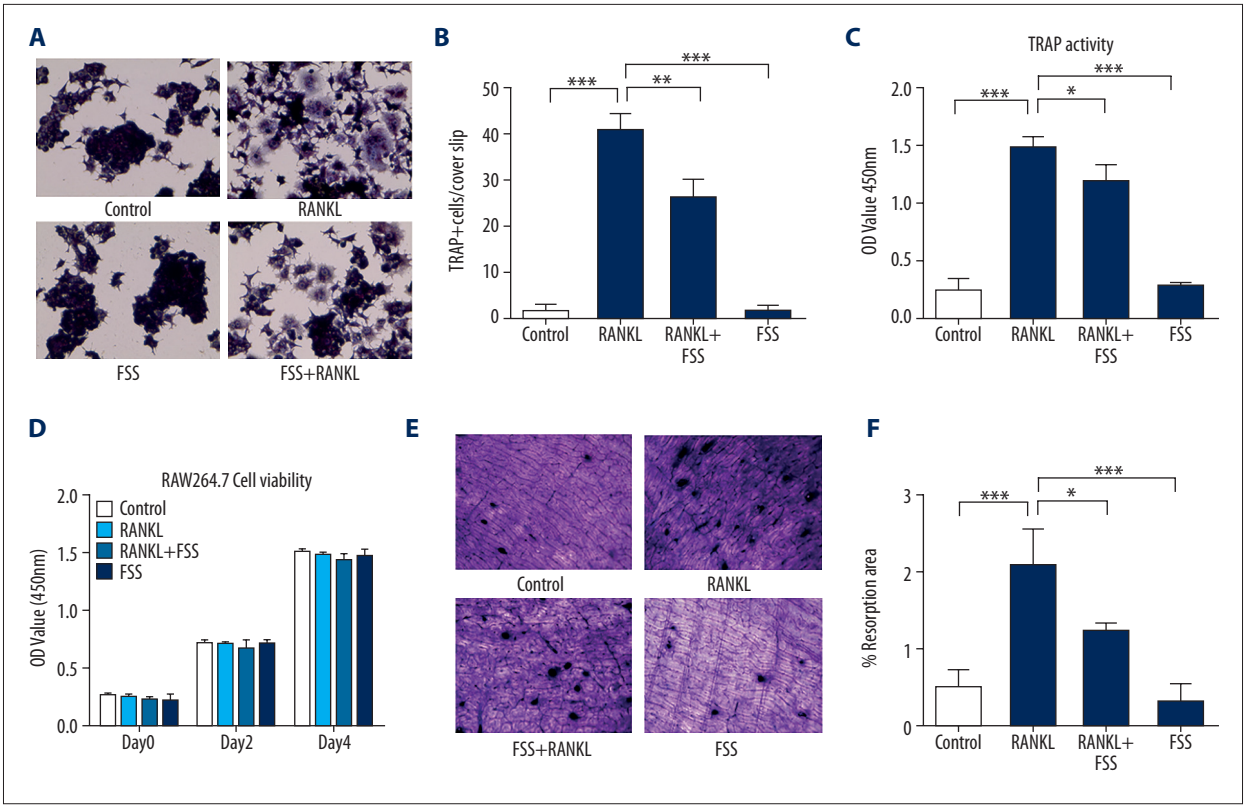

Figure 2. FSS inhibited osteoclastic differentiation without changing the cell viability. (**A, B**) RAW264.7 cells were loaded FSS (12 dyn/cm^2, 30 min/day) with or without RANKL for 4 days, then stained for TRAP and counted TRAP-positive multinuclear cells by ImageJ, magnification 40×. (**C, D**) Measured cell viability at 450 nm and TRAP activity at 540 nm. (**E, F**) Osteoclasts bone resorption capacity was measured by analyzing resorption areas using ImageJ, magnification 40×. Data are shown as mean±standard deviation; n=3,* P<0.05, ** P<0.01, *** P<0.001. FSS – fluid shear stress; TRAP – tartrate-resistant acid phosphatase.

electrophoresis) and transferred onto PVDF (polyvinylidene difluoride) membranes. After blocking in bovine serum albumin (BSA) in nonfat milk (1: 5) for 1.5 hours, the membranes were incubated with appropriate primary antibodies overnight at 4°C, followed by incubation with respective secondary antibodies for 1.5 hours. The ImageQuant LAS 500 system captured the immunoreaction. Results were analyzed and quantified using Image-Pro Plus 6.0 software.

Statistical analysis

All experiments were repeated at least 3 times, and all data are expressed as mean±standard deviation (SD). One-way ANOVA and Student's *t*-test analyzed data, *P*-value <0.05 was considered statistically significant. SPSS 17.0 was used to analyze statistical data.

Results

ERK5 expression was increased by FSS but not by RANKL

RANKL did not cause intracellular activation of ERK5 in RAW264.7 cells at any time points tested (Figure 1A). However, exposure to 12 dyn/cm^2 FSS resulted in phosphorylation of ERK5, which peaked at 30 minutes. Phosphorylation of ERK5 inhibited by XMD8-92 (Figure 1B–1E).

FSS inhibited osteoclast differentiation without affecting the viability

RAW264.7 cells were subjected to FSS (12 dyn/cm^2, 30 min/day) with or without RANKL for 4 days, and TRAP-positive multinucleated cells were significantly reduced in the FSS group compared with the RANKL group (Figure 2A, 2B), and TRAP activity was decreased in the FSS group as well (Figure 2C). Moreover, the FSS did not affect cell viability (Figure 2D). We observed that a minimal number of cells were washed away by FSS, but there was no substantial impact on the experimental results.

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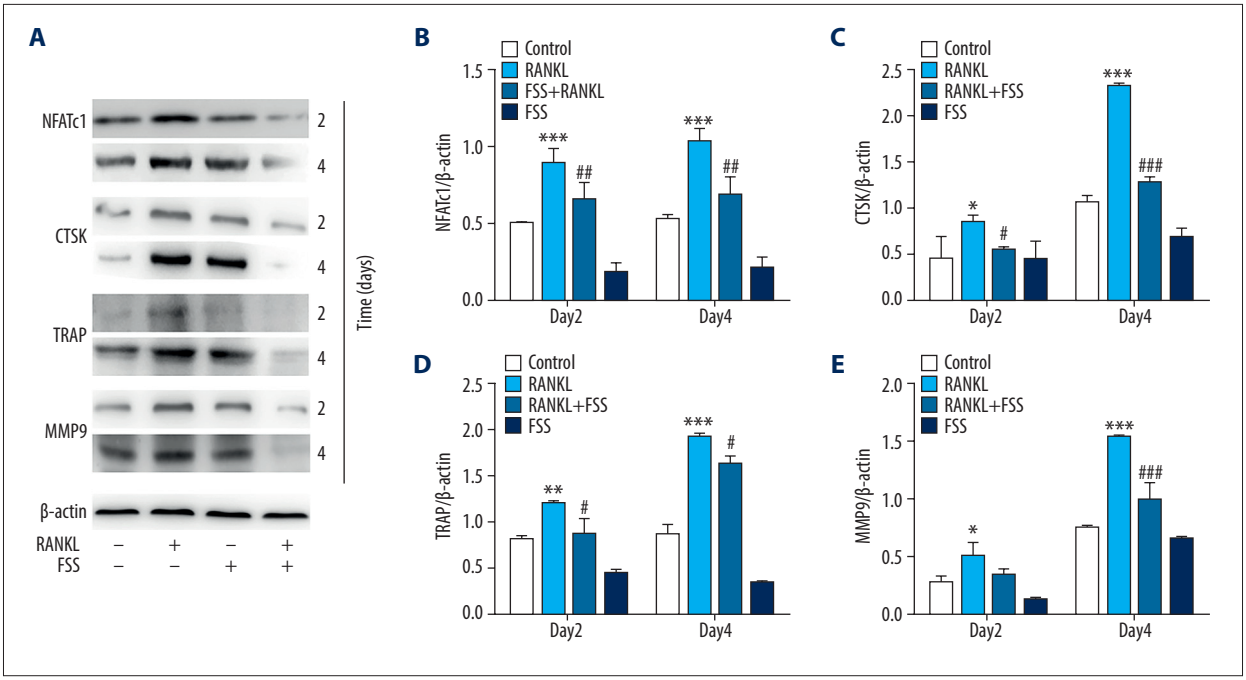

Figure 3. FSS inhibited NFATc1, CTSK, TRAP, and MMP-9 expression. (**A–E**) RAW264.7 cells were loaded FSS (12 dyn/cm^2, 30 min/day) with or without RANKL for 4 days, extracted protein in second day and fourth day, then detected by western blot and measured the protein expression. Data are shown as mean±standard deviation; n=3, * P<0.05, ** P<0.01, *** P<0.001 compared to control, # P<0.05, ## P<0.01, ### P<0.001 compared to RANKL alone. FSS – fluid shear stress; NFATc1 – nuclear factor of activated T cells c1; CTSK – cathepsin K; TRAP – tartrate-resistant acid phosphatase; MMP-9 – matrix metalloproteinases-9.

Furthermore, bone resorption in the FSS group was lower than the RANKL group (Figure 2E, 2F).

FSS inhibited NFATc1, CTSK, TRAP and MMP-9 expression

To examine the effect of FSS on osteoclast-related protein expression, under the condition of changing RANKL every 2 days and loading FSS for 30 minutes per day, expression of NFATc1, CTSK, TRAP, and MMP-9 were reduced in the FSS-treated group (Figure 3A–3E). The results proved that FSS could significantly reduce the expression of the NFATc1 and downstream factors, which were activated by RANKL.

ERK5 mediated NFATc1 activation in response to FSS

To investigate the effect of FSS on ERK5 and NFATC1 expression, we designed a study and found that XMD8-92 inhibited ERK5 activation in all the subgroups (Figure 4A, 4B). Addition of XMD8-92 significantly reversed the inhibitory effect of FSS on NFATc1 activation by RANKL (Figure 4A, 4C) and increased the NFATc1 and osteoclast differentiation-specific proteins CTSK, MMP-9, and TRAP expression (Figure 4D–4G). Hence, these results indicated that FSS inhibited RANKL-induced osteoclast differentiation and function by activating the ERK5 pathway (Figure 5).

Meanwhile, we found an increase in NFATc1 as well as CTSK, TRAP, and MMP-9 expression in the FSS+XMD8-92 group compared with the FSS group. The expression of CTSK, MMP-9, and TRAP in the FSS+XMD8-92 group was similar to that of the RANKL-FSS group, indicating that FSS may promote osteoclast differentiation through pathways other than the ERK5 pathway. Therefore, FSS can promote activation of ERK5, which will suppress RANKL-activated NFATc1 and downstream proteins, including CTSK, TRAP and MMP-9. Inhibition of ERK5, on the other hand, will allow RANKL-mediated activation of NFATc1.

Discussion

In recent years, our laboratory has demonstrated the role of ERK5 signaling pathways in FSS induced proliferation of osteoblast [11,12,14,15,30], but there is a scarcity of reports on osteoclasts. Amano et al. observed that inhibition of the MEK5/ERK5 pathway by the inhibitors blocked the differentiation of RAW264.7D cells and M-CSF-dependent bone marrow macrophages into osteoclasts [25]. However, Loveridg et al. found that inhibited ERK5 pathway could increase osteoclast numbers in RANKL-stimulated primary bone marrow-derived macrophage, and reduction of ERK5 expression increased osteoclast numbers and expression of Rank, Cathepsin K and

