

Original Paper

Periodic Mechanical Stress Activates Integrin β 1-Dependent Src-Dependent PLC γ 1-Independent Rac1 Mitogenic Signal in Rat Chondrocytes through ERK1/2

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Key Words

Periodic mechanical stress • Chondrocyte proliferation and matrix synthesis • Rac1 • Integrin β 1 • Integrin β 3

Abstract

The effects of periodic mechanical stress on the mitogenesis of chondrocytes have been studied extensively in recent years. However, the mechanisms underlying the ability of chondrocytes to sense and respond to periodic mechanical stress remain a matter of debate. We explored the signal transduction pathways of proliferation and matrix synthesis when chondrocytes were exposed to periodic mechanical stress. We observed that periodic mechanical stress statistically and significantly enhanced the phosphorylation and activation of Rac1 ($p < 0.05$ for each). Pre-treatment with the Rac1 selective inhibitor NSC23766 attenuated periodic mechanical stress-induced chondrocyte proliferation and matrix synthesis ($p < 0.05$ for each) and abrogated ERK1/2 signal activation ($p < 0.05$), but did not block periodic mechanical stress-induced Src and PLC γ 1 phosphorylation in this context. In addition, inhibition of Src with its selective inhibitor PP2 and shRNA targeted to Src blocked Rac1 signal activation ($p < 0.05$ for each), but inhibition of the activity of PLC γ 1 did not affect the phosphorylation and activation levels of Rac1 under conditions of periodic mechanical stress. The up-regulation of proliferation and matrix synthesis was inhibited in chondrocytes in response to periodic mechanical stress after pretreatment with blocking antibody against integrin β 1 ($p < 0.05$ for each) but not after pretreatment with blocking antibody against integrin β 3. The phosphorylation levels of ERK1/2, Rac1, PLC γ 1 and Src, and Rac1 activation level were also reduced when integrin β 1 was blocked in this context ($p < 0.05$ for each). These findings suggest that periodic mechanical stress promotes chondrocyte proliferation and matrix synthesis in part by activating the ERK1/2 mitogenic signal through the integrin β 1-Src-PLC γ 1/Rac1-ERK1/2 pathway, which links these important signaling molecules into mitogenic cascades.

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Introduction

Periodic mechanical stress could significantly promote chondrocyte proliferation and matrix synthesis [1-4]. These mechanical stimuli are, in fact, important factors in maintaining the normal structure and function of articular cartilage. However, the mechanisms underlying the ability of chondrocytes to detect and respond to periodic mechanical stress have not been well delineated.

Several lines of evidence suggest that Rac1, known as one of the small GTP-binding proteins of the Rho family, is responsible for controlling many downstream signaling cascades, including ERK1/2 signals [5-8]. Rac1 signaling pathways could be activated by mechanical stimulation in numerous non-chondrocytic cell types, and these pathways can mediate multiple cell activities and functions [9-12]. Work from Chaturvedi has demonstrated that repeated deformation-induced intestinal epithelial cell proliferation is dependent on ERK1/2, whereas ERK1/2 activity is mediated by activated Rac1 [11]. Following periodic mechanical stress, the role of the small GTPase Rac1 in mechanosensing, signaling transduction, and final mitogenic effects in chondrocytes, including the nature of any functional association between Rac1 and ERK1/2 in this context, has yet to be explored.

Our previous study found that Src and PLC γ 1 are both activated following periodic mechanical stress, and they mediate an ERK1/2 signaling pathway in that system that is responsible for chondrocyte proliferation and matrix synthesis. The upstream and downstream relationships between Src, PLC γ 1, and Rac1 have been mentioned many times in studies concerning signaling transduction [13-16]. However, different systems, different cell types, and different physical forces may be associated with different mechanical stimulation-activated signaling pathways. Moreover, the functional association between PLC γ 1 and Rac1 is particularly complex and uncertain. Thus, the nature of the relationship between Src, PLC γ 1, and Rac1 in chondrocytes in this setting has yet to be elucidated.

Integrins are cell-surface adhesion receptors that regulate cell viability and function in response to cues derived from the extracellular environment [17-19]. As the main cell-surface mechanosensors, integrins transform mechanical stimuli into chemical signals in chondrocytes and other various cell types [20-22]. Increasing evidence confirms that, in various non-chondrocytic cell types, mechanical stimuli activate downstream signals and cell proliferation mainly by initiating integrin β 1 and/or integrin β 3, but it is not clear whether the same occurs in chondrocytes [23, 24]. Integrins have no kinase activity, and those signaling pathways initiated by integrins must be transmitted into cells through the activation of downstream signaling molecules, such as Src and ERK1/2 [23]. Thus, we have reason to speculate that, in chondrocytes, the activation of Src, PLC γ 1, Rac1, and ERK1/2 induced by periodic mechanical stress depends on integrin β 1 and/or integrin β 3 in this context.

We therefore sought to determine whether Rac1 activates ERK1/2 mitogenic signals in chondrocytes under periodic mechanical stress, and the nature of the relationship between Src, PLC γ 1, and Rac1 in this setting. Our present study was also designed to confirm whether integrin β 1 and/or integrin β 3 mediates ERK1/2 mitogenic signals and to identify the signaling pathways through which integrin β 1 and/or integrin β 3 modulates ERK1/2 in chondrocytes following periodic mechanical stress, in order to trace the mechanotransduction pathways and link these signals into mitogenic cascades.

Materials and Methods

Materials

Two-week-old Sprague-Dawley (SD) rats of either sex were provided by the Animal Center of Nanjing Medical University. Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd (China). DMEM-F12, 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-Src, anti-phospho-Src (Tyr⁴¹⁸), anti-PLC γ 1, anti-phospho-PLC γ 1 (Tyr⁷⁸³), anti-Rac1-cdc42, anti-phospho-Rac1-cdc42 (Ser⁷¹), anti-ERK1/2, anti-phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴), HRP-goat anti-rabbit IgG, and PD98059 were supplied by CST Co., Ltd. (USA). ECL, PP2, and U73122 were purchased from Amersham (UK), BIOMOL (USA), and Santa Cruz (USA), respectively. Blocking antibodies against integrin β 1 and integrin β 3, and NSC23766 were supplied by BD Biosciences (USA), Chemicon International (USA) and Calbiochem (USA), respectively. RNAiso Plus, PrimeScript RT Reagent Kit, and SYBR Premix Ex Taq II were purchased from TaKaRa (Japan). Src shRNA (r) Lentiviral Particles, Control shRNA Lentiviral Particles and Polybrene were supplied by Santa Cruz (USA).

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 [25]. Cells were purified by repeated adherence, and morphology was observed under an inverted phase contrast microscope by staining collagen type II according to the conventional ABC method. Cells of the second generation were seeded on a glass slide (25 mm \times 25 mm) coated with type II collagen at a density of 10^5 cells. Experiments were performed when cells were approximately 70-80% confluent.

Inhibitors

Blocking antibodies against integrin β 1 and integrin β 3, PP2, U73122, NSC23766, and PD98059 were specific inhibitors of integrin β 1, integrin β 3, PP2, PLC γ 1, Rac1, and ERK1/2, respectively. Blocking antibodies against integrin β 1 and integrin β 3, and Rac1 were dissolved in DMEM, and the other inhibitors were dissolved in anhydrous dimethylsulfoxide (DMSO) to form 1,000 \times concentrated solution. Aliquots of all inhibitors were stored at -20°C. Each concentrated solution was diluted by 1,000 \times immediately prior to use; PP2, U73122, and PD98059 pretreatment groups contained 0.1% (v/v) DMSO, which was the concentration used for the control group. Cells were pre-treated with PP2 (10 μ M), U73122 (10 μ M), NSC23766 (50 μ M), PD98059 (50 μ M), or an equivalent amount of DMEM or DMSO (0.1% v/v) for 1 h. The remaining cells were pre-treated with blocking antibodies against integrin β 1 or integrin β 3 (10 μ g/mL for each) or an equivalent amount of DMEM for 5 h.

Construction of a periodical mechanical stress field

A periodic stress field encompassing the perfusion culture system with adjustable stress intensity and frequency was constructed by connecting the reciprocating intensifier pump to the air-tight cell culture device through a barrier-type pressure transducer, as previously described [26]. Our previous study found that tissue-engineered cartilage exhibited the best quality when the rabbit chondrocytes underwent stress varying from 0 kPa to 200 kPa at 0.1 Hz. Therefore, this pressure range (0-200 kPa) and frequency (0.1 Hz) were used in the experiment.

Experimental groups

Two steps were undertaken in the experiment.

In the first step, cells were divided into a non-pressure group and a pressure group. Cells were maintained under static conditions or periodic mechanical stress for 0 h, 0.5 h, 1 h, or 2 h prior to Western blot analysis and Racc1 GTPase activity assay.

In the second step, cells were pretreated with blocking antibodies against integrin β 1 or integrin β 3 (10 μ g/mL for each), PP2 (10 μ M), U73122 (10 μ M), NSC23766 (50 μ M), or PD98059 (50 μ M), respectively. Or cells were pretreated with shRNA targeted to Src or control scrambled shRNA, respectively. Cells were maintained under static conditions or periodic mechanical stress for 1 h prior to Western blot analysis and Rac1 GTPase activity assay, for 8 h prior to qPCR analysis (aggrecan and type II collagen gene expression), and for 3 d (8 h per d mechanical stress) prior to direct cell counting, CCK-8 assay and Western blot analysis (aggrecan and type II collagen protein expression).

All groups of cells involved in the experiments were incubated at 37°C with 5% CO₂.

Table 1. Primer sequences and product sizes in real-time PCR.

Genes	Sequence of primers (5'-3')	AS(bp)	AT(degrees)
Aggrecan	F GAAGTGATGCATGGCATTGAGG R ATGATGGCGCTGTTCTGAAGG	146	60
Typr II collagen	F GAGGGCAACAGCAGGTTTAC R TGTGATCGGTACTCGATGATGG	95	60
GAPDH	F GGCACAGTCAAGGCTGAGAATG R ATGGTGGTGAAGACGCCAGTA	143	60

F: forward; R: reverse; AS: amplicon size; bp: base pairs; AT: annealing temperature

Western blot analysis

Total protein was prepared and Western blot analyses were performed as previously described [27]. Total protein was prepared using RIPA buffer and the Bradford assay was used to determine protein concentration. Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Following blocking for 1 h with 5% milk in TBST, the membranes were incubated with antibodies (1:1,000 dilutions for three antibodies) overnight at 4°C. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody at ambient temperature for 1 h and the colors were developed with ECL. The results were scanned using a Gel Imaging System (UVP Company, USA) and measured using Gel-Pro Analyzer software (Media Cybernetics, US).

Quantitative real-time PCR (qPCR) analysis

Total RNA was extracted using RNAiso Plus and was reverse transcribed into cDNA with the PrimeScript RT Reagent Kit according to the manufacturer's protocol. qPCR analysis was performed with the LightCycler System (Roche Diagnostics) using SYBR Premix Ex Taq II as previously described [28]. The reaction was performed in a 20- μ L mixture containing 2 μ L of the above cDNA. Each cDNA sample was amplified using specific primers (Table 1, TaKaRa, Japan). The cycling conditions were a 30 s initial denaturation step at 95°C followed by 40 cycles at 95°C for 5 s and 60°C for 20 s. Gene expression for AGC and Col2 was normalized against that for GAPDH.

Proliferation studie

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

Direct cell counting

Cells were trypsinized and counted as previously described [11]. Second generation chondrocytes were seeded on glass slides (25 mm \times 25 mm) coated with type II collagen at a density of 10^5 cells and were randomly divided into different groups. Each group included cells on six glass slides. Experiments were performed when cells had reached approximately 70-80% confluence. Chondrocytes were cultured for 3 days under non-pressure conditions or conditions of periodic mechanical stress 8 h per day prior to direct cell counting. Cells were trypsinized and counted. Cell number was determined by counting cells from each glass slide independently. The experiments were repeated five times.

CCK-8 assay

Cell proliferation was determined by using CCK-8 solution according to the manufacturer's instructions as previously described [29]. Cells were added to 10 μ L CCK-8 solution in each well of five 96-well plates (n=5) and incubated for 4 h at 37°C. The absorbance of each well was finally determined at 450 nm by using a microplate reader.

Rac1 GTPase activity assay

Rac1 activity was assessed by a pulldown assay as described previously [11]. Cells were lysed in a buffer containing 20 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 2 mM NaF, 1% Triton X-100, 10% glycerol, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, and 1 mM PMSF at pH 7.6. Extracts were sonicated and clarified by centrifugation (10 min, 14,000 rpm at 4°C), and the protein concentration was determined by the BCA

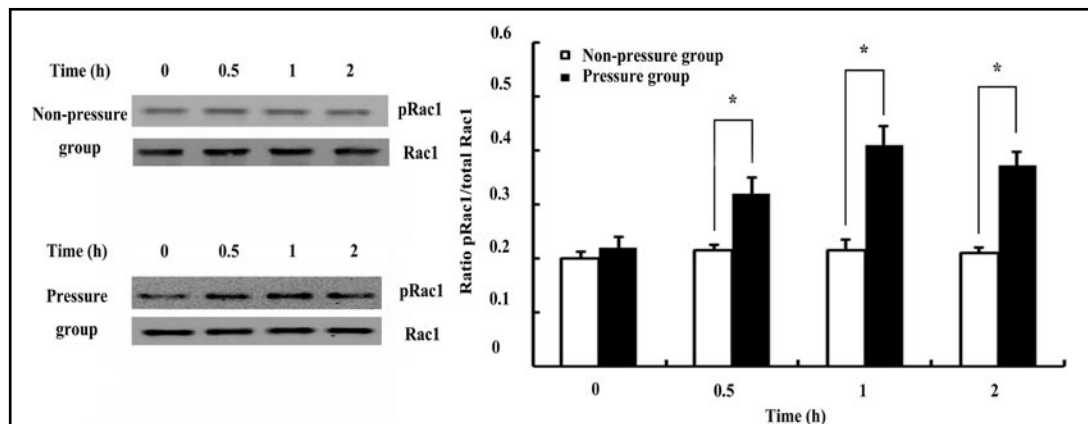


Fig. 1. Effects of periodic mechanical stress on the expression and phosphorylation of Rac1. Rat chondrocytes were cultured *in vitro* for 0 h, 0.5 h, 1 h and 2 h with or without periodic mechanical stress. The expression and phosphorylation levels of Rac1 were detected by western blotting and the total quantity of Rac1 served as the control. Results are represented in the histogram ($n=5$, *, $p<0.05$ for each). The images above are representative results of western blotting. The phosphorylation levels of Rac1 in the pressure groups were significantly increased in comparison with those in the non-pressure groups ($n=5$, $p<0.05$ for each, Student unpaired *t*-test).

method. Active Rac1 levels were determined by glutathione *S*-transferase-conjugated p21 binding domain (GST-PBD) of p21-activated kinase 1 pulldown assays. The cell lysates were incubated with PAK-PBD beads for 1 h at 4°C on a rotator, and the beads were pelleted by centrifugation at 5000 $\times g$ for 3 min at 4°C. The resulting pellet was then resuspended in Laemmli buffer, resolved electrophoretically, transferred to nitrocellulose, and immunoblotted with monoclonal anti-Rac1 antibody. 15 to 20 μg of cell lysates were used for Western blots for total Rac1 antibody.

Statistical analysis

Statistical analyses were performed using SPSS 14.0 software and the results are expressed as the mean \pm standard deviation. Student unpaired *t*-tests and one-way analysis of variance (ANOVA) followed by post-hoc Fisher's least significance difference (LSD) tests were used to determine the statistical significance. A *p* value of 0.05 was considered to be significant.

Results

Periodic mechanical stress stimulates Rac1 phosphorylation

We observed that periodic mechanical stress statistically and significantly increased Rac1-cdc42-Ser⁷¹ phosphorylation levels relative to those in chondrocytes cultured under static conditions ($p<0.05$ for each, Fig. 1, $n=5$).

Periodic mechanical stress stimulates Rac1 activation

To further confirm that Rac1 is activated in response to periodic mechanical stress, we performed a Rac1 pulldown assay. We observed that periodic mechanical stress statistically and significantly increased Rac1 activity relative to that in chondrocytes under static conditions ($p<0.05$ for each, Fig. 2, $n=5$).

Rac1 is required for periodic mechanical stress-initiated chondrocyte proliferation and matrix synthesis

We then investigated whether Rac1 was involved in periodic mechanical stress-induced chondrocyte proliferation and matrix synthesis. Chondrocytes were pretreated with NSC23766 (50 μM) or with control vehicle (DMEM) for 1 h, and then chondrocyte

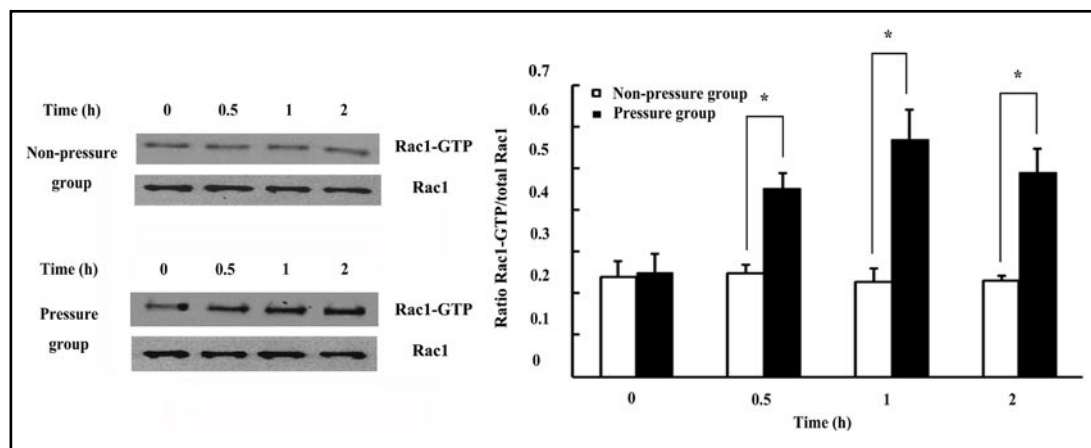


Fig. 2. Effects of periodic mechanical stress on the activation of Rac1. Rat chondrocytes were cultured *in vitro* for 0 h, 0.5 h, 1 h and 2 h with or without periodic mechanical stress. Rac1 activation was quantified by pulldown assays of the GST-PBD domain of p21-activated kinase 1, and the total quantity of Rac1 served as the control. Results are represented in the histogram (n=5, *, $p < 0.05$ for each). The images above are representative results. The activation levels of Rac1 in the pressure groups were significantly increased in comparison with those in the non-pressure groups (n=5, $p < 0.05$ for each, Student unpaired *t*-test).

proliferation and matrix synthesis were measured. Rac1 inhibition blocked periodic mechanical stress-induced chondrocyte proliferation, aggrecan and type II collagen gene and protein expressions in comparison with the control group (DMEM-pretreated chondrocytes) ($p < 0.05$ for each, Fig. 3, n=5).

Integrin β 1 but not integrin β 3 is required for periodic mechanical stress-stimulated chondrocyte proliferation and matrix synthesis

We sought to determine whether integrin β 1 and/or integrin β 3 signaling pathways were also required to stimulate chondrocyte proliferation and matrix synthesis during periodic mechanical stress. Pretreatment with blocking antibodies against integrin β 1 (10 μ g/mL) for 5 h before the initiation of periodic mechanical stress prevented the up-regulation of chondrocyte proliferation and aggrecan and type II collagen gene expression ($p < 0.05$ for each, Fig. 4, n=5). Pretreatment with blocking antibodies against integrin β 3 (10 μ g/mL) did not elicit the same response in chondrocytes, compared with the control group (DMEM-pretreated chondrocytes), subjected to periodic mechanical stress ($p > 0.05$ for each, Fig. 4, n=5).

Rac1 is required for periodic mechanical stress-induced ERK1/2 phosphorylation

NSC23766 (50 μ M) blocked periodic mechanical stress-induced ERK1/2 phosphorylation in comparison with the control group (DMEM-pretreated chondrocytes) growing under the same conditions ($p < 0.05$ for each, Fig. 5, n=5).

Rac1 is not required for periodic mechanical stress-induced Src and PLC γ 1 phosphorylation

Inhibition of Rac1 with its selective inhibitor NSC23766 (50 μ M) did not block periodic mechanical stress-induced Src or PLC γ 1 activation in comparison with the control group (DMEM-pretreated chondrocytes) growing under the same conditions ($p > 0.05$ for each, Fig. 5, n=5).

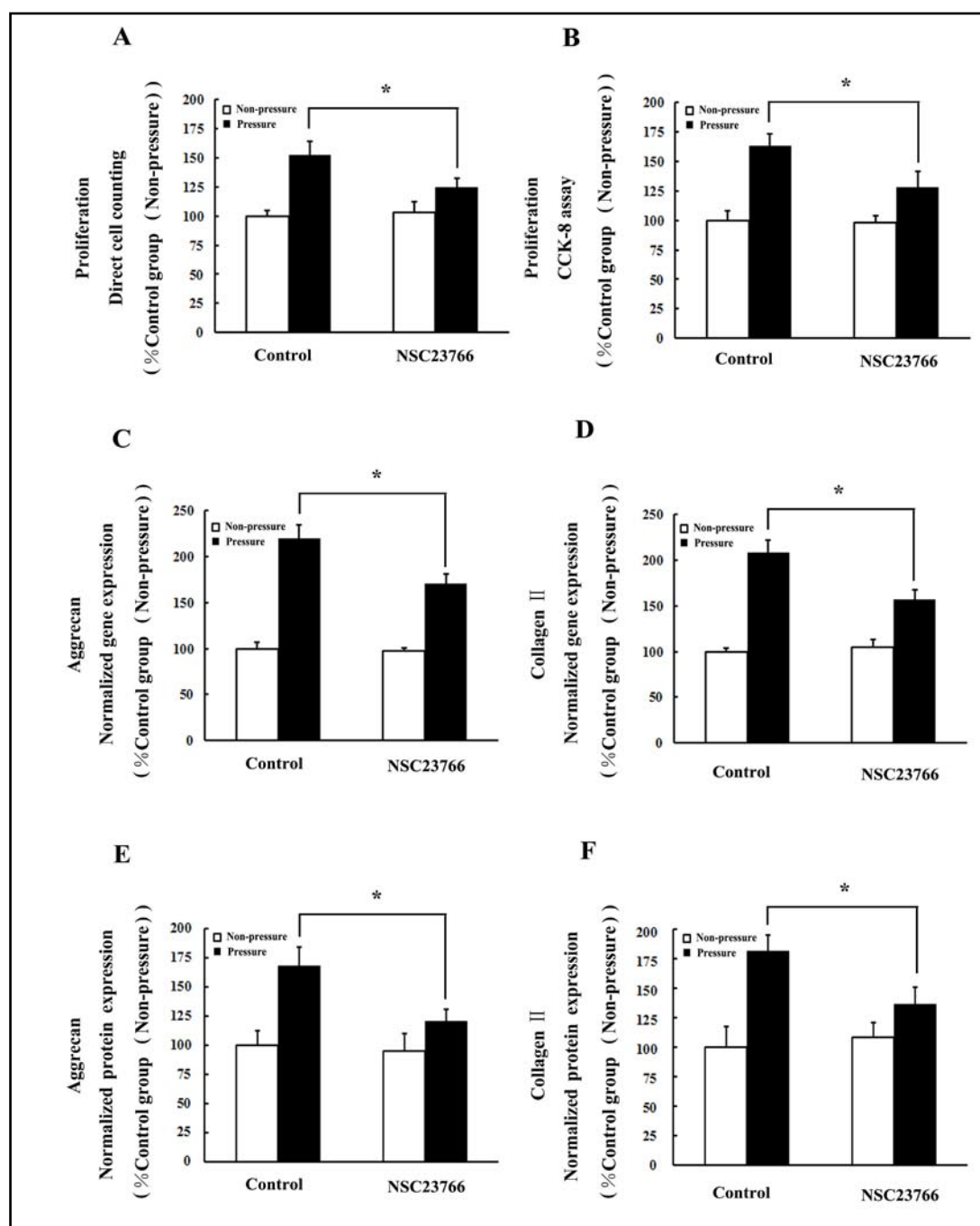


Fig. 3. Effects of Rac1 on chondrocyte proliferation and matrix synthesis under conditions of periodic mechanical stress. After pretreatment with control vehicle (DMEM) or Rac1 selective inhibitor (NSC23766), rat chondrocytes were cultured for 3 d under static conditions or conditions of periodic mechanical stress for 8 h per day prior to proliferation studies, or chondrocytes were cultured *in vitro* for 8 h with or without periodic mechanical stress prior to matrix synthesis studies. Chondrocyte proliferation was analyzed using direