

Original Paper

Periodic Mechanical Stress Stimulates GIT1-Dependent Mitogenic Signals in Rat Chondrocytes Through ERK1/2 Activity

Kewei Ren^a Jilei Tang^b Xuefeng Jiang^a Huiqing Sun^a Luming Nong^c
Nan Shen^d Yanqing Gu^e

^aDepartment of Orthopedics, the Affiliated Jiangyin Hospital of Southeast University Medical School, Jiangyin, ^bDepartment of Orthopedics, Qidong People's Hospital, Nantong, ^cDepartment of Orthopedics, The Affiliated Changzhou No. 2 People's Hospital with Nanjing Medical University, Changzhou, ^dDepartment of Clinical Pharmacy, the Affiliated Jiangyin Hospital of Southeast University Medical School, Jiangyin, ^eDepartment of Orthopedics, Nanjing First Hospital, Nanjing Medical University, Nanjing, China

Key Words

Periodic mechanical stress • Chondrocyte proliferation • GIT1 • FAK • Src • Integrin β 1

Abstract

Background/Aims: The mitogenic effects of periodic mechanical stress on chondrocytes have been studied extensively, but the mechanisms whereby chondrocytes sense and respond to mechanical stimuli remain to be determined. We explored the question and verified the key role of G protein coupled receptor kinase interacting protein 1 (GIT1) signaling in periodic mechanical stress-induced chondrocyte proliferation. **Methods:** Two steps were undertaken in the experiment. In the first step, the cells were maintained under non-pressure conditions or periodic mechanical stress for 1 h prior to Western blot analysis. In the second step, the cells were pretreated with short hairpin RNA (shRNA) targeted to GIT1 or Src or control scrambled shRNA, or transfected with GIT1 wild-type or GIT1 mutant Y321F, or focal adhesion kinase (FAK) wild-type or FAK mutants Y397F or Y576F/Y577, respectively. Moreover, the cells were pretreated with blocking antibody against integrin β 1 or PP2. Then the cells were maintained under non-pressure conditions or periodic mechanical stress for 1 h prior to Western blot analysis, and for 3 days, 8 h per day, prior to direct cell counting and CCK-8 assay, respectively. **Results:** Periodic mechanical stress significantly induced sustained phosphorylation of GIT1 at Tyr³²¹. Reduction of GIT1 with shRNA targeted to GIT1 and GIT1 mutant Y321F inhibited periodic mechanical stress-promoted chondrocyte proliferation, accompanied by attenuated extracellular signal-regulated kinase (ERK)1/2 and FAK phosphorylation at Tyr^{576/577}. However, activation of Src and FAK-Tyr³⁹⁷ was not prevented upon GIT1 suppression. Furthermore, pretreatment with blocking antibody against integrin β 1, Src-selective inhibitor, PP2, and shRNA targeted to Src blocked GIT1 activation under periodic mechanical stress. In addition,

Luming Nong, Nan Shen
and Yanqing Gu

Department of Orthopedics, The Affiliated Changzhou No. 2 People's Hospital
with Nanjing Medical University, Changzhou 213003 (China)
E-Mail lumingnong@hotmail.com; keweiren@hotmail.com; gyqjmu@163.com

GIT1 phosphorylation at Tyr³²¹ was not reduced upon pretreatment with FAK mutants Y397F or Y576F/Y577 under conditions of periodic mechanical stress. **Conclusion:** These findings collectively suggested that periodic mechanical stress promoted chondrocyte proliferation through at least two separate pathways, integrin β 1-Src-GIT1-FAK(Tyr^{576/577})-ERK1/2, and the other parallel GIT1-independent integrin β 1-FAK(Tyr³⁹⁷)-ERK1/2.

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Introduction

Periodic mechanical stress is advantageous to simulate *in vivo* physiological mechanical conditions and the mitogenic effects on chondrocytes have been demonstrated [1, 2]. However, the mechanisms of the transduction of mechanical stimuli and biochemical signaling events are still poorly understood in chondrocytes.

GIT1 is a multidomain protein with intrinsic tyrosine kinase activity. Structurally, GIT1 consists of an N-terminal ARF-GAP domain, ankyrin repeats, Spa homology domain (SHD), synaptic localization domain (SLD), and paxillin-binding site (PBS) [3]. GIT1, as a scaffold protein, is involved in controlling downstream signaling cascades transduction including ERK1/2 MAPKs, and the biological functions of GIT1 are diverse [4-7]. Several researchers and our collaborator Professor Yin have established that GIT1 can be activated by mechanical stimuli besides biochemical stimulation [8, 9]. Kim-Kaneyama and co-workers reported that GIT1 is essential for regulating the contractile capability of smooth-muscle cells in the stress fibers under uni-axial cyclic stretching [10]. Zhao et al. confirmed dependence of Methyl-CpG binding protein 2 (MeCP2)-induced the activation of ERK1/2 signals and cell proliferation on GIT1 in gastric cancer cells [11]. Furthermore, a recent study by another Dr. Zhao proved that chemical stimuli (platelet-derived growth factor, PDGF)-enhanced chondrocyte activity and functions was dependent on activated GIT1 [12]. However, it is similarly unclear whether GIT1 is stimulated, and the specific functions of GIT1 involved in modulating mechanical stress-initiated mitogenic effects, including the nature of any functional association between GIT1 and ERK1/2 in this context, remain to be elucidated in chondrocytes.

Accumulating evidence supports that GIT1 is tightly linked to integrin signaling transduction pathways in some cell types [13, 14]. The activation of GIT1 by integrin has recently been demonstrated to be a signaling transduction mechanism in breast tumor cells and perivascular mural cells for the detection and response to various extracellular stimuli [15, 16]. And our previous study found periodic mechanical stress activates integrin β 1-initiated signaling to promote mitogenic effects in chondrocytes [17]. The coincidence raises the possibility that there may be some causal relationship between integrin β 1 and GIT1 in chondrocytes in this context. However, different cell types, different systems, and even different mechanical stimuli may result in different activation patterns of signaling pathways.