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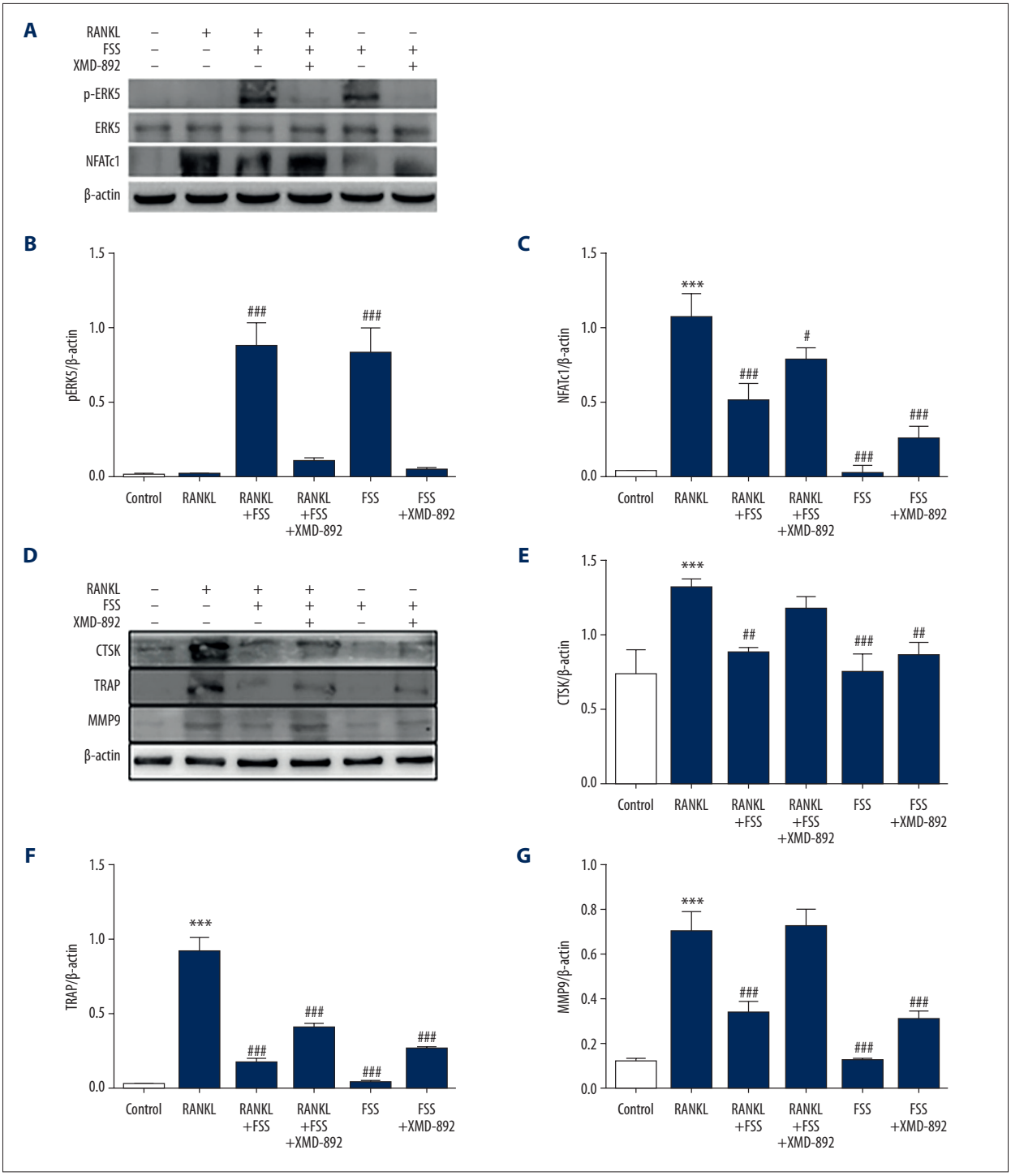

