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Original Paper

Periodic Mechanical Stress Induces Extracellular Matrix Expression and Migration of Rat Nucleus Pulposus Cells Through Src-GIT1-ERK1/2 Signaling Pathway

Gongming Gao[a] Haibo Li[a] Yongjing Huang[b] Jianjian Yin[a] Yuqing Jiang[a] Nanwei Xu[a] Dong Zhou[a] Luming Nong[a] Kewei Ren[c]

[a]Department of Orthopedics, The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University, Changzhou, [b]Department of Orthopedics, Wuxi Ninth People's Hospital Affiliated to Soochow University, Wuxi, [c]Department of Orthopedics, The Affiliated Jiangyin Hospital of Southeast University Medical School, Jiangyin, China

Key Words
Periodic mechanical stress • Extracellular matrix • Nucleus pulposus cells • Src • GIT1 • ERK1/2

Abstract
Background/Aims: Periodic mechanical stress has been shown to promote extracellular matrix (ECM) synthesis and cell migration of nucleus pulposus (NP) cells, however, the mechanisms need to be fully elucidated. The present study aimed to investigate the signal transduction pathway in the regulation of NP cells under periodic mechanical stress. ***Methods:*** Primary rat NP cells were isolated and seeded on glass slides, and then treated in our self-developed periodic stress field culture system. To further explore the mechanisms, data were analyzed by scratch-healing assay, quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis, western blotting, and co-immunoprecipitation assay. ***Results:*** Under periodic mechanical stress, the mRNA expression of ECM collagen 2A1 (Col2A1) and aggrecan, and migration of NP cells were significantly increased ($P < 0.05$ for each), associating with increases in the phosphorylation of Src, GIT1, and ERK1/2 ($P < 0.05$ for each). Pretreatment with the Src inhibitor PP2 reduced periodic mechanical stress-induced ECM synthesis and cell migration of NP cells ($P < 0.05$ for each), while the phosphorylation of GIT1 and ERK1/2 were inhibited. ECM synthesis, cell migration, and phosphorylation of ERK1/2 were inhibited after pretreatment with the small interfering RNA for GIT1 in NP cells under periodic mechanical stress ($P < 0.05$ for each), whereas the phosphorylation of Src was not affected. Pretreatment with the ERK1/2 inhibitor PD98059 reduced periodic mechanical stress-induced ECM synthesis and cell migration of NP cells ($P < 0.05$ for each). Co-immunoprecipitation assay showed that there

G. Gao and H. Li contributed equally to this work

Luming Nong
and Kewei Ren

Dept. of Orthopedics, The Aff. Changzhou No. 2 People's Hospital of Nanjing Med.Univ.
29 Xinglong Road, Changzhou (China)
E-Mail nongluming1977@hotmail.com; keweiren@hotmail.com

was a direct interaction between Src and GIT1 and between GIT1 and ERK1/2. ***Conclusion:*** In conclusion, periodic mechanical stress induced ECM expression and migration of NP cells via Src-GIT1-ERK1/2 signaling pathway, playing an important role in regulation of NP cells.

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Introduction

The Intervertebral disc contains two main components, including the nucleus pulposus (NP) and the annulus fibrosus. *In vivo*, NP cells in the center of intervertebral disc (IVD) are exposed to mechanical stress. The periodic mechanical load is advantageous to maintain the physiologic functions of the disc [1, 2]. However, few studies have already elucidated the mechanism of this mechanical-biochemical signal transduction in the NP cells lineage.

Src-family kinases (SFKs) consist of nine tyrosine kinases in the cytoplasmic domain. Among them, Src protein not only is currently the most studied member, but also the most closely protein associated with human diseases. Wu et al. reported that pressure alone induced hepatic stellate cells proliferation involving the activation of Src-dependent signaling [3]. Our previous study also revealed that periodic mechanical stress partly promoted chondrocyte area expansion and migration through the Src-dependent signaling [4]. However, no study demonstrated the effects of Src on NP cells in response to mechanical stimulation.

GIT1 is a protein kinase involved in controlling the activation of downstream signaling proteins [5]. A study conducted by Kaneyama et al. demonstrated that GIT1 was localized to stress fibers during the cyclic stretching, and participated in regulating the contractile capability of cells in the stress fibers [6]. Meanwhile, our previous study confirmed that periodic mechanical stress could promote the expression of extracellular matrix of rat nucleus pulposus (NP) cells by regulation of GIT1 [7]. However, it is presently unclear which proteins are involved in the upstream and downstream regulation of GIT1 during signaling transduction.

ERK1/2 is a serine and threonine kinase, virtually existing in all eukaryotic cells [8]. ERK1/2 is one of the core proteins in Mitogen-activated protein kinase (MAPK) signaling pathway and is closely associated with cell activity [9]. ERK1/2 activation has been shown to be capable of regulating the mitogenic responses under mechanical stimulation in rat chondrocytes [10, 11]. However, the regulation of ERK1/2 signaling needs to be elucidated in NP cells under mechanical stimulation.

Based on the above-mentioned studies, we therefore attempted to explore the mechanisms by which periodic mechanical stress is translated into biochemical signals, and also verified the nature of the relationship among Src, GIT1, and ERK1/2 in extracellular matrix expression and migration of NP cells.

Materials and Methods

Cell culture

NP cells were harvested using the method described previously [12]. In brief, four-week-old Sprague-Dawley (SD) rats of either sex from Animal Center of Nanjing Medical University (Nanjing, Jiangsu province, China) were sacrificed by cervical dislocation. NP cells collected from thoracic and lumbar spine were digested by 1.5% type II collagenase (Gibco; Thermo Fisher Scientifc, Inc., Waltham, MA, USA) at 37°C for 2 h, followed by filtration through a 200 mm Medium Mesh Strainer. The seed cells were cultured in DMEM-F12 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences Hyclone Laboratories, Logan, UT, USA) in a BB5060 incubator (Hereus, Hanau, Germany) at 37 ˚C and 5% CO2. Cells of the second generation were used for the following experiments in presence of approximately 80% confluent.