Our previous findings collectively suggested that periodic mechanical stress promotes rat chondrocyte proliferation through at least two pathways, integrin β 1-Src-FAK(Tyr^{576/577})-ERK1/2 and integrin β 1-FAK (Tyr³⁹⁷)-ERK1/2 [18]. And GIT1 can be stimulated by integrin signal-associated proteins and interact with the latter, and anchor potential downstream signaling molecules, activating intracellular signaling cascades [12, 19, 20]. Interaction between FAK and GIT1 within signal transduction has been analyzed in several non-chondrocytic cell types, however, the conclusions are controversial [21-23]. Furthermore, the association between Src and GIT1 has not been determined in chondrocytes subjected to mechanical stimuli. Thus, the elucidation of the relationship between GIT1 and Src, FAK are essential in chondrocytes in this setting.

This study aimed to determine whether GIT1 activates ERK1/2 mitogenic signals in chondrocytes under periodic mechanical stress. Additionally, the nature of the association between GIT1 and integrin β 1, Src, FAK was analyzed in this system, in order to establish the mechanotransduction pathways and link these signals into mitogenic cascades.

Materials and Methods

Materials

Two-week-old Sprague-Dawley rats of either sex were provided by the Animal Center of Nanjing Medical University. Dulbecco's modified Eagle's medium–nutrient mixture (DMEM) F-12, fetal bovine serum, trypsin, collagenase II, and anti-collagen monoclonal antibody II were purchased from Gibco (USA). Type II collagen was purchased from Sigma (USA). Cell counting kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology (China). Anti-GIT1, anti-phospho-GIT1 (Tyr³²¹), anti-Src, anti-phospho-Src (Tyr⁴¹⁸), anti-FAK, anti-phospho-FAK (Tyr³⁹⁷), anti-FAK, anti-phospho-FAK (Tyr^{576/577}), anti-ERK1/2, anti-phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴), horseradish peroxidase-goat anti-rabbit IgG, and enhanced chemiluminescence (ECL) assay kit were supplied by Cell Signaling Technology Co., Ltd. (USA). Blocking antibody against integrin β 1 and PP2 were purchased from BD Biosciences (USA) and BIOMOL (USA), respectively. GIT1 short hairpin RNA (shRNA) lentiviral particles, Src shRNA lentiviral particles, Control shRNA lentiviral particles, and Polybrene were supplied by Santa Cruz (USA). The GV287 vector was obtained from GENECHM (China). The GIT1 mutant (Tyr³²¹ to Phe³²¹) and FAK mutants (Tyr³⁹⁷ to Phe³⁹⁷ and Tyr⁵⁷⁶-Tyr⁵⁷⁷ to Phe⁵⁷⁶-Phe⁵⁷⁷) were generated via site-directed mutagenesis using overlapping PCR and subcloned into the GV287 vector. The successful introduction of mutations was verified by sequencing. Wild-type GIT1 and FAK cDNA was subcloned into the GV287 vector served as the control.

A cell incubator (Hereus BB 5060), air-tight cell culture device and reciprocating pressure pump, barrier-type pressure transducer and inversion microscope equipped with camera system were bought from Hereus (Germany), Taixing Experimental Instrument Factory (China), Tianjin Plastics Research Institute (China) and Olympus (Japan), respectively.

Cell culture

Chondrocytes were harvested using the method described by Séguin and Bernier [24]. The cells were purified by repeated adherence, and the morphology was observed under an inverted phase contrast microscope by staining for collagen type II according to the conventional ABC method. The cells of the second generation were seeded on a glass slide (25 × 25 mm²) coated with type II collagen at a density of 10⁵. Experiments were performed when the cells were approximately 70–80 % confluent. After experiments, cells were collected for proliferation studies, and total protein was extracted and prepared for Western blot analysis.

Inhibitors

Blocking antibody against integrin β 1 and PP2 were used as specific inhibitors against integrin β 1 and Src, respectively. Blocking antibody against integrin β 1 was dissolved in DMEM, and PP2 was dissolved in anhydrous dimethylsulfoxide (DMSO). Cells were pretreated with PP2 (10 μ M) or an equivalent amount of DMSO (0.1 % v/v) for 1 h. The remaining cells were pretreated with blocking antibodies against integrin β 1 (10 μ g/mL) or an equivalent amount of DMEM for 5 h.

Construction of a periodical mechanical stress field

A periodic stress field of perfusion culture system with adjustable stress intensity and frequency was built by connecting the reciprocating intensifier pump to the air-tight cell culture device through a barrier-type pressure transducer, as previously described [25]. The pressure in this system ranged from 0 to 300 kPa and the frequency from 0 to 1 Hz. Earlier, it was shown that rabbit chondrocytes subjected to stress varying from 0 to 200 kPa at 0.1 Hz yielded tissue-engineered cartilage of the best quality [26, 27]. Accordingly, a pressure range of 0–200 kPa and 0.1 Hz frequency were used in the current study.

Experimental grouping

Two steps were undertaken in the experiment. In the first step, the cells were maintained under non-pressure conditions or periodic mechanical stress for 1 h prior to Western blot analysis. In the second step, the cells were pretreated with shRNA targeted to GIT1 or Src or control scrambled shRNA, respectively, or transfected with GIT1 wild-type or GIT1 mutant Y321F, or FAK wild-type or FAK mutant Y397F or Y576F/Y577. Moreover, the cells were pretreated with blocking antibody against integrin β 1 or PP2. Then the cells

were maintained under non-pressure conditions or periodic mechanical stress for 1 h prior to Western blot analysis, and for 3 days, 8 h per day, prior to direct cell counting and CCK-8 assay, respectively. All groups of cells involved in the experiments were incubated at 37 °C in the incubator with 5 % CO₂.

Western blot analysis

Total protein was prepared and Western blot analyses were performed as previously described [28]. Total protein was analyzed for protein concentration using Bradford assay. Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Following blocking for 1 h with 5 % milk in Tris-buffered saline and Tween 20, the membranes were incubated with antibodies (1:1000 dilution for three antibodies) overnight at 4 °C. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h and developed colors with ECL. The results were scanned using Gel Imaging System (UVP Company, USA) and measured using Gel-Pro Analyzer software (Media Cybernetics, USA).

Proliferation Studies

Proliferation studies were assessed by two different methods: direct cell counting and CCK-8 assay.

Direct cell counting

The cells were trypsinized and counted as previously described [29]. The cell number was determined by counting each piece of glass slide independently. Each group included six random pieces of glass slides and the experiments were repeated five times.

CCK-8 assay

Cell proliferation was determined by using CCK-8 solution according to the manufacturer's instruction as previously described [30]. The cells were kept in five 96-well plates (n = 5); 10 µL of CCK-8 solution was added to each well and incubated for 4 h at 37 °C. The absorbance of each well was finally determined at 450 nm using a microplate reader.

Statistical analysis

Statistical analyses were performed using SPSS 14.0 software (SPSS, IL, USA). All the experimental steps were repeated 5 times, and the results were expressed as mean ± standard deviation. Student's unpaired *t* tests were used to determine the statistical significance. A *P* value of 0.05 was considered to be significant.

Results

Effects of Periodic Mechanical Stress on GIT1 Phosphorylation

The phosphorylation levels of GIT1 at Tyr³²¹ at 1 h (0.85 ± 0.054) increased significantly in the pressure group compared to the non-pressure group (0.36 ± 0.045) (Fig. 1).

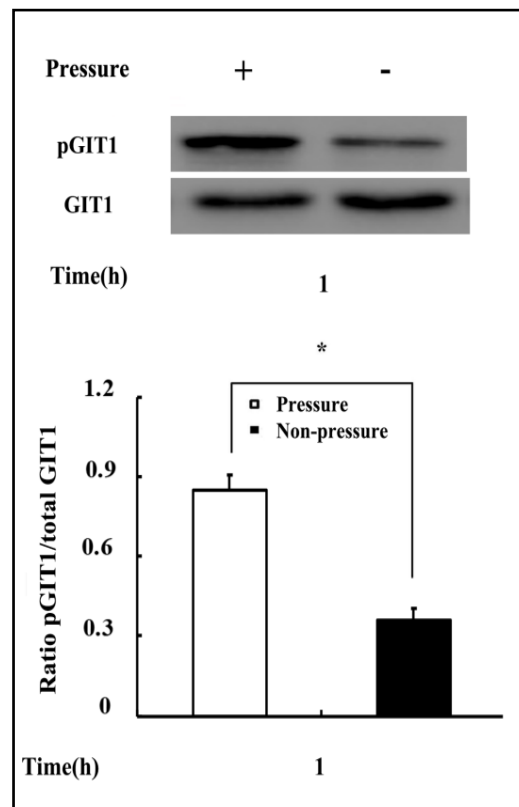


Fig. 1. Effects of periodic mechanical stress on the phosphorylation of GIT1 at Tyr³²¹ (pGIT1-Y321). Rat chondrocytes were cultured in vitro for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of GIT1 (pGIT1-Y321) were detected by Western blotting. The total amount of protein served as a control. Gray values are represented with a histogram (n = 5, *P<0.05 for each). The aforementioned images are representative results of Western blotting.