Figure 4. ERK5 mediates NFATc1 activation in response to FSS. (**A**) Before loaded FSS (12 dyn/cm^2, 30 min/day) with or without RANKL(50 ng/mL, 1 hour), RAW264.7 cells were added XMD8-92 or not and then extracted protein and detected by western blot. (**B, C**) Quantified the expression of pERK5 and ERK5, NFATc1 and β-actin. (**D**) RAW264.7 cells were loaded FSS (12 dyn/cm^2, 30 min/day) with or without pre-incubation of RANKL(50 ng/mL, 1 hour), and inhibitor group added in XMD8-92, and then extracted protein and detected by western blot. (**E–G**) Quantified the results of CTSK/β-actin, MMP-9/β-actin, and TRAP/β-actin separately. Data are shown as mean±standard deviation; n=3, * P<0.05, ** P<0.01, *** P<0.001 compared to control, $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, $^{\#\#\#}$ P<0.001 compared to RANKL alone. ERK5 – extracellular signal-regulated kinase 5; NFATc1 – nuclear factor of activated T cells c1; FSS – fluid shear stress; CTSK – cathepsin K; MMP-9 – matrix metalloproteinases-9.

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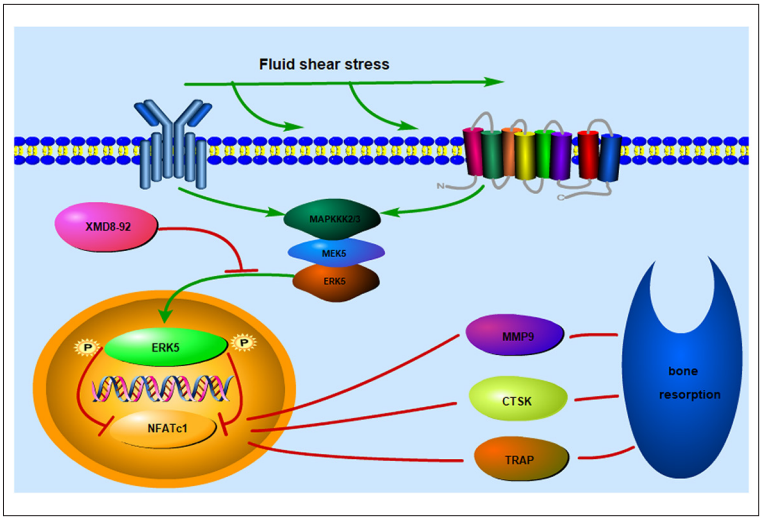

Figure 5. Schematic diagram of FSS regulating osteoclasts bone resorption function via ERK5 signaling pathway (green arrows are positively adjusted; red arrows are negatively adjusted; red lines are decreased due to inhibition of upstream proteins). FSS – fluid shear stress; ERK5 – extracellular signal-regulated kinase 5.

NFATc1 [24]. The aforementioned results are inconclusive about the role of the ERK5 pathway in osteoclast differentiation. Previous studies have indicated that FSS plays an essential role in osteoclast differentiation [16–19]. Therefore, we loaded FSS *in vitro* and observed the effect of FSS on osteoclast differentiation through the ERK5 pathway, which helps to further improve the study of the relationship between ERK5 and osteoclast. We explored the impact of FSS on osteoclast differentiation and function via the ERK5 pathway, and we demonstrated that FSS enhanced phosphorylation of ERK5 in RAW264.7 cells in a time-dependent manner while inhibiting RANKL-dependent osteoclast differentiation, including decreased osteoclastogenesis and osteoclast differentiation-specific proteins expression.

We found that 12 dyn/cm^2 FSS for 30 minutes significantly increased the phosphorylation of ERK5 in RAW264.7 cells. On the other hand, RANKL did not cause phosphorylation of ERK5, and XMD8-92 inhibited the FSS induced activation of ERK5 in RAW264.7 cells. In this study, we conducted new observations that further explain the relationship between FSS and differentiation of osteoclasts.

FSS loading did not affect cell proliferation or death, suggesting that FSS was not cytotoxic to RAW264.7 cells during RANKL-induced osteoclastogenesis. Increased TRAP expression is a hallmark of osteoclast differentiation [31]. Our observation of decreased staining for TRAP-positive cells and the number of bone resorption pits indicated that FSS induced inhibition of RANKL-dependent osteoclast differentiation was a biological rather than a cytotoxic response.

RANKL is indispensable to the process of osteoclastogenesis [32]. Previous reports from our laboratory have shown that the NFATc1-ERK5 pathway regulates proliferation of osteoblasts [33]. NFATc1, a major osteoclast-specific transcription factor, is required for pre-osteoclast differentiation [34,35], and acts by upregulating the expressions of TRAP, CTSK, and beta3 integrin [36]. FSS was shown to inhibit gene expression of TRAP and CTSK in osteoclasts [37]. Mature osteoclasts have poor adhesion ability, and it is challenging to culture mature osteoclasts and inoculate them into bone slices for FSS studies. Therefore, we have changed our mind when verifying the bone resorption capacity of osteoclasts under FSS. Osteoclasts secrete H+ and proteolytic enzymes through the osteoclast ruffled border to the absorption chamber (e.g., CTSK, MMP) that dissolve minerals and degrade bone matrix [38]. CTSK, a selective and highly expressed in osteoclasts, can degrade type I collagen in the bone matrix. *In vitro* and *in vivo* studies have proved that inhibiting CTSK activity is a feasible way to treat osteoporosis [39,40]. Considering the direct regulation of CTSK by osteoclast differentiation-specific protein NFATc1 [41], we found that FSS inhibited the osteoclasts differentiation by decreasing the NFATc1 along with the expression of CTSK, MMP-9 and TRAP expression. Therefore, FSS can inhibit the osteoclasts differentiation and bone resorption not by toxic effects, but by inhibiting NFATc1 and downstream factors expression. We found that in FSS or FSS-RANKL group, ERK5 was significantly activated in parallel with decreased expression of NFATc1. However, inhibiting ERK5 by using a specific inhibitor XMD8-92 rescued the expression of NFATc1, TRAP, CTSK, and MMP-9, which proved that FSS could inhibit the expression of RANKL-activated osteoclast-related proteins via activation of ERK5, while XMD8-92 reverted the inhibition by FSS.

We found that phosphorylation of ERK5 and activation of NFATc1 showed a significant negative correlation effect. However, after the addition of XMD8-92 inhibited the phosphorylation of ERK5, NFATc1, and TRAP expression were significantly decreased than that of the RANKL group, indicating that inhibition of ERK5 activation does not entirely offset the inhibition of FSS on these 2 proteins expression, suggesting

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that other unknown mechanisms are also affecting the expression of these proteins. There was no significant difference in expression of NFATc1 and downstream factors between the FSS group and the control group, but there was a significant increase in protein expression after the addition of the ERK5 inhibitor, indicating that FSS can activate proteins involved in osteoclast differentiation through other signaling pathways, but this effect may be inhibited by phosphorylation of ERK5. Meanwhile, we found that no significant changes in CTSK expression, which may be affected by other mechanism that remains unknown.

Previous studies have proved that FSS promoted osteoclast differentiation and function [20,21]. Our current understanding of the regulatory molecules and pathways between osteoclast biology and fluid mechanics is still in its infancy. We agree that differences in the methods of osteoclast induction and FSS can generate variation in response patterns between different preparations. In this study, a constant 12 dyn/cm^2 cyclic FSS was applied at a constant temperature of 36.5°C. Moreover, in this study cells were starved to eliminate any effects of serum and secreted factors on protein expression. Relevant variables were strictly controlled to improve the accuracy of the experiments. There are still some shortcomings in this research, the major one being the single choice of RAW264.7 cells. Our preliminary experiments showed that FSS studies required cells with strong adhesion. We concur that selecting RAW264.7 cells for FSS studies was appropriate given their adhering ability. Despite these limitations, this model provides new directions for future studies on osteoclast differentiation.

Conclusions

Our study revealed that FSS could inhibit osteoclastogenesis by activating ERK5 in RAW264.7 cells. FSS acts as mechanical stress to inhibit NFATc1 regulation by activating ERK5, which inhibits osteoclast differentiation. Phosphorylated ERK5 inhibits the activation of NFATc1 and its downstream proteins, such as CTSK, MMP-9, and TRAP. Our study revealed a novel finding in the molecular mechanism of osteoclasts and provides new ideas for the future on osteoclast-related research.

Conflicts of interest

None.

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