Construction of a periodical mechanical stress field

A periodic stress field of perfusion culture system with adjustable stress intensity was constructed by connecting the reciprocating intensifier pump to the air-tight cell culture device through a barrier-type pressure transducer (Taixing Experimental Instrument Factory, Jiangsu province, China), which provided a periodic mechanical stress with a pressure of 0-0.3 MPa and frequency of 0-1 Hz, as previously described [13]. Our previous study revealed that NP cells had a proper response to stress varying from 0 to 200 kPa at 0.1 Hz. Therefore, this range of pressure (0-200 kPa) and frequency (0.1 Hz) were used in the experiments [14, 15].

Cell treatment

Cells were plated on slides (25 mm × 25 mm), and then underwent periodic mechanical stress treatment of 0-0.2 MPa and 0.1 Hz (designated as pressure group), and also control group (without treatment) was considered. In some experiments, NPs were transfected with GIT1 SiRNA (SiRNA group) or SiRNA negative control (NC group) or without treatment (Control group) before the administration of periodic mechanical stress. NP cells were also pretreated with PP2, PD98059, or without treatment (Control group) before the administration of periodic mechanical stress. After stress treatment, cells were collected for various assays.

Inhibitors

PP2 (Amersham, UK) and PD98059 (Cell Signaling Technology Inc., Danvers, MA, USA) are specific inhibitors against Src and ERK1/2, respectively. Each inhibitor was dissolved in anhydrous dimethyl sulfoxide (DMSO) to 5 mmol/L storage concentration solution, and stored at −20°C. Each storage concentration solution was immediately diluted prior to use; each pretreatment group contained 0.1% (v/v) DMSO, which also contained the concentration used for the control group. NP cells were pre-treated with PP2 (10 uM), PD98059 (10 uM), or an equivalent amount of DMSO (0.1%, v/v) for 30 min, respectively. Then periodic mechanical stress was applied immediately after treatment with inhibitors.

Cell transfection

The SiRNA sequence for GIT1 was as follows: Sense 5'- CCCAUCAAGUCAGAGUUCATT-3' and antisense 5'-UGAACUCUGACUUGAUGGGTT-3'; the NC SiRNA sequence was as follows: Sense 5'-UUCUCCGAACGUGUCACGUdTdT-3' and antisense 5'-ACGUGACACGUUCGGAGAAdTdT-3'. The SiRNA and the NC sequence were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). For transfection, 75 pM of SiRNA or NC, and 7.5 μl lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) were resolved in 50 μl Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), respectively, mixed for 5 min, and then added to the cells on slides (100 μl for each slide; $1x10^5$ cells/ml). After 6 h at 37 ˚C, the cells were collected for following periodic mechanical stress treatment.

Western blot analysis

The cells were collected and prepared for total protein using radio immunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, China). Then, Western blot analysis was performed as previously described [16]. The concentration of the resultant total protein was determined using a bicinchoninic acid assay (BCA) (Beyotime Institute of Biotechnology, China). Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Primary antibodies used for incubating overnight at 4 °C were as follows: anti-Src (cat. no.2108; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phospho-Src (Tyr418) (cat. no. ab40660; Abcam, USA), anti-GIT1(cat. no.2919; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phospho-GIT1 (Tyr554) (cat. no. 0301412; PL Laboratories, Inc., British Columbia, Canada), anti-ERK1/2(cat. no.9102; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phospho-ERK1/2 (Thr202/Tyr204) (cat. no.4370; Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-GAPDH (cat. no. AP0063; Bioworld Technology, Inc., MN, USA). Following washing the membrane with phosphate buffered saline with Tween 20 (PBST), blots were then incubated with horseradish peroxidase (HRP) conjugated secondary antibody at ambient temperature for 1 h, and the colors were developed with ECL. The secondary antibodies used were as follows: goat anti-rabbit IgG-HRP (cat. no. BS13278; Bioworld Technology, Inc., MN, USA), goat anti-mouse IgG-HRP (cat. no. BS12478; Bioworld Technology, Inc., MN, USA) or rabbit anti-goat IgG-HRP (cat. no. sc-2768; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The blots were scanned with Bio-Rad Gel Doc Imaging System (Bio-

Rad Laboratories, Inc., Hercules, CA, USA), and the band densities were quantified and compared using "Quantity One" software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Expression of the target protein was calculated as the band density of the target protein normalized to that of GAPDH.

Co-immunoprecipitation

For cell samples, immunoprecipitation was performed according to a previous study [17]. Briefly, NP cells were collected, rinsed twice with cold phosphate buffered saline (PBS), and lysed in Lysis/Wash buffer (Beyotime Institute of Biotechnology, China), and supplemented with phenylmethylsulfonyl fluoride (PMSF; Beyotime Institute of Biotechnology, China). Lysates were cleared by centrifugation at 11, 000 g at 4 °C for 10 min. Supernatants were incubated with 2μg of the specified antibody overnight at 4 °C followed by 30 μl of protein A/G beads (cat. no. sc-2003; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 4 h at 4°C with gentle rotation. The beads were then pelleted at 1, 000 g for 1 min, and washed three times in 1 ml ice-cold Lysis/Wash buffer containing 1 mM PMSF and 50 g/ml aprotinin. Antibody-protein conjugates were eluted by boiling (10 min), and samples were then subjected to immunoblotting as described above.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted using TRIzol regent (Invitrogen, Carlsbad, CA, USA), and transcribed into cDNA using a PrimeScript RT Master Mix kit (Takara Bio, Inc., Shiga, Japan). The primers for aggrecan and Col2a1 were as follows: sense 5'-CCCTACCCTTGCTTCTCCA-3' and antisense5'-CTTGAGAGGCACTCATCAATGT-3' for aggrecan; sense 5'-GACCCCCAGGTTCTAATGG-3' and antisense 5'-GCACCTTTGGGACCATCTT-3' for Col2A1; and sense 5'-GCAGAAGGAGATTACTGCCCT-3' and antisense 5'-GCTGATCCACATCTGCTGGAA-3' for β-actin. The primers were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). RT-qPCR analysis was performed using a SYBR Premix Ex Taq II kit (Takara Bio, Inc., Shiga, Japan) and a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The reaction was performed in a 20-μL mixture containing 2 μL of the cDNA. The cycling conditions were as follows: at 95°C for 30 s; by 40 cycles at 95°C for 5 s, and at 60°C for 30 s. The results were quantified using the $2^{-\Delta\Delta Ct}$ method [18].