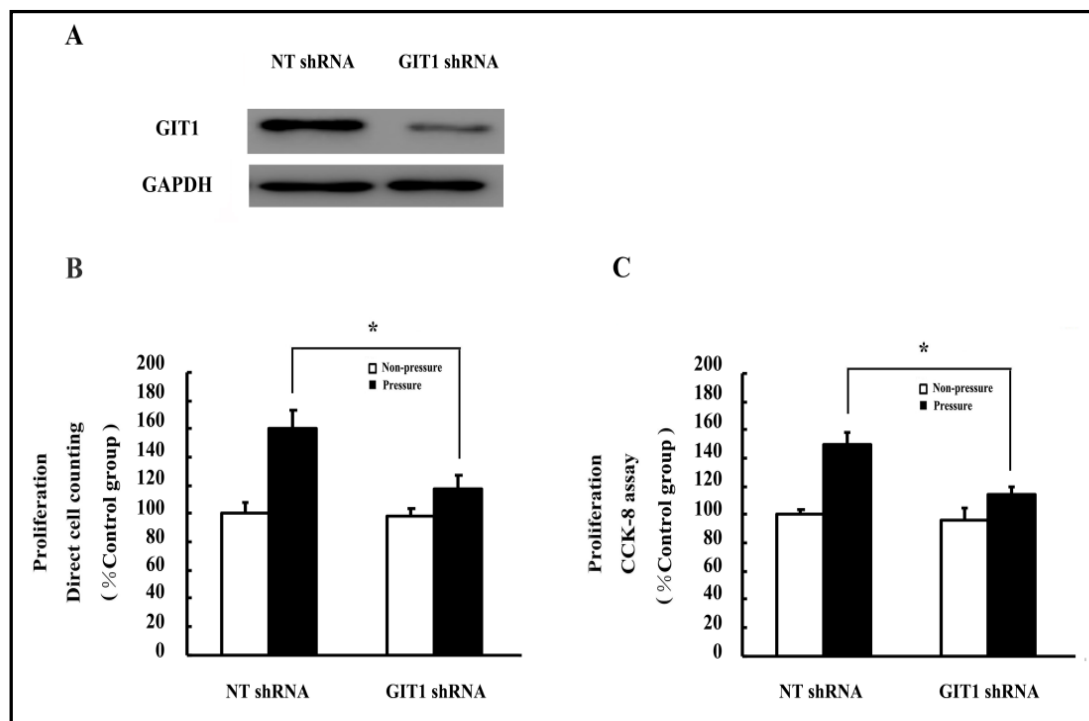


Fig. 2. Effects of GIT1 on chondrocyte proliferation under conditions of periodic mechanical stress. Rat chondrocytes were transfected with shRNA targeted to GIT1 or with nontargeting sequences prior to lysis and Western blotting for GIT1 protein. Transfection with shRNA for GIT1 achieved about a 50% reduction in GIT1 protein level (A). After pretreatment with control or GIT1 shRNA, rat chondrocytes were cultured in vitro for 3 days under non-pressure conditions or conditions of periodic mechanical stress 8 h per day prior to proliferation studies. Chondrocyte proliferation was analyzed using direct cell counting (B) and CCK-8 assay (C). The data were expressed as mean \pm standard deviation. We divided the data of all groups by the mean of the control group, and got a percentage to complete the normalization. Chondrocyte proliferation results are shown in the histogram ($n = 5$, $*P < 0.05$ for each).

GIT1 and GIT1-Tyr³²¹ Are Required for Periodic Mechanical Stress-initiated Chondrocyte Proliferation

GIT1 inhibition with targeted shRNA blocked periodic mechanical stress-induced chondrocyte proliferation significantly (118 ± 9 (Direct cell counting); 114 ± 6 (CCK-8 assay)) compared with chondrocytes in the control group (160 ± 13 (Direct cell counting); 150 ± 8 (CCK-8 assay)) in response to periodic mechanical stress (Fig. 2). Periodic mechanical stress-induced chondrocyte proliferation significantly reduced in chondrocytes transfected with GIT1 mutant-expressing plasmid (Phe³²¹) (130 ± 4 (Direct cell counting); 124 ± 4 (CCK-8 assay)), compared with those transfected with GIT1 wild-type (165 ± 11 (Direct cell counting); 153 ± 5 (CCK-8 assay)) (Fig. 3).

Integrin $\beta 1$ and Src Are Required for Periodic Mechanical Stress-induced GIT1 Phosphorylation

Pretreatment with blocking antibody against integrin $\beta 1$ attenuated significantly periodic mechanical stress-induced GIT1-Tyr³²¹ phosphorylation (0.51 ± 0.039) in chondrocytes under conditions of periodic mechanical stimulation, compared with those in the control groups (0.81 ± 0.044) (Fig. 4A). Src inhibition by PP2 pretreatment and shRNA targeted to Src abrogated significantly the levels of GIT1-Tyr³²¹ phosphorylation (0.56 ± 0.051 (PP2); 0.48 ± 0.039 (Src shRNA)) in chondrocytes under conditions of mechanical stress, compared with those in the control groups (0.83 ± 0.038 (DMSO); 0.81 ± 0.041 (NT shRNA)) (Fig. 4B and 4C).

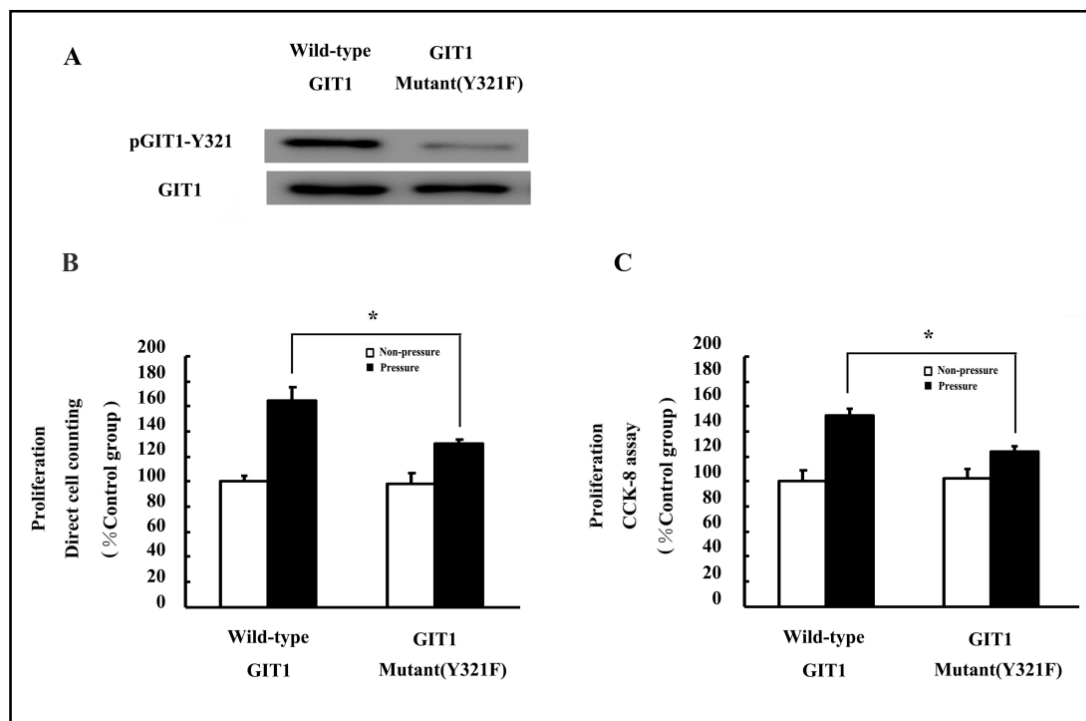


Fig. 3. Effects of GIT1-Tyr³²¹ on chondrocyte proliferation under conditions of periodic mechanical stress. Rat chondrocytes were transfected with a GFP-tagged GIT1 wide-type GV287 vector or GV287 expression mutant GIT1 (Tyr³²¹ to Phe³²¹). Phosphorylation of GIT1-Tyr³²¹ in the GIT1-Phe³²¹ group was significantly abolished, compared with that in the wide-type group (A). Rat chondrocytes were cultured for 3 days under non-pressure conditions or conditions of periodic mechanical stress 8 h per day prior to proliferation studies. Chondrocyte proliferation was analyzed using direct cell counting (B) and CCK-8 assay (C). Chondrocyte proliferation results are shown in the histogram (n = 5, *P < 0.05 for each).

FAK-Tyr³⁹⁷ and FAK-Tyr^{576/577} Are Not Required for Periodic Mechanical Stress-induced GIT1 Phosphorylation

Periodic mechanical stress-induced GIT1-Tyr³²¹ activation was not affected in chondrocytes transfected with FAK mutant-expressing plasmids (Phe³⁹⁷ and Phe^{576/577}) (0.83 ± 0.031 (Phe³⁹⁷); 0.78 ± 0.042 (Phe^{576/577})), compared with those transfected with FAK wild-type (0.79 ± 0.049 (Wild type); 0.82 ± 0.033 (Wild type)) (Fig. 5).

GIT1 Are Not Required for Periodic Mechanical Stress-induced Src and FAK-Tyr³⁹⁷ Phosphorylation

Periodic mechanical stress-induced Src-Tyr⁴¹⁸ and FAK-Tyr³⁹⁷ activation did not decrease in chondrocytes transfected with GIT1 mutant-expressing plasmids (Phe³²¹) (0.45 ± 0.019 (Src); 0.74 ± 0.033 (FAK-Tyr³⁹⁷)), compared with those transfected with FAK wild-type (0.48 ± 0.033 (Src); 0.76 ± 0.015 (FAK-Tyr³⁹⁷)) (Fig. 6A and 6B).

GIT1 Inhibition Prevents Periodic Mechanical Stress-induced FAK-Tyr^{576/577} and ERK1/2 Phosphorylation

Inhibition of GIT1 with shRNA targeted to GIT1 prevented significantly periodic mechanical stress-induced FAK-Tyr^{576/577} and ERK1/2 activation in chondrocytes under mechanical conditions (0.42 ± 0.028 (FAK-Tyr^{576/577}); 0.58 ± 0.036 (ERK1/2)), compared with those in the control groups (0.68 ± 0.039 (FAK-Tyr^{576/577}); 0.94 ± 0.042 (ERK1/2)) (Fig. 6C and 6D).

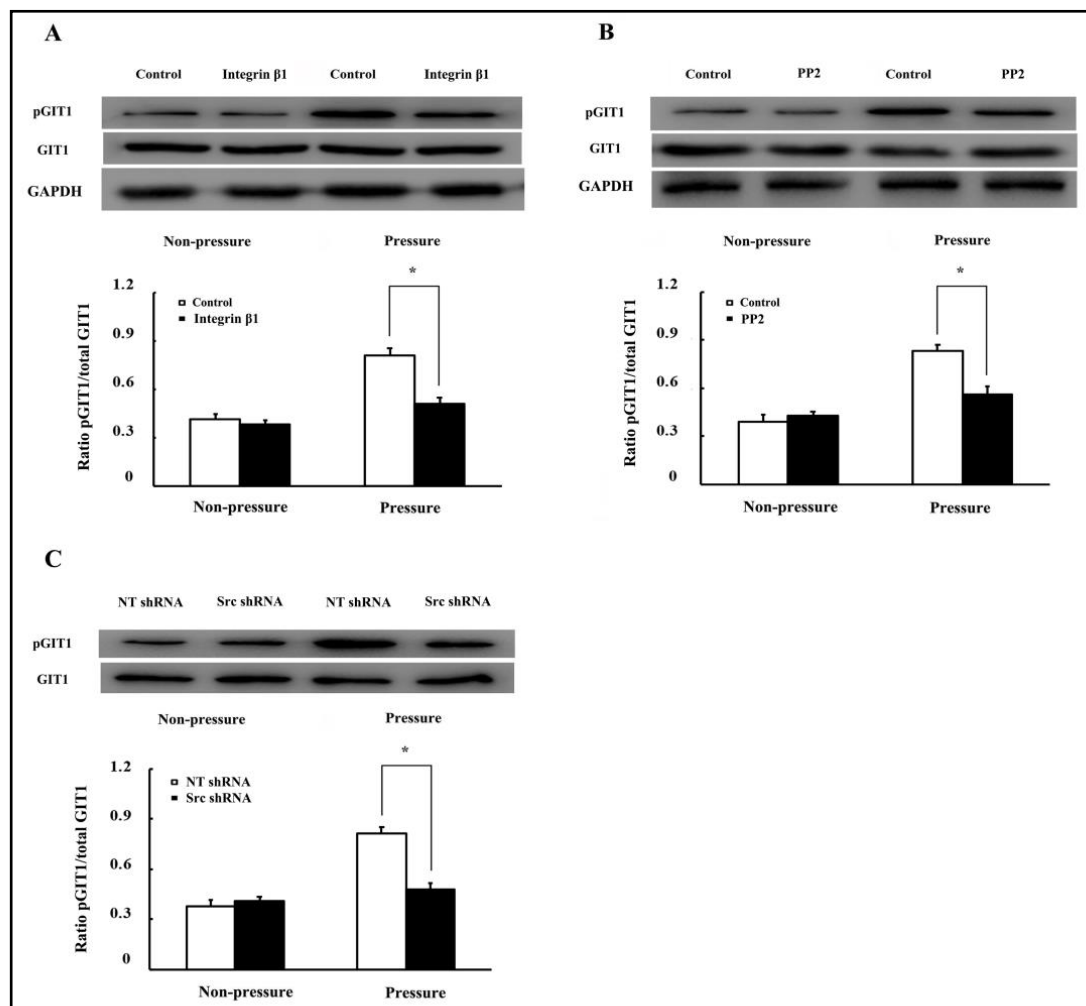


Fig. 4. Effects of integrin β 1 and Src on the expression and phosphorylation of GIT1 at Tyr³²¹ under conditions of periodic mechanical stress. After pretreatment with DMEM or blocking antibody against integrin β 1 (A), or DMSO or Src inhibitor PP2 (B), or NT sequence or Src shRNA (C), rat chondrocytes were cultured in vitro for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of GIT1 (pGIT1-Y321) were detected by Western blotting. The total amount of each protein served as one control (GAPDH served as the other control in A and B, to eliminate the influence of inhibitors on the expression of GIT1). Gray values are represented with a histogram (n = 5, *P<0.05 for each). The aforementioned images are representative results of Western blotting.

GIT1-Tyr³²¹ Inhibition Prevents Periodic Mechanical Stress-induced FAK-Tyr^{576/577} and ERK1/2 Phosphorylation

Inhibition of GIT1-Tyr³²¹ with GIT1 mutant-expressing plasmid (Phe³²¹) prevented significantly periodic mechanical stress-induced FAK-Tyr^{576/577} and ERK1/2 activation in chondrocytes under mechanical conditions (0.47 ± 0.023 (FAK-Tyr^{576/577}); 0.62 ± 0.041 (ERK1/2)), compared with those in the control groups (0.66 ± 0.019 (FAK-Tyr^{576/577}); 0.89 ± 0.024 (ERK1/2)) (Fig. 7).

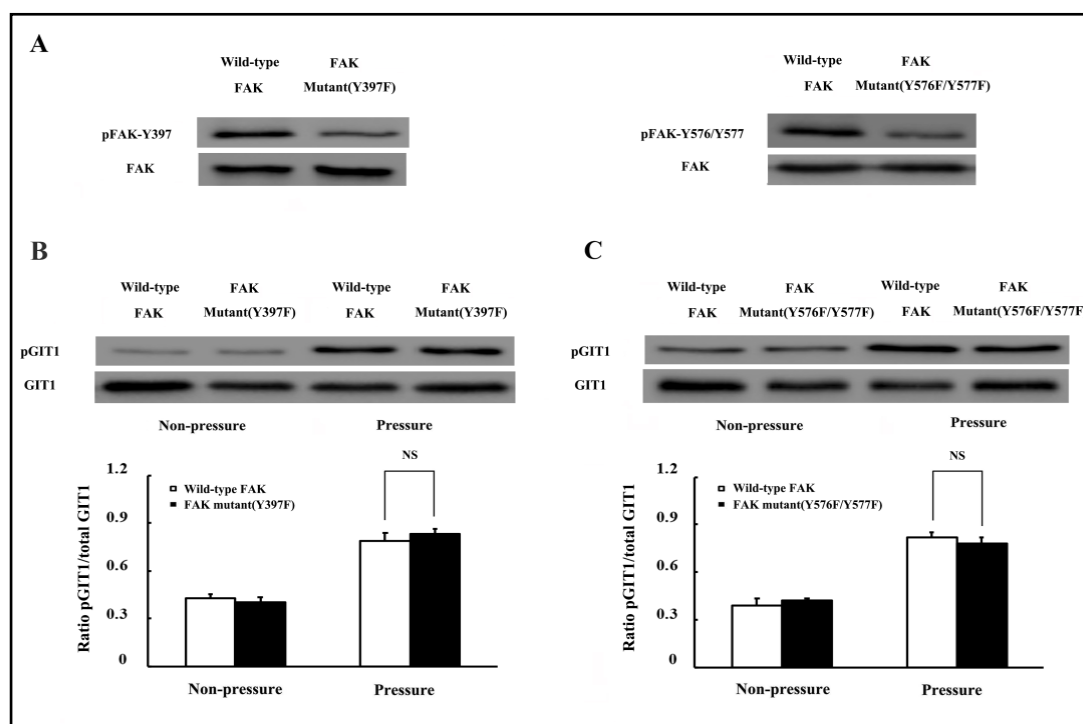


Fig. 5. Effects of FAK-Tyr³⁹⁷ and FAK-Tyr^{576/577} on the expression and phosphorylation of GIT1 under conditions of periodic mechanical stress. Rat chondrocytes were transfected with a GFP-tagged FAK wide-type GV287 vector or GV287 expression mutants FAK (Tyr³⁹⁷ to Phe³⁹⁷ and Tyr^{576/577} to Phe^{576/577}). Phosphorylation of FAK-Tyr³⁹⁷ in the FAK-Phe³⁹⁷ group and phosphorylation of FAK-Tyr^{576/577} in the FAK-Phe^{576/577} group were significantly abolished, respectively, compared with those in the wide-type group (A). Rat chondrocytes were cultured in vitro for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of GIT1 (pGIT1-Y321) were detected by Western blotting (B: FAK Mutant (Y397F); C: FAK Mutant (Y576F/Y577F)). The total amount of each protein served as a control. Gray values are represented with a histogram n = 5, *P<0.05 for each). The aforementioned images are representative results of Western blotting.

