Fluid shear stress promotes osteoblast proliferation and suppresses mitochondrial-mediated osteoblast apoptosis through the miR-214-3p-ATF4 signaling axis

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Abstract: MicroRNAs (miRNAs) play vital roles in bone metabolism and participate in the

mechanically induced bone alterations. The underlying molecular mechanisms by which fluid shear stress (FSS) regulate the proliferative and apoptotic phenotypic changes of osteoblasts remain elusive. The study aimed to investigate the regulatory effects of FSS on osteoblast proliferative and apoptotic phenotypes and the roles of miR-214-3p-ATF4 (activating transcription factor 4) signaling axis in the mechanomodulation processes. FSS promoted the proliferative activity of osteoblasts and suppressed mitochondrial-mediated osteoblast apoptosis. FSS decreased miR-214-3p expression and increased ATF4 expression in MC3T3-E1 osteoblasts. MiR-214-3p inhibited osteoblast proliferative activity and promoted mitochondrial-mediated osteoblast apoptosis. Overexpression of miR-214-3p attenuated FSS-enhanced osteoblast proliferation and FSS-suppressed mitochondrial-mediated osteoblast apoptosis. We validated that ATF4 acted as a target gene of miR-214-3p. Moreover, miR-214-3p regulated osteoblast proliferation and apoptosis through targeting ATF4. Taken together, our study proved that FSS could suppress mitochondrial-mediated osteoblast apoptosis and promote osteoblast proliferation through the miR-214-3p-ATF4 signaling axis.
Keywords: fluid shear stress, miR-214-3p, activating transcription factor 4, osteoblast, proliferation, apoptosis

Introduction

Although bone forming cells and meniscus fibrochondrocytes could sense and respond to mechanical forces, the underlying molecular mechanisms by which bone forming cells converted extracellular mechanical stimuli to intracellular biological signals were poorly understood [1, 2]. Macroscopic forces such as muscle strain, blood pressure, lymphatic drainage and body movements could generate microcosmic fluid flow inside the lacunar-canalicular system called fluid shear stress (FSS) that was critical to maintain the skeletal health [3, 4], and FSS modulated the phenotypic changes of bone forming cells [1, 5, 6]. Microcosmic forces applied on osteoblasts included FSS, strain, membrane stretching induced by hypoosmotic solution, pressure and extracellular matrix stiffness [7, 8], which could be simulated by different kinds of apparatuses [9-13]. Physiological (beneficial) and superphysiological (harmful) mechanical stimulation both modulated the metabolism of bone tissue and cells and influenced the balance of osteogenesis and osteoclastogenesis [14]. Physiological (beneficial) mechanical forces were vital for osteogenesis and bone formation and promoted fracture healing and osteointegration between the implant and surrounding bone [14-17].

MicroRNAs(miRNAs) are small noncoding RNAs that participated in diverse biological activities such as the differentiation, proliferation, apoptosis and senescence of bone cells and the development and canceration of bone tissue [18, 19]. The homeostasis of endogenous miRNAs was critical to these biological activities of bone forming cells and skeletal development [20-22]. Mechanosensitive miRNAs could sense and respond to mechanical stimulation by influencing their downstream target genes, which modulated bone metabolism through osteogenic and osteoclastogenic signaling pathways [19, 23-32]. MiR-214 belonging to the former family of small, highly conservative noncoding RNA and its downstream targets played roles in the diverse biological activities such as osteogenesis and osteoclastogenesis [18, 19, 27, 33-39]. Immature miR-214 could be cleaved into mature miR-214-3p and miR-214-5p, and the expression levels of miR-214 (including miR-214-3p and miR-214-5p) could significantly influence osteogenesis and bone

formation. Moreover, the dysregulation of miR-214 and its upstream and downstream targets participated in the onset and development of osteonecrosis of femoral head, postmenopausal osteoporosis and disuse osteoporosis [33, 34, 40-49].

This study aimed to investigate the influence of FSS on the miR-214-3p expression and confirm the regulatory effects of FSS on the proliferative and apoptotic phenotypes of MC3T3-E1 osteoblasts. Online publicly available algorithms including TargetScan and miRWalk were used to identify the miR-214-3p binding sites for activating transcription factor 4 (ATF4). We hypothesized that miR-214-3p-ATF4 signaling axis, if present, contributed to the modulatory effects of FSS on the proliferative and apoptotic phenotypes of MC3T3-E1 osteoblasts.

Materials and methods

Cell culture and cell transfection

The α-MEM with 10% FBS was prepared to feed MC3T3-E1 cells. The DMEM with 10% FBS was prepared to feed HEK 293T cells. The incubated environment was 37℃ and 5% CO_2. Lipofectamine 2000 (Invitrogen, USA) was used to transfect MC3T3-E1 cells with miR-214-3p mimic or inhibitor and control groups (RiboBio, China). The transfection concentration of miR-214-3p mimic or control group was 50 nM, the transfection concentration of miR-214-3p inhibitor or control group was 100 nM. Lipofectamine 2000 (Invitrogen, USA) was used to transfect MC3T3-E1cells with ATF4 overexpression vector or siRNA-ATF4 and control groups (GenePharma, China). The transfection concentration of siRNA-ATF4 was 100 nM, and the transfection concentration of pcDNA3.1-ATF4 plasmid vector was 250 ng/μl.

FSS experiment

We applied FSS experiments on MC3T3-E1 cells according to our previous study [50]. MC3T3-E1 osteoblasts were seeded on 20x50 mm cover slips. When cellular density were nearly 80% confluence, MC3T3-E1 osteoblasts were loaded under 12 dyn/cm^2 FSS for 0, 30, 60 or 90 min, or MC3T3-E1 osteoblasts were incubated under 0, 3, 6, 9, 12, 15, 18 dyn/cm^2 for 60min.

Luciferase reporter assay

HEK 293T cells with few endogenous miRNA were chosen to conduct transfection experiments. We purchased the reporter vector that contained the wild-type 3'UTR of murine ATF4 and the reporter vector that contained the mutant 3'UTR of murine ATF4 from GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, USA) was used to co-transfect HEK 293T cells with the 3'UTR reporter vector of ATF4 WT or ATF4 MUT and miR-214-3p mimic. Finally, HEK 293T cells were harvested for the luciferase reporter assay.

qRT-PCR annlysis

The cells were harvested to obtain total RNA by TRIzol extracting method. To obtain the cDNA, we first used reverse transcription kit to reverse the mRNA. For the cDNA synthesis of miRNA, a specific reverse transcription kit was utilized. Then, SYBR fluorescence reagent was utilized to conduct qRT-PCR on a Roche LC96 instrument. GAPDH and U6 were used for normalization. The primers are detailed in Table 1.

Table 1. Sequences of the primers for qRT-PCR

Name	Sequence(5'-3')
mmu-miR-214-3p	ACAGCAGGCACAGACAGGCAGT
mmu-ATF4-F	CCTGAACAGCGAAGTGTTGG
mmu-ATF4-R	TGGAGAACCCATGAGGTTTCAA
mmu-GAPDH-F	TGTGTCCGTCGTGGATCTGA
mmu-GAPDH-R	TTGCTGTTGAAGTCGCAGGAG
U-6-F	CTCGCTTCGGCAGCACA
U-6-R	AACGCTTCACGAATTTGCGT

EdU labeling assay

The proliferative activity of MC3T3-E1 osteoblasts was assessed by using the EdU staining kit (RiboBio, China). First, cells were fed and cultured in α-MEM medium (10%FBS) containing 50 μM EdU working fluid for 4h. 4% paraformaldehyde for fixation (15min) and 0.5% Triton X-100 for permeation (15 min). After that, the cells were probed with working fluid for 30 min. Finally, hoechst 33342 was used to treat the cells for 30 min.

Mitochondrial Membrane Potential Assay

The JC-1 fluorescence probe (Yeasen, China) was used to measure the level of mitochondrial membrane potential (ΔΨm) and detect early apoptosis detection through the analysis of fluorescence microscopic imaging. After experimental treatments, JC-1 probe was used to load MC3T3-E1 cells in the dark environment. After loading with JC-1 at 37 °C for 20 min, JC-1 staining buffer was used to wash the cells three times and the pictures were get from the cells. ImageJ software was used to measure the average fluorescence intensity. A decreased red/green fluorescence intensity means the decreased ratio of the JC-1 aggregate to the monomer, which suggested the loss of ΔΨm and normal mitochondrial activity.