Cell migration assay

NP cells of the second generation were used to perform a scratch-healing assay [19]. Cells were grown on a glass slide (25 mm × 25 mm) at a density of 10^5 cells/slide. When cell growth reached 80% confluency, scratch wounds were created with the fine end of 200 μl tips. After experiments, the slides were observed under a CKX31 microscope (Olympus, Tokyo, Japan), and images were captured under three optimal fields at 0 and 24 h following the scratch. Cell migration was analyzed using ImageJ 1.43 software. The cell migration distance (μm) was calculated as the scratch width at 0 h minus the scratch width at 24 h following the scratch.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD), and analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The data between groups were analyzed using an unpaired t-test. P-value < 0.05 was statistically considered significant.

Results

Periodic mechanical stress significantly induces the mRNA expression of extracellular matrix (ECM) Col-2A1 and aggrecan, and promotes the migration of NP cells.

Compared with the control group, periodic mechanical stress (0.2 MPa; 0.1 Hz; 6 h) significantly induced the mRNA expression of ECM Col2A1 and aggrecan, as determined using RT-qPCR analysis ($P < 0.05$, Fig. 1A), promoted the migration of NP cells, which determined in scratch experiments ($P < 0.05$, Fig. 1B).

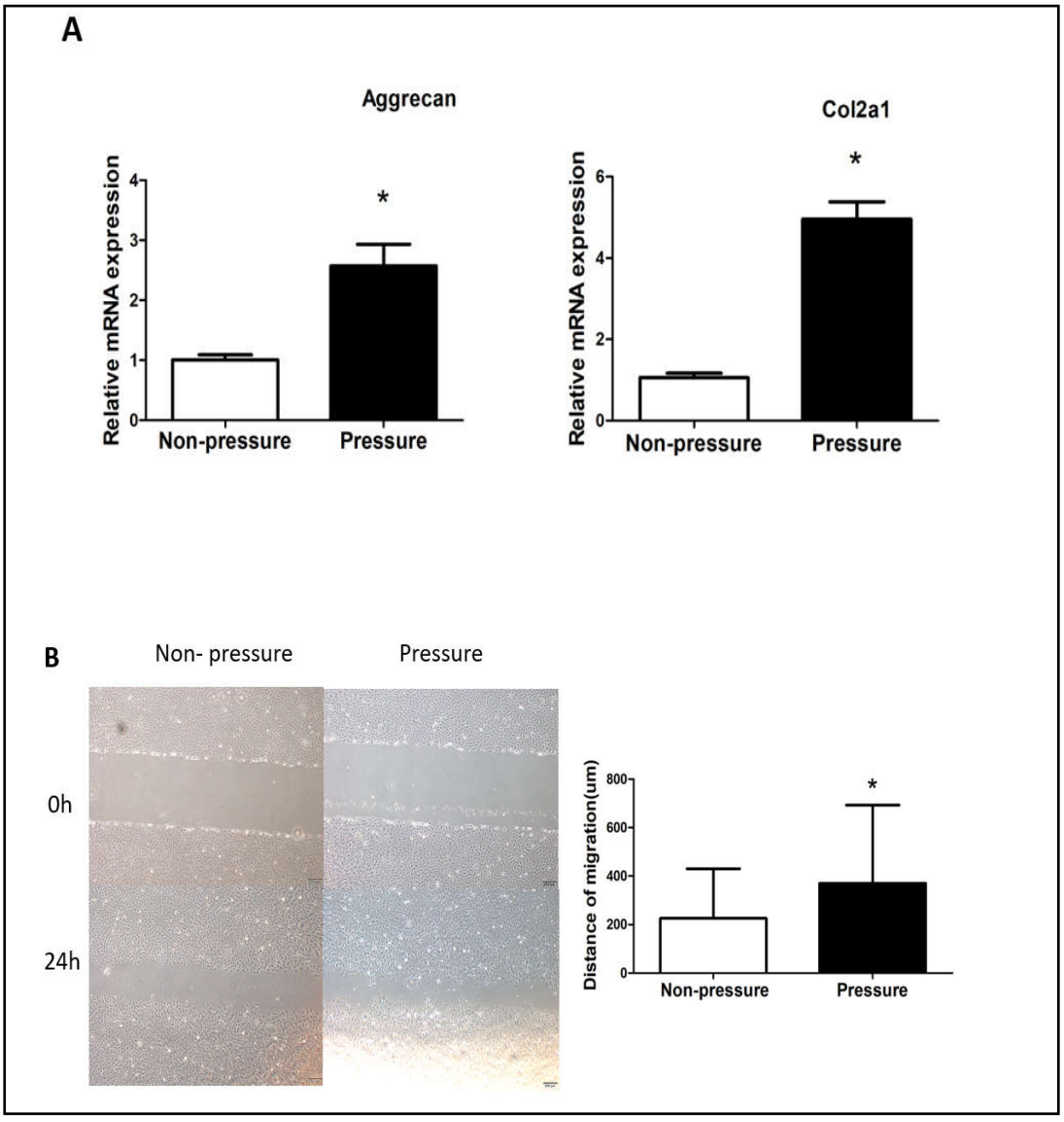

Fig. 1. Periodic mechanical stress significantly induces the mRNA expression of Col2A1 and aggrecan, and promotes migration of NP cells. Cells were exposed to periodic mechanical stress (pressure) or remained untreated (non-pressure). (A) mRNA expression levels of Col2A1 and aggrecan were analyzed by quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis after 6 h stress treatment. (B) Migration was determined by scratch experiments after 24 h stress treatment. The scratch width at 0 h minus the scratch width at 24 h was equal to the cell migration distance (magnification, x200). Data are expressed as mean ± standard deviation (SD). *P<0.05, compared with the non-pressure group (n=3). Col2A1, collagen 2A1; NP, nucleus pulposus.

Periodic mechanical stress significantly induces the phosphorylation of Src, GIT1, and ERK1/2 in NP cells.

The Western blot analysis showed that periodic mechanical stress significantly induced the phosphorylation of Src, GIT1, and ERK1/2 ($P < 0.05$; Fig. 2).

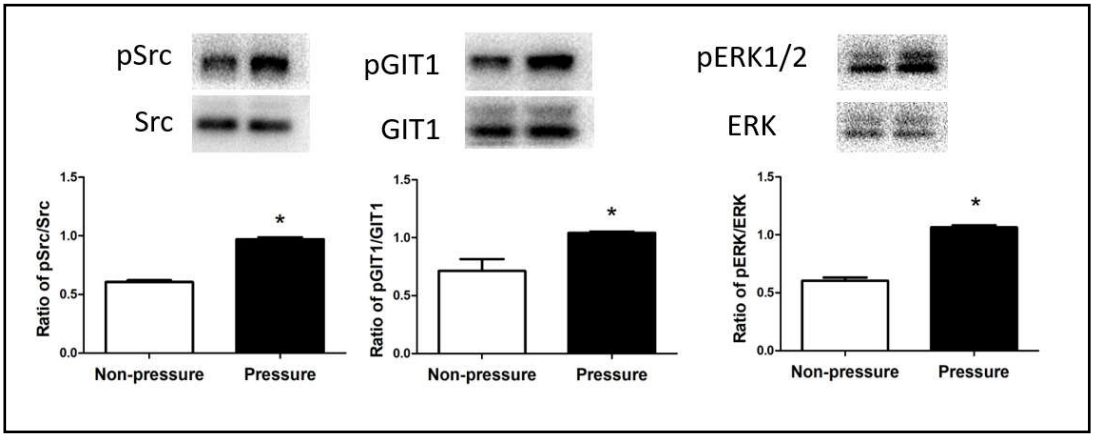

Fig. 2. Periodic mechanical stress significantly induces the phosphorylation of Src, GIT1 and ERK1/2 in NP cells. Phosphorylation of of Src, GIT1 and ERK1/2 exposed to periodic mechanical stress for 1 h (pressure) or not (non-pressure) were analyzed by Western blot analysis. Data are expressed as mean ± standard deviation (SD). *P<0.05, compared with the control (n=3). pSrc, phosphorylated Src; pGIT1, phosphorylated GIT1; NP, nucleus pulposus; pERK1/2, phosphorylated ERK1/2.

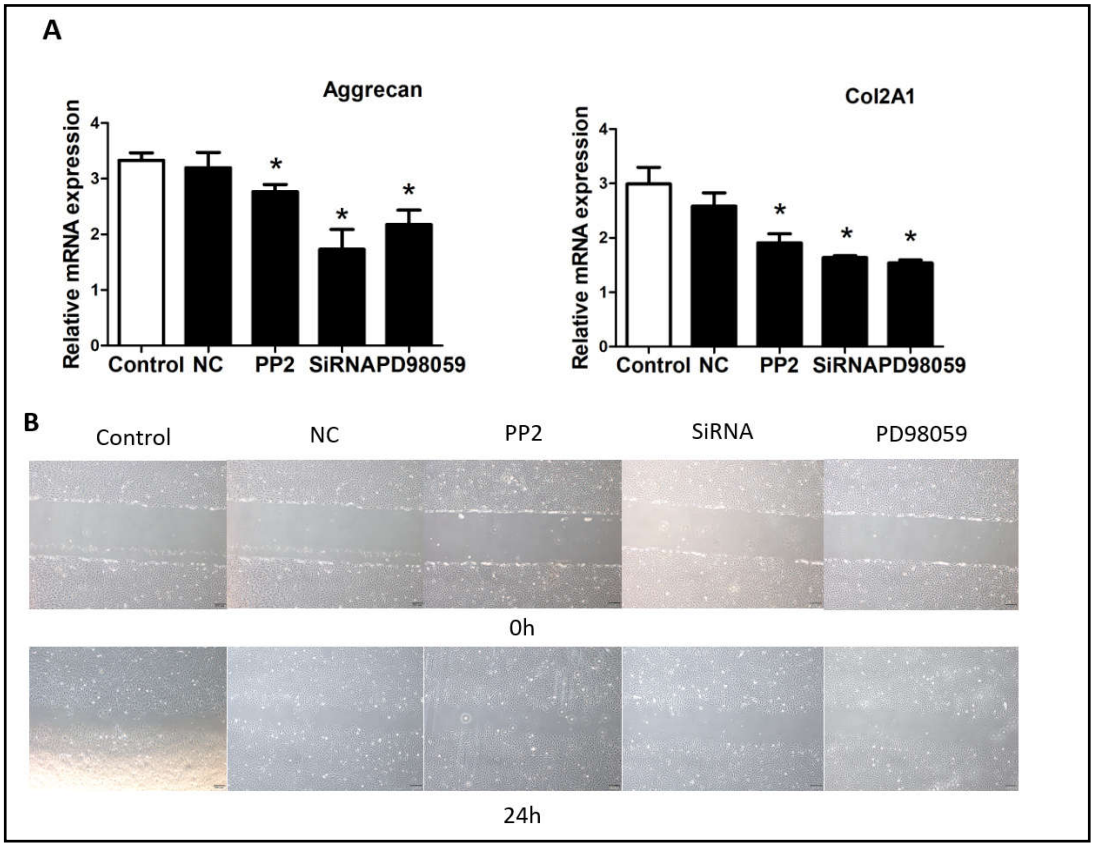

Fig. 3. PP2, PD98059, and GIT1 siRNA respectively suppress the mRNA expression levels of Col2A1 and aggrecan, and migration of NP cells under periodic mechanical stress. NP cells treated with GIT1 SiRNA (SiRNA), NC SiRNA(NC), PP2, PD98059 or untreated cells (control) were collected. The NP cells from SiRNA, NC, PP2, PD98059, and control groups were collected to measure the (A) mRNA expression levels of Col2A1 and aggrecan, (B) migration by scratch experiments (magnification, x200). Data are expressed as mean ± standard deviation (SD). *P<0.05, compared with the control group (n=3). SiRNA, small interfering RNA; Col2A1, collagen 2A1; NP, nucleus pulposus; NC, negative control.

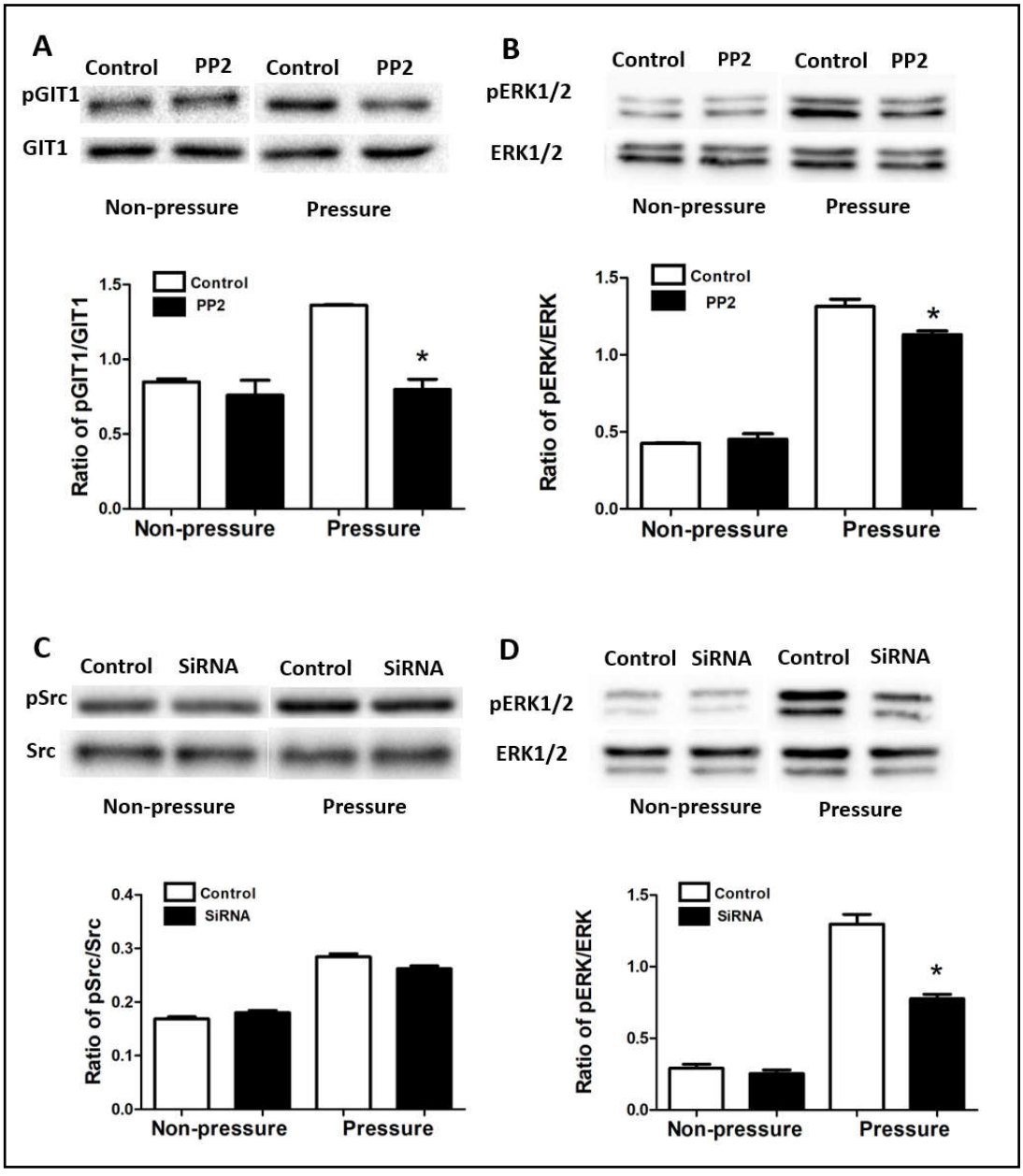

Fig. 4. Src-GIT1-ERK1/2 is a sequential chained reaction in signal transduction of NP cells under periodic mechanical stress. After pretreatment with DMSO (control), PP2 or SiNRA, cells were cultured in vitro for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of Src, GIT1, and ERK1/2 were detected by Western blotting. Results are represented in the histogram (n = 3, *, P<0.05 for each). The above-illustrated images are representative results. (A,B) The phosphorylation levels of GIT1 and ERK1/2 in the PP2 pretreatment group were significantly diminished relative to those of the control group in response to periodic mechanical stress (n = 3, P<0.05 for each). (C, D) The phosphorylation levels of ERK1/2 in the GIT1 SiRNA pretreatment group were significantly decreased relative to those of the control group (n = 3, P<0.05 for each), whereas the phosphorylation level of Src in the GIT1 SiRNA pretreatment group was not affected (n =3, P<0.05).

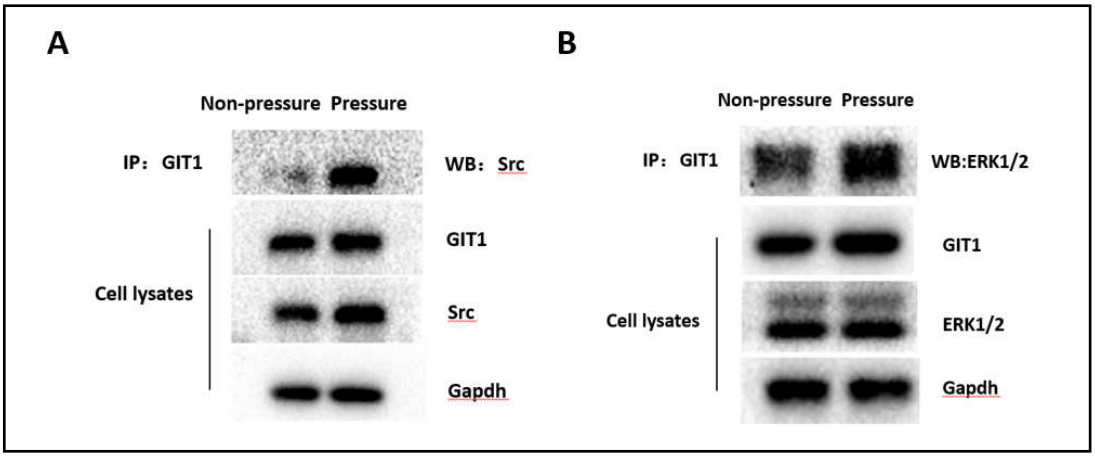

Fig. 5. Periodic mechanical stress enhances the interaction among Src, GIT1, and ERK1/2. The expression of Src, GIT1, and ERK1/2 in NP cells were verified by Western blot analysis. Cell lysates were subjected to co-immunoprecipitation with the antibody against GIT1 followed by Western blotting using indicated antibodies. (A) Co-immunoprecipitation showed that GIT1 interacted with Src in both non-pressure and pressure groups (stress treatment for 1 h). In pressure group, the interaction between GIT1 and Src was stronger than the non-pressure group. (B) Co-immunoprecipitation showed that GIT1 interacted with ERK1/2. In pressure group (stress treatment for 1 h), the interaction between GIT1 and ERK1/2 was stronger than the non-pressure group.

Phosphorylation of Src, GIT1, and ERK1/2 is required for the periodic mechanical stress-induced expression of ECM and the migration of NP cells

To examine whether the phosphorylation of *Src, GIT1 or ERK1/2* is involved in the periodic mechanical stress-induced expression of ECM, the NP cells were pretreated with PP2, GIT1 SiRNA, or PD98059 prior to the administration of periodic mechanical stress. Pretreatment with PP2, GIT1 SiRNA or PD98059 significantly suppressed the mRNA expression levels of Col2A1 and aggrecan in the NP cells ($P<0.05$; Fig. 3A), and the migration of NP cells under the periodic mechanical stress ($P < 0.05$; Fig. 3B).

Src-GIT1-ERK1/2 is a sequential chained reaction in signal transduction of NP cells under periodic mechanical stress

To explore the relationship among Src, GIT1, and ERK1/2 in NP cells' signaling pathway under mechanical stress, the NP cells were pretreated with PP2 or GIT1 SiRNA prior to the administration of periodic mechanical stress. Pretreatment with PP2 suppressed the phosphorylation of GIT1 and ERK1/2 in the NP cells ($P < 0.05$; Fig. 4A-B). Pretreatment with GIT1 SiRNA suppressed the phosphorylation of ERK1/2, while failed to affect the phosphorylation of Src in the NP cells ($P < 0.05$; Fig. 4C-D).

Periodic mechanical stress enhances the interaction among Src, GIT1, and ERK1/2

As shown in Fig. 5, co-immunoprecipitation confirmed that there were clear relationships between Src and GIT1 (Fig. 5A), and between GIT1 and ERK1/2 (Fig. 5B) in both non-pressure and pressure groups. The interaction in pressure group was stronger than that in non-pressure group.

Discussion

This study was designed to determine the mechanism of signal transduction by which periodic mechanical stress promotes NP cells migration and ECM synthesis. We demonstrated that periodic mechanical stress partly activates NP cells migration and ECM synthesis through Src-GIT1-ERK1/2 signaling pathway.

Src participates in regulation of cell growth, and proliferation and differentiation related to multiple signaling pathways [20-22]. One study has reported that Src plays crucial roles in shear stress-induced cellular processes [23]. A report published by Hum et al. revealed that mechanical loading may induce the formation of a Src/Pyk2/MBD2 complex in the nucleus, that functions to suppress anabolic gene expression [24]. Moreover, it has been demonstrated that SFKs, the upstream regulators of ERK1/2, regulate ERK1/2 in nociceptive neurons during H_2O_2 [25]. We found that periodic mechanical stress promotes ECM expression and migration of NP cells. Simultaneously, the phosphorylation level of Src significantly increased. When the phosphorylation level of Src was inhibited, the activation of GIT1 and ERK1/2 were suppressed, besides, ECM expression and migration of NP cells were also significantly reduced, suggesting that Src regulates the activation of GIT1 and ERK1/2 to promote ECM expression and migration under stress environment.

GIT1 is a type of bridging protein within the cell's local adhesion that regulates downstream signaling molecules and cell activity [26]. A study conducted by Chen et al. indicated that GIT1 is an independent prognostic biomarker and facilitates HCC progression via activating ERK/MMP9 signaling [27]. Xiao et al. found that Platelet-derived growth factor (PDGF) is able to activate the GIT1-PLCγ1-mediated ERK1/2 pathway to control chondrocyte proliferation. However, the role of GIT1 in signaling pathways under stress environment is less studied. The results of our study confirmed that periodic mechanical stress not only causes ECM expression and cell migration, but also activates the phosphorylation level of GIT1. ECM expression and cell migration were inhibited after SiRNA transfection of GIT1, which indicated that GIT1 mediates ECM expression and migration of NP cells induced by periodic mechanical stress. Additionally, when the activation of GIT1 was inhibited, the phosphorylation level of ERK1/2 induced by periodic mechanical stress was suppressed as well, while the activation of Src was not affected, which suggested that GIT1 was located upstream of ERK1/2 and downstream of Src under conditions of periodic mechanical stress.

ERK1/2 is one of the core proteins of the MAPK cascade, and plays an important role in cell proliferation, differentiation, and migration [28-32]. In addition, increasing evidence showed that ERK1/2 plays an important role in the signal transduction pathway under mechanical stress environment. Sun et al. demonstrated that mechanical stress can promote matrix synthesis of mandibular condylar cartilage via the RKIP-ERK pathway [33]. Li et al. found that chronic mechanical stress can enhance MUC5AC expression in human bronchial epithelial cells through the ERK signal transduction pathway [34]. Mu et al. suggested that mechanical stress is an important factor affecting the proliferation and differentiation of stem cells from apical papilla via the activation of ERK and JNK signaling pathways [35]. In the present study, we observed that periodic mechanical stress significantly enhances ECM expression and migration of NP cells through activation of ERK1/2, relative to that in NP cells under static conditions. Moreover, blocking ERK1/2 with PD98059 prevented the up-regulation of ECM expression and cell migration following periodic mechanical stress. Our findings indicate that ERK1/2 appears to play a key role in cellular adaptation of NP cells to periodic mechanical stress.

In addition, our study for the first time demonstrated that there is a direct interaction between the three proteins of Src, GIT1, and ERK1/2 in rat NP cells through co-immunoprecipitation, and this effect was significantly enhanced under periodic mechanical stress.

Conclusion

In conclusion, the present study confirmed that periodic mechanical stress can stimulate the phosphorylation level of Src, which then can activate downstream of GIT1. Downstream activation of GIT1 led to ECM expression and migration of rat NP cells via regulating the activity of ERK1/2. Thus, Src-GIT1-ERK1/2 constitutes at least a key signal transduction pathway regulated by periodic mechanical stress in rat NP cells (Fig. 6).

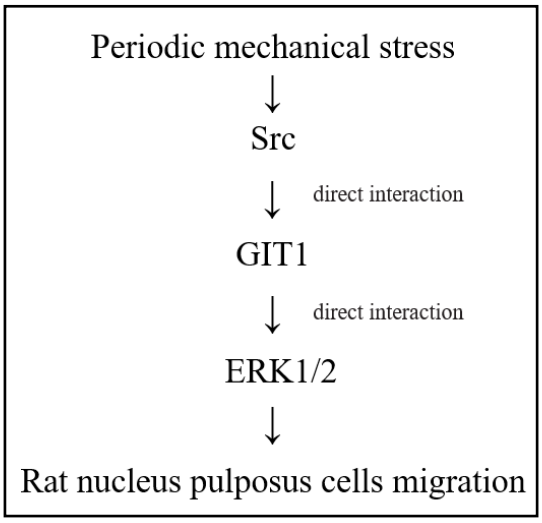

Fig. 6. Schematic diagram of the signal transduction pathway of rat nucleus pulposus cells migration induced by periodic mechanical stress.

Acknowledgements

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Disclosure Statement

The authors declare no competing interests.

References

1 Kasra M, Goel V, Martin J, Wang ST, Choi W, Buckwalter J: Effect of dynamic hydrostatic pressure on rabbit intervertebral disc cells. J Orthop Res 2003;21:597-603.
2 Maclean JJ, Lee CR, Alini M, Iatridis JC: Anabolic and catabolic mRNA levels of the intervertebral disc vary with the magnitude and frequency of *in vivo* dynamic compression. J Orthop Res 2004;22:1193-1200.
3 Wu HJ, Zhang ZQ, Yu B, Liu S, Qin KR, Zhu L: Pressure activates Src-dependent FAK-Akt and ERK1/2 signaling pathways in rat hepatic stellate cells. Cell Physiol Biochem 2010;26:273-280.
4 Nong L, Yin G, Ren K, Tang J, Fan W: Periodic mechanical stress enhances rat chondrocyte area expansion and migration through Src-PLCgamma1-ERK1/2 signaling. Eur J Cell Biol 2010;89:705-711.
5 Premont RT, Claing A, Vitale N, Perry SJ, Lefkowitz RJ: The GIT family of ADP-ribosylation factor GTPase-activating proteins. Functional diversity of GIT2 through alternative splicing. J Biol Chem 2000;275:22373-22380.
6 Kim-Kaneyama JR, Suzuki W, Ichikawa K, Ohki T, Kohno Y, Sata M, Nose K, Shibanuma M: Uni-axial stretching regulates intracellular localization of Hic-5 expressed in smooth-muscle cells *in vivo*. J Cell Sci 2005;118:937-949.
7 Xie H, Nong L, Gao G, Zhou D, Huang Y: Regulation of periodic mechanical stress on extracellular matrix of nucleus pulplsus cells through G protein coupled receptor kinase interacting protein 1. Chin J Exp Surg 2015;32:1116-1118.

8 Marshall CJ: Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179-185.
9 Ryan JA, Eisner EA, DuRaine G, You Z, Reddi AH: Mechanical compression of articular cartilage induces chondrocyte proliferation and inhibits proteoglycan synthesis by activation of the ERK pathway: implications for tissue engineering and regenerative medicine. J Tissue Eng Regen Med 2009;3:107-116.
10 Liang W, Ren K, Liu F, Cui W, Wang Q, Chen Z, Fan W: Periodic mechanical stress stimulates the FAK mitogenic signal in rat chondrocytes through ERK1/2 activity. Cell Physiol Biochem 2013;32:915-930.
11 Ren K, Ma Y, Huang Y, Liang W, Liu F, Wang Q, Cui W, Liu Z, Yin G, Fan W: Periodic mechanical stress activates MEK1/2-ERK1/2 mitogenic signals in rat chondrocytes through Src and PLCγ1. Braz J Med Biol Res 2011;44:1231-1242.
12 Gao G, He J, Nong L, Xie H, Huang Y, Xu N, Zhou D: Periodic mechanical stress induces the extracellular matrix expression and migration of rat nucleus pulposus cells by upregulating the expression of intergrin α1 and phosphorylation of downstream phospholipase Cγ1. Mol Med Rep 2016;14:2457-2464.
13 Gao G, Shen N, Jiang X, Sun H, Xu N, Zhou D, Nong L, Ren K: Periodic mechanical stress activates EGFR-dependent Rac1 mitogenic signals in rat nucleus pulpous cells via ERK1/2. Biochem Biophys Res Commun 2016;469:723-730.
14 Gao G, Xu N, Jiang Y, Lu Y, Nong L, Zhou D: The gene expression of different integrin subunits in rat nucleus pulposus cells under periodic mechanical stress. Chin J Exp Surg 2013;30:596-598.
15 Gao G, Nong L, Zhou D, Huang Y, Zhao S, Xu N: Proliferation and migration of nucleus pulposus cells regulated by interleuifin-6 through p38 protein. Chin J Exp Surg 2015;32:117-119.
16 Wang P, Cao X, Nagel DJ, Yin G: Activation of ASK1 during reperfusion of ischemic spinal cord. Neurosci Lett 2007;415:248-252.
17 Hu M, Wang C, Li W, Lu W, Bai Z, Qin D, Yan Q, Zhu J, Krueger BJ, Renne R, Gao SJ, Lu C: A KSHV microRNA Directly Targets G Protein-Coupled Receptor Kinase 2 to Promote the Migration and Invasion of Endothelial Cells by Inducing CXCR2 and Activating AKT Signaling. PLoS Pathog 2015;11:e1005171.
18 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-408.
19 Bron JL, Mulder HW, Vonk LA, Doulabi BZ, Oudhoff MJ, Smit TH: Migration of intervertebral disc cells into dense collagen scaffolds intended for functional replacement. J Mater Sci Mater Med 2012;23:813-821.
20 Lowell CA, Fumagalli L, Berton G: Deficiency of Src family kinases p59/61hck and p58c-fgr results in defective adhesion-dependent neutrophil functions. J Cell Biol 1996;133:895-910.
21 Turkson J: STAT proteins as novel targets for cancer drug discovery. Expert Opin Ther Targets 2004;8:409-422.
22 Eum SY, Rha GB, Hennig B, Toborek M: c-Src is the primary signaling mediator of polychlorinated biphenyl-induced interleukin-8 expression in a human microvascular endothelial cell line. Toxicol Sci 2006;92:311-320.
23 Liu B, Lu S, Hu YL, Liao X, Ouyang M, Wang Y: RhoA and membrane fluidity mediates the spatially polarized Src/FAK activation in response to shear stress. Sci Rep 2014;4:7008.
24 Hum JM, Day RN, Bidwell JP, Wang Y, Pavalko FM: Mechanical loading in osteocytes induces formation of a Src/Pyk2/MBD2 complex that suppresses anabolic gene expression. PLoS One 2014;9:e97942.
25 Singh AK, Vinayak M: Activation of ERK signalling by Src family kinases (SFKs) in DRG neurons contributes to hydrogen peroxide (H2O2)-induced thermal hyperalgesia. Free Radic Res 2017;51:838-850.
26 Schmalzigaug R, Garron ML, Roseman JT, Xing Y, Davidson CE, Arold ST, Premont RT: GIT1 utilizes a focal adhesion targeting-homology domain to bind paxillin. Cell Signal 2007;19:1733-1744.
27 Chen J, Yang P, Yang J, Wen Z, Zhang B, Zheng X: GIT1 is a novel prognostic biomarker and facilitates tumor progression via activating ERK/MMP9 signaling in hepatocellular carcinoma. Onco Targets Ther 2015;8:3731-3742.
28 Zhao LY, Tong DD, Xue M, Ma HL, Liu SY, Yang J, Liu YX, Guo B, Ni L, Liu LY, Qin YN, Wang LM, Zhao XG, Huang C: MeCP2, a target of miR-638, facilitates gastric cancer cell proliferation through activation of the MEK1/2-ERK1/2 signaling pathway by upregulating GIT1. Oncogenesis 2017;6:e368.
29 Jin E, Ren M, Liu W, Liang S, Hu Q, Gu Y, Li S: Effect of boron on thymic cytokine expression, hormone secretion, antioxidant functions, cell proliferation, and apoptosis potential via the ERK1/2 signaling pathway. J Agric Food Chem 2017;65:11280-11291.

30 Lan H, Wang N, Chen Y, Wang X, Gong Y, Qi X, Luo Y, Yao F: Macrophage migration inhibitory factor (MIF) promotes rat airway muscle cell proliferation and migration mediated by ERK1/2 and FAK signaling. Cell Biol Int 2018;42:75-83.
31 Cheng X, Wang Y, Chen H, Xu Y, Xiong W, Wang T: Claudin-1 regulates pulmonary artery smooth muscle cell proliferation through the activation of ERK1/2. Biomed Pharmacother 2017;89:983-990.
32 Lim W, Jeong M, Bazer FW, Song G: Coumestrol Inhibits Proliferation and Migration of Prostate Cancer Cells by Regulating AKT, ERK1/2, and JNK MAPK Cell Signaling Cascades. J Cell Physiol 2017;232:862-871.
33 Sun L, Zhao J, Wang H, Pan Y, Wang L, Zhang WB: Mechanical stress promotes matrix synthesis of mandibular condylar cartilage via the RKIP-ERK pathway. J Mol Histol 2017;48:437-446.
34 Li N, Li Q, Zhou XD, Kolosov VP, Perelman JM: Chronic mechanical stress induces mucin 5AC expression in human bronchial epithelial cells through ERK dependent pathways. Mol Biol Rep 2012;39:1019-1028.
35 Mu C, Lv T, Wang Z, Ma S, Ma J, Liu J, Yu J, Mu J: Mechanical stress stimulates the osteo/odontoblastic differentiation of human stem cells from apical papilla via erk 1/2 and JNK MAPK pathways. Biomed Res Int 2014;2014:494378.