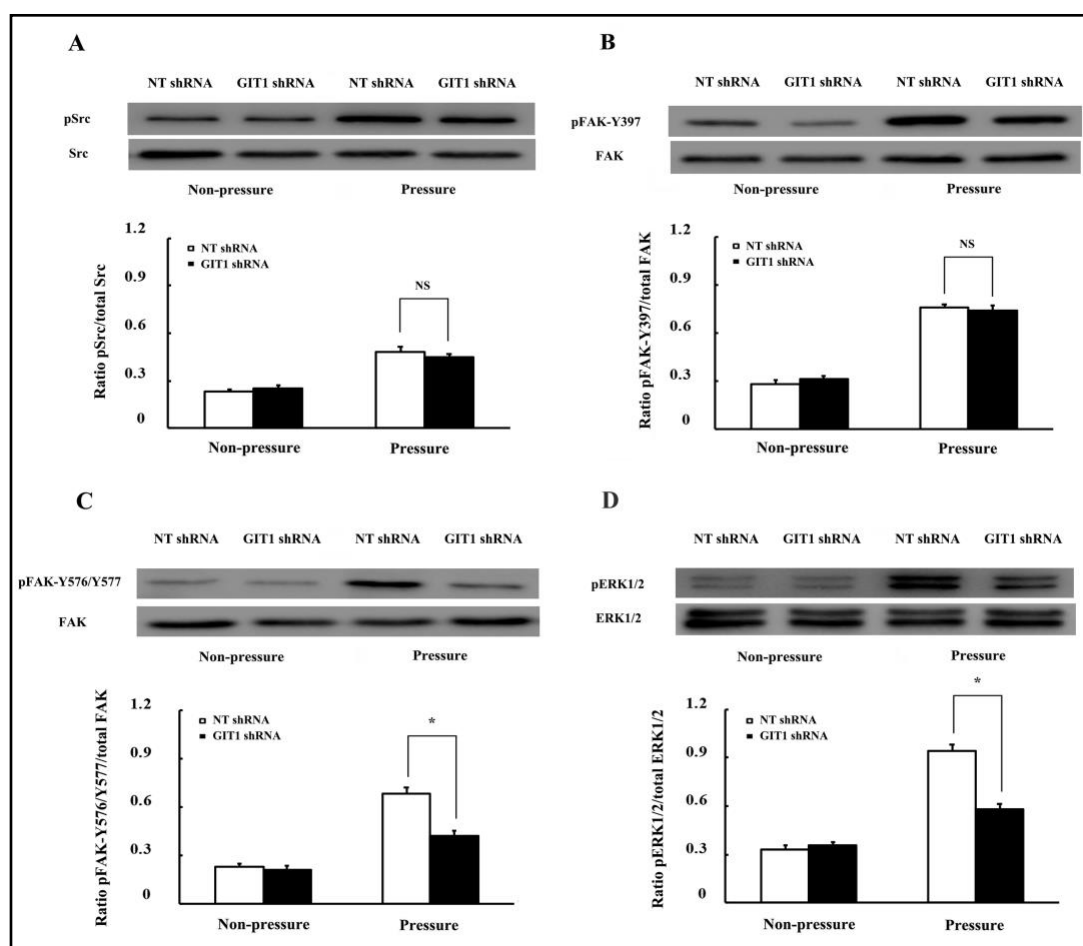


Fig. 6. Effects of GIT1 on the expression and phosphorylation of Src, FAK (Tyr³⁹⁷ and Tyr^{576/577}) and ERK1/2 under conditions of periodic mechanical stress. After pretreatment with NT sequence or GIT1 shRNA, rat chondrocytes were cultured in vitro for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of Src (A), FAK (Tyr³⁹⁷ (B) and Tyr^{576/577} (C)) and ERK1/2 (D) were detected by Western blotting. The total amount of each protein served as a control. Gray values are represented with a histogram n = 5, *P<0.05 for each). The aforementioned images are representative results of Western blotting.

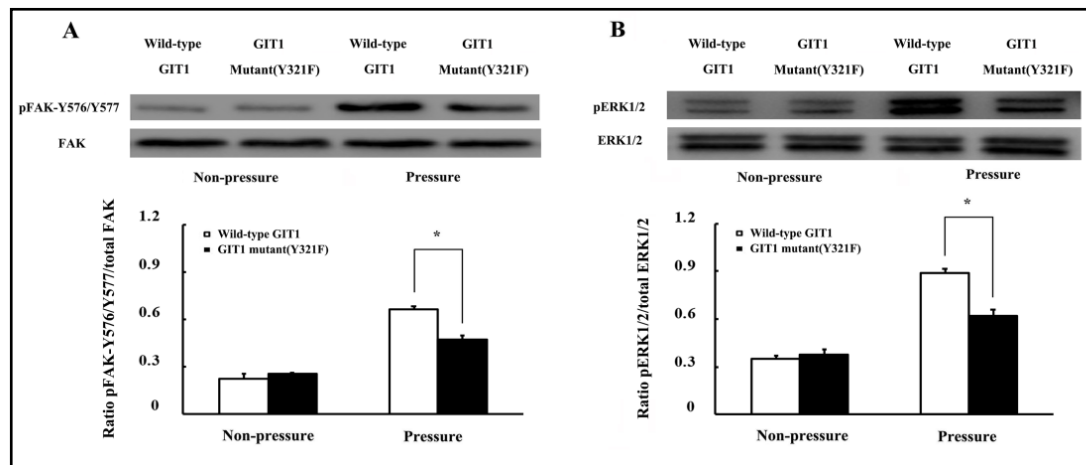


Fig. 7. Effects of GIT1-Tyr³²¹ on the expression and phosphorylation of FAK (Tyr^{576/577}) and ERK1/2 under conditions of periodic mechanical stress. Rat chondrocytes were transfected with a GFP-tagged GIT1 wide-type GV287 vector or GV287 expression mutant GIT1 (Tyr³²¹ to Phe³²¹). Rat chondrocytes were cultured in vitro for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of FAK (Tyr^{576/577}) (A) and ERK1/2 (B) were detected by Western blotting. The total amount of each protein served as a control. Gray values are represented with a histogram n = 5, *P<0.05 for each. The aforementioned images are representative results of Western blotting.

Discussion

Periodic mechanical stress induction of chondrocyte proliferation may be mediated through a process of signaling transduction, integrating these signals into cellular responses. Our present findings demonstrated that GIT1 and GIT1-Tyr³²¹ are required for integrin β 1-ERK1/2 mitogenic signaling cascade in chondrocytes in response to periodic mechanical stress.

The catalytic functions of GIT1 are dependent on the phosphorylation of tyrosine residues, which affects various cellular activities [31]. Webb used mass spectrometry to generate a phosphorylation map of GIT1, among potential tyrosine residues, Tyr³²¹ was suggested to be a possible activated phosphorylation site [32]. And Yin has previously shown that GIT1-Tyr³²¹ is a key regulator of bone mass and osteoblast cell migration [6]. We observed that periodic mechanical stress statistically and significantly enhanced the phosphorylation of GIT1 at Tyr³²¹ in chondrocytes. Moreover, depletion of GIT1 with shRNA abolished chondrocyte proliferation following periodic mechanical stimuli. These results provide definitive evidence to support the participation of GIT1 signaling in the regulation of the mitogenic responses to periodic mechanical stimulation in chondrocytes. Meanwhile, GIT1 Y321F mutant resulted in blockage of cell proliferation in this system. This is a novel discovery that clearly highlighted the significance of site-specific tyrosine residue within GIT1 in chondrocytic adaption to physical mechanical stimuli. Despite the importance of GIT1-Tyr³²¹ in mediating the mechanotransduced mitogenic effects, it is possible that other phosphorylation residues within GIT1 may also play an indispensable role in chondrocytes.

The activation sites in the domain of GIT1 allow for specific binding, which is essential for several downstream transduction signal events [11, 33, 34]. A study by Pang and his colleagues documented that vascular endothelial growth factor (VEGF)-initiated ERK1/2 phosphorylation is dependent on the activation of GIT1 modulating pulmonary vascular behavior and functions [5]. In the present study, the inhibition of GIT1 by targeted shRNA was found to abrogate the activation of ERK1/2, suggesting that GIT1 activation of ERK1/2 contributes to the mechanism by which chondrocytes respond to physical mechanical stimulation. Moreover, pretreatment with GIT1 Y321F mutant prevented ERK1/2 phosphorylation stimulated by periodic mechanical stress. Therefore, it could

be concluded that activation of GIT1 at Tyr³²¹ seemed to mediate at least some mitogenic mechanotransduced signals through ERK1/2 MAPKs in chondrocytes.

Increasing evidence has concluded that the relationship between GIT1 and Src, FAK is complex and still controversial in diverse complex signaling pathways [35-38]. Zhang et al. demonstrated that endothelins stimulation of endothelial nitric-oxide synthase (eNOS) in sinusoidal endothelial cells is initiated by activated GIT1 through a Src signaling transduction mechanism [19]. Instead of being activated by a GIT1-dependent mechanism, the inhibition of cyclic mechanical stress-stimulated GIT1-Tyr³²¹ by PP2 or shRNA targeted to Src, suggested that Src might be responsible for the initial GIT1 activation in the present study. This was consistent with other observation of Src modulation and interaction with GIT1 in other cell types in different systems [22, 39]. However, this may not be universally true, and Src and GIT1 signals can also act in parallel in human liver and colon cancer cells and in other settings [35].

GIT1 may also be activated by other signaling proteins, such as FAK, and mechanosensors following mechanical stimulation. Tyrosine phosphorylation at different sites within FAK is catalyzed in distinct ways and affects various cellular functions. Our previous study and works by others regarding FAK activity mainly concentrate on the Tyr³⁹⁷ and Tyr^{576/577} phosphorylation sites in chondrocytes and other cell types under mechanical stimulation [18, 40]. In our experiments, transfected with FAK Y397F or Y576/577F mutants did not decrease periodic mechanical stress-enhanced GIT1 activation in chondrocytes, while GIT1 inhibition by shRNA targeted to GIT1 and GIT1 Y321F mutant attenuated FAK phosphorylation at Tyr^{576/577}, but not Tyr³⁹⁷. Based on the results, we can provide definitive evidence to support that GIT1 activation of FAK at Tyr^{576/577}, but not Tyr³⁹⁷, is responsible for the signaling transduction mechanisms in chondrocytes under conditions of cyclic mechanical stimulation. Meantime, based on our present and previous results, periodic mechanical stress-activated GIT1 and Tyr³⁹⁷ signals may act in parallel in this setting.

Recently, GIT1 was viewed as an important integrin-associated kinase that receives and transmits various integrin-initiated intracellular biochemical signals [16, 41, 42]. The activation of GIT1 by integrin has been reported to be a signaling transduction mechanism. However, this is still obscure in chondrocytes under mechanical stimulation. Our present study revealed that pretreatment with an integrin β 1 functional blocking antibody abrogated the activation of GIT1-Tyr³²¹ in chondrocytes under periodic mechanical stress conditions. These data above demonstrate the concept that integrin β 1, as a proximal mechanosensor, play a key role in the signals that lead to the increased GIT1 activation and subsequent chondrocyte proliferation in response to physical mechanical stress. Our present observation seems consistent with a study on vascular stability, cerebral angiogenesis and endothelial cell proliferation in the developing embryo disclosed that GIT1 phosphorylation is mediated by activation of cell-surface integrin [15].

Conclusion

In conclusion, GIT1 is a crucial decision-making protein participating in chondrocytic ERK1/2 mitogenic signals initiated by periodic mechanical stress. Specifically, based on the current and previous results, two activated signaling pathways converge on ERK1/2: one requires activation of Src and is GIT1-dependent, which stimulates activated FAK-Tyr^{576/577}, and the other whereby separate phosphorylation at Tyr³⁹⁷ occurs independently of Src and GIT1, although both pathways are initiated by integrin β 1.

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Disclosure Statement

The authors declare that they have no competing interests.

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