Western blot analysis

MC3T3-E1 cells were harvested by using RIPA buffer. Lysate samples were then centrifuged at 12000 rpm for 15 min, and the supernatants were collected. And the loading buffer was added in the supernatants. Protein samples were loaded and the gel electrophoresis was conducted and protein samples were transferred onto PVDF bands. After blocking with quick blocking buffer, the bands were incubated with primary antibodies including ATF4 (1:1000, Affinity, USA), (PCNA; 1:1000, Abcam, USA), CDK4 (1:2000, Abcam, USA), CDK6 (1:2000, Affinity, USA), Cyclin D1 (1:10,000, Abcam, USA), Bax (1:1000, Affinity, USA), Bim (1:1000, Affinity, USA), cleaved caspase-3 (1:1000, Abcam, USA) and β-actin (1:1000, Abcam, USA) overnight at 4°C. Then, the bands were probed with secondary antibodies (1:1500, Affinity, USA) were applied for 2h. Finally, the protein bands were observed on ECL system (BioRad, USA).

Immunofluorescence

After experimental treatments, 4% paraformaldehyde for fixation and 0.1% Triton X-100 for permeation. Then, 10% normal goat serum was used to block the cells for 60 min, and the cells were probed with primary antibody ATF4 (1:100, Affinity, USA) overnight at 4°C. After treating with green fluorescence secondary antibody (1:300, ProteinTech) and subsequently staining with DAPI. We got the needed pictures through manipulating the fluorescence microscope.

Statistical analysis

All statistical analyses were performed using graphpad prism 8.0.1 software. All data are presented as the mean ± SD of at least three independent experiments. Statistical significance was analyzed using a two-tailed t test or a oneway ANOVA. p-values < 0.05 were considered statistically significant.

Results

FSS decreases the expression level of endogenous miR-214-3p

To explore the effects of FSS on the expression levels of miR-214-3p in MC3T3-E1 cells, FSS was applied on MC3T3-E1 cells and the cDNA of miRNA was obtained for qRT-PCR. After different FSS experiments, the qRT-PCR analysis showed 60 min of 12dyn/cm^2 FSS significantly decreased the expression of miR-214-3p (Fig 1).

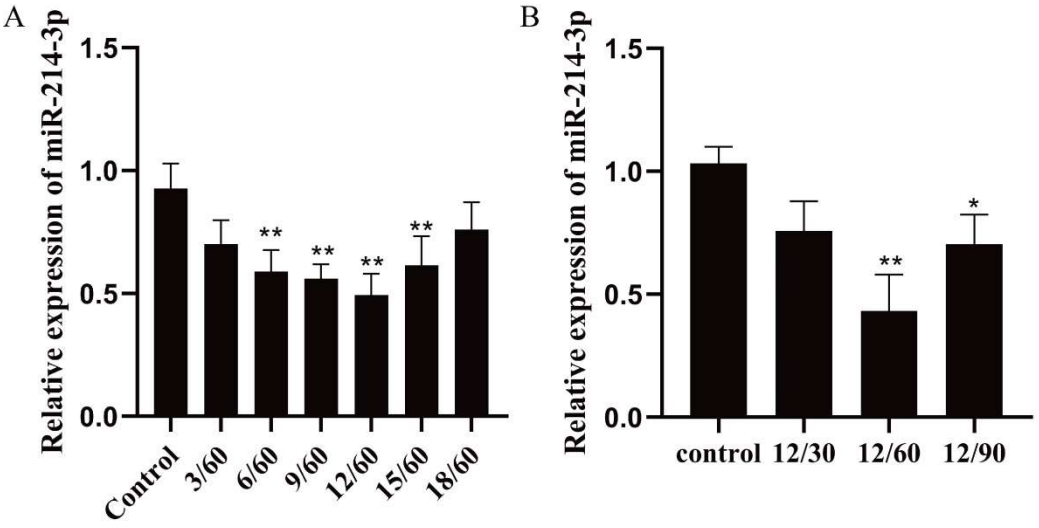

Fig 1. FSS decreases the intracellular content of endogenous miR-214-3p. Detecting intracellualr miR-214-3p expression levels in MC3T3-E1 cells responding to FSS (12 dyn/cm^2) for different times or treated under different scales of FSS (dyn/cm^2) for 60min. Data are shown as the mean ±SD. (*p < 0.05, **p <0.01)

The miR-214-3p-ATF4 signaling axis participated in regulating the proliferative activity of osteoblasts and mitochondrial-mediated osteoblast apoptosis

To investigate the influence of miR-214-3p on the apoptotic and proliferative phenotypes of osteoblasts, we respectively increased and decreased the intracelluar content of miR-214-3p in MC3T3-E1 osteoblasts by transfection with miR-214-3p mimic and miR-214-3p inhibitor. After transfection with miR-214-3p mimic, the protein expression of Bax, Bim and caspase3 was increased and the level of ΔΨm was decreased (Fig 2(A)). Moreover, transfection with miR-214-3p mimic suppressed the protein expression of CDK4, CDK6, PCNA and CyclinD1 and decreased the number of EdU-positive cells (Fig 2(B)). Moreover, inhibition of miR-214-3p had opposite effects in MC3T3-E1 osteoblasts (Fig 2(A) and Fig 2(B)). Taken together, miR-214-3p impaired mitochondrial activities and promoted osteoblast apoptosis, meanwhile, miR-214-3p inhibited

osteoblast proliferation.

To explore the effects of ATF4 on the apoptosis and proliferation of MC3T3-E1 cells, the gene expression of ATF4 was respectively overexpressed and downregulated in MC3T3-E1 osteoblasts by transfection with the overexpression vector (pcDNA 3.1-ATF4) and the RNA interference (siRNA-ATF4). After transfection with the siRNA-ATF4, the protein expression of Bax, Bim and caspase3 was increased and the level of (ΔΨm) was decreased (Fig 2(C)). Moreover, transfection with the siRNA-ATF4 suppressed the protein expression of CDK6, PCNA, CyclinD1 (Fig 2(D)). Moreover, transfection with pcDNA 3.1-ATF4 had opposite effects on the apoptosis and proliferation of MC3T3-E1 cells. These results suggested that ATF4 protected and maintained normal mitochondrial activities and inhibited apoptosis, meanwhile, ATF4 was critical for osteoblast proliferative activity and promoted osteoblast proliferation.

We constructed co-transfection groups to conform whether miR-214-3p influenced the proliferative and apoptotic phenotypes of MC3T3-E1 cells through its target ATF4 or not. Western blot analysis showed that upregulation of ATF4 partially reversed the effects of miR-214-3p-mimic on the protein expression of Bax, Bim and cleaved-caspase3(Fig 2(E)). JC-1 staining assays showed that upregulation of ATF4 attenuated the effects of miR-214-3p-mimic on ΔΨm (Fig 2(E)). Upregulation of ATF4 miR-214-3p partially reverse mimic-induced downregulation of CDK4, CDK6, PCNA and CyclinD1 and attenuated miR-214-3p mimic-induced suppression of osteoblast proliferation (Fig 2(F)). Western blot analysis showed that knock-down of ATF4 partially reversed the effects of miR-214-3p inhibitor on the protein expression of Bax, Bim and cleaved-caspase3 (Fig 2(G)). JC-1 staining assays showed that knock-down of ATF4 attenuated the effects of miR-214-3p inhibitor on ΔΨm (Fig 2(G)). Western blot analysis showed that knock-down of ATF4 partially reversed the effects of miR-214-3p inhibitor on the protein expression of CDK4, CDK6, PCNA and CyclinD1 (Fig 2(H)). EdU staining assays showed that knock-down of ATF4 attenuated the effects of miR-214-3p inhibitor on the proliferative activity of osteoblasts (Fig 2(H)).

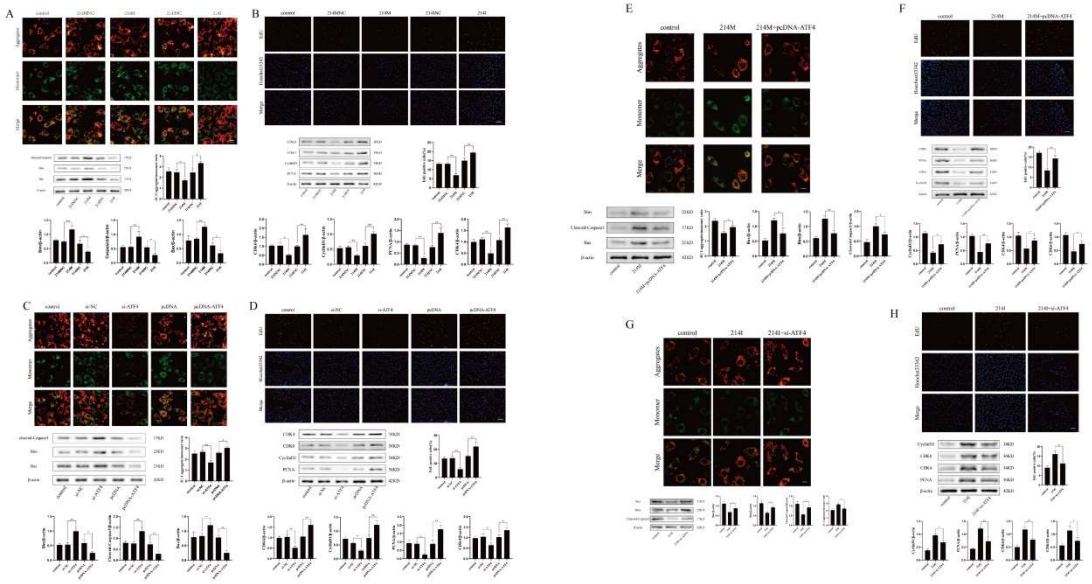

Fig 2. MiR-214-3p compromises the proliferative activity of osteoblasts and promotes mitochondrial-mediated osteoblast apoptosis (A, B). ATF4 enhances the proliferative activity of osteoblasts and suppresses osteoblast apoptosis (C, D). Upregulation of ATF4 partially reverses the regulatory effects of miR-214-3p mimic on the proliferative activity of osteoblasts and

mitochondrial-mediated osteoblast apoptosis (E, F). Knock-down of ATF4 partially reverses miR-214-3p inhibitor-induced suppression of osteoblast apoptosis and promotion of osteoblast proliferation (G, H). Treating MC3T3-E1 cells with miR-214-3p mimic, miR-214-3p inhibitor and negative controls (A, B). Treating MC3T3-E1 cells with pcDNA 3.1-ATF4, siRNA-ATF4 and negative controls. (C, D). Western blot analysis of Bax, Bim and caspase-3 and analysis of ΔΨm by measuring the aggregate(red)/monomer(green) fluorescence intensity ratio in MC3T3-E1 cells (Scale bar = 10 μm (A/C/E/G)). Western blot analysis of CDK4, CDK6, PCNA and CyclinD1 and EdU staining of MC3T3-E1 cells (Scale bar = 50 μm(B/D/F/H)). Data are shown as the mean ±SD. (*$p < 0.05$, **$p < 0.01$)

FSS promoted osteoblast proliferation and suppressed mitochondrial-mediated osteoblast apoptosis through the miR-214-3p-ATF4 signaling axis

Our former FSS-related studies have indicated that FSS could enhance the proliferative activity of osteoblasts and suppress osteoblast apoptosis [50-54]. We investigated whether miR-214-3p participated in the modulatory effects of FSS on the proliferative and apoptotic phenotypes of MC3T3-E1 osteoblasts. Therefore, MC3T3-E1 osteoblasts were treated with miR-214-3p mimic and its negative control before loading 12 dyn/cm^2 FSS for 1 h. Overexpression of miR-214-3p partially attenuated FSS-induced upregulation of CDK4, CDK6, PCNA and CyclinD1 (Fig 3(A)) and FSS-induced downregulation of Bax, Bim and cleaved-caspase3 (Fig 3(B)). Moreover, upregulation of miR-214-3p partially attenuated FSS-induced increased number of EdU-positive cells (Fig 3(A)) and FSS-induced increased level of ΔΨm (Fig 3(B)). These results indicated that transfection with miR-214-3p mimic partially reversed FSS-enhanced proliferative activity of osteoblasts and FSS-suppressed mitochondrial-mediated osteoblast apoptosis.

To unveil the underlying mechanism in the modulatory effects of miR-214-3p on osteoblast proliferation and osteoblast apoptosis, we used TargetScan and miRWalk to predict the potential target of miR-214-3p. Based on these analysis, we chosen ATF4, an important actor in osteoblast proliferation, differentiation and apoptosis [55-57], as the potential target gene of miR-214-3p to verify their relationship. The results of dual-luciferase reporter assays showed that miR-214-3p mimic decreased ATF4 3'UTR WT luciferase reporter activity, but not ATF4 3'UTR MUT reporter activity (Fig 3(D)). And western blot analysis, qRT-PCR analysis and immunofluorescence experimental results all proven that miR-214-3p mimic and inhibitor respectively downregulated and upregulated ATF4 expression (Fig 3(D)). Moreover, FSS promoted the mRNA and protein expression of ATF4 in MC3T3-E1 osteoblasts compared with the control group (Fig 3(C)). Transfection with miR-214-3p mimic attenuated FSS-induced upregulation of ATF4 (Fig 3(C)).

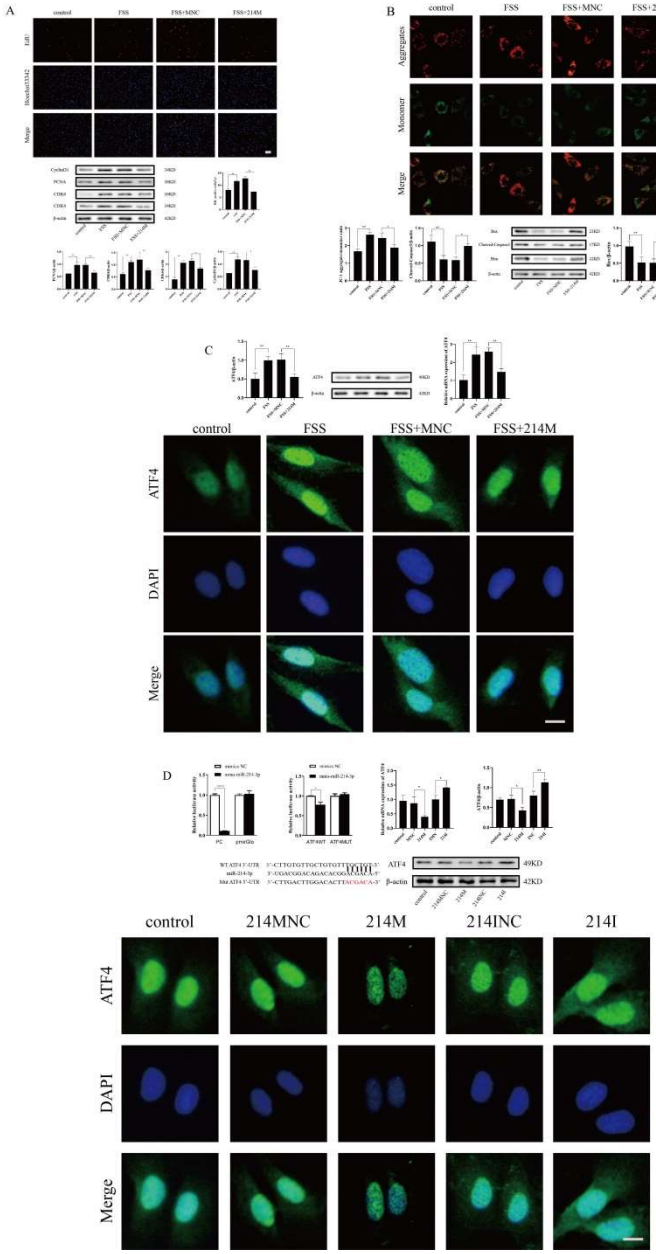

Fig 3. Transfection with miR-214-3p mimic partially reverses FSS-enhanced osteoblast proliferation and FSS-induced repression of osteoblast apoptosis (A, B). Upregulation of miR-214-3p attenuates FSS-induced upregulation of ATF4. ATF4 acts as a target of miR-214-3p (C). ATF4 acts as a target of miR-214-3p (D). Western blot analysis of Bax, Bim and caspase-3 and analysis of ΔΨm by measuring the aggregate(red)/monomer(green) fluorescence intensity ratio in MC3T3-E1 cells (Scale bar = 10 μm(B)). Western blot analysis of CDK4, CDK6, PCNA and CyclinD1 and EdU staining of MC3T3-E1 cells (Scale bar = 50 μm(A)). Immunofluorescence of ATF4 (Scale bar = 10μm (C/D)). Data are shown as the mean ±SD. (*p < 0.05, **p <0.01)

Discussion

Mechanosensitive miRNAs could sense and respond to mechanical stimulation by influencing their downstream target genes, which modulated bone metabolism through osteogenic and

osteoclastogenic signaling pathways [19, 23-32]. Just as shown in Fig 4, the present study demonstrated that: First, FSS downregulated miR-214-3p expression, enhanced the proliferative activity of osteoblasts and suppressed mitochondrial-mediated osteoblast apoptosis. Second, miR-214-3p compromised the proliferative activity of osteoblasts and promoted osteoblast apoptosis. Upregulation of miR-214-3p attenuated the regulatory effects of FSS on the proliferative activity of osteoblasts and mitochondrial-mediated osteoblast apoptosis. Third, ATF4 acted as a target of miR-214-3p and miR-214-3p participated in FSS-induced modulation of osteoblast through targeting ATF4.

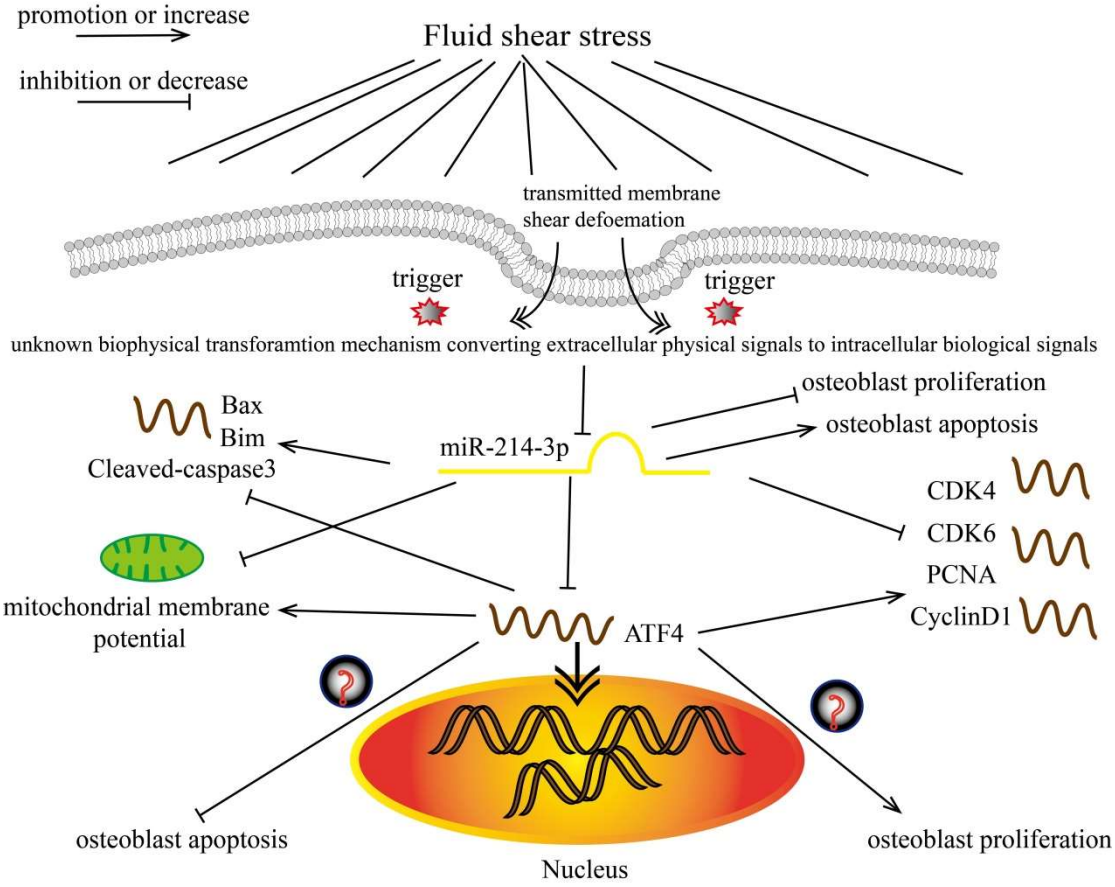

Fig 4. The molecular mechanisms mediated by the miR-214-3p-ATF4 signaling axis and phenotypic changes in osteoblasts responding to FSS.

Physiological (beneficial) mechanical stimulation such as FSS resulted in increased osteogenesis and decreased osteoclastogenesis [14]. FSS maintained the normal metabolism of bone forming cells [14]. Our previous studies confirmed FSS enhanced the proliferative activity of osteoblasts and attenuated TNF-α-induced osteoblast apoptosis [50, 51]. As an important actor in musculoskeletal system [24, 58], the disbalance of endogenous miR-214-3p was correlated with osteoblast proliferation, osteogenic differentiation and osteonecrosis and the mechanosensitivity of miR-214-3p is involved with mechanically induced bone alterations [24, 34, 59]. MiR-214-3p inhibited osteoblast activity and suppressed bone formation [19]. The expression of miR-214-3p was decreased in exercised mice and in osteoblasts exposed to mechanical strain and transfection with miR-214-3p mimic attenuated mechanical strain-enhanced osteogenesis in osteoblasts [27]. Knock-down or overexpression of some mechanosensitive miRNAs could attenuate or deteriorate physiopathological phenotypic changes of osteoblasts responding to FSS [60, 61], which proven

mechanical stimulation could modulate bone metabolism through influencing the expression of mechanosensitive miRNAs. After treating with 12dyn/cm2 FSS for 1 h, the content of endogenous miR-214-3p was decreased in osteoblasts. Moreover, we found that upregulation of miR-214-3p compromised the proliferative activity of osteoblast and deteriorated programmed osteoblast dearth. And upregulation of miR-214-3p extended the proliferative period of osteoblasts and impaired the homeostasis of ΔΨm. The maintenance of ΔΨm is vital for mitochondrial health and the decreased (impaired) ΔΨm could trigger mitochondrial-mediated apoptosis. Knock-down of miR-214-3p exerted opposite effects. Based on our previous studies, therefore, we hypothesized that the altered expression levels of mechanosensitive miR-214-3p may participate in proliferative and apoptotic phenotypic changes of osteoblasts responding to FSS. Based on our experimental results, we proven that FSS enhanced the proliferative activity of osteoblasts and suppressed mitochondrial-mediated osteoblast apoptosis through decreasing miR-214-3p expression.

Acting as important post-transcriptional regulators, intracellular miRNAs can directly target sites of mRNAs of numerous signals to influence the biological activities of signaling pathways [62]. MiR-214-3p directly targeted ATF4 to compromise the proliferative activity of osteoblasts [19]. We validated that ATF4 acted as a target gene of miR-214-3p. Therefore, it was valuable to investigate the modulatory effects of ATF4 on the proliferative activity of osteoblasts and mitochondrial-mediated osteoblast apoptosis. The study confirmed that ATF4 was vital for the anabolic actions of PTH on the skeleton [56]. Knock-down of ATF4 significantly impaired the proliferative activity of primary bone marrow stromal cells and calvarial osteoblasts [55]. Consistent to these reports, we confirmed that knock-down of ATF4 suppressed osteoblast proliferative activity and induced mitochondrial dysfunction in osteoblasts, and overexpression of ATF4 had opposite effects. The modulatory effects of miR-214-3p on the proliferative activity of osteoblasts and mitochondrial-mediated osteoblast apoptosis probably be achieved through targeting ATF4. Therefore, we constructed co-transfection groups to verify if miR-214-3p exerted above effects through targeting ATF4. Upregulation of miR-214-3p impaired the proliferative activity of osteoblasts and promoted osteoblast apoptosis through decreasing the expression of ATF4, downregulation of miR-214-3p enhanced the proliferative activity of osteoblasts and suppressed mitochondrial-mediated osteoblast apoptosis through increasing the expression of ATF4, which suggested the miR-214-3p-ATF4 signaling axis participated in regulating the proliferative and apoptotic phenotypes of osteoblasts. Our former experimental results have proven that FSS enhanced the proliferative activity of osteoblasts and repressed mitochondrial-mediated osteoblast apoptosis through downregulating miR-214-3p expression. Taken together, we hypothesized that FSS could exerted above effects through miR-214-3p targeting ATF4. Considering that transfection with miR-214-3p mimic attenuated FSS-induced downregulation of ATF4, we concluded that FSS enhanced the proliferative activity of osteoblasts and suppressed mitochondrial-mediated osteoblast apoptosis through the miR-214-3p-ATF4 signaling axis.

In summary, the present study has proven the physiological (beneficial) FSS enhances the proliferative activity of osteoblasts and protects osteoblasts against mitochondrial-mediated apoptosis, which provides theoretical basement for mechanotherapy of osteopenia. Based on the phenomenon of mechanically induced bone formation and microgravity-induced bone loss, therefore, it is valuable to explore the potential molecular mechanisms in the mechanically induced bone anabolism or catabolism, which may sharpen insights into the field of mechnaotherapy and provide more possible therapeutic targets for curing delayed fracture healing, osteoporosis and

aseptic implant loosening.

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Author Contributions: Kun Zhang and Yayi Xia conceived and designed the idea and drafted this paper; Bin Geng supervised the framework of the article. Kun Zhang, Xuening Liu, Yuchen Tang, Lifu Wang, Qiong Yi and Zhongcheng Liu collected and analyzed the data. All authors read and approved the final version of the manuscript. Kun Zhang was the first author of this article. Kun Zhang, Xuening Liu and Yuchen Tang contributed equally to this work. Bin Geng and Yayi Xia were co-corresponding authors.

Conflicts of interest: The authors have no conflicting interests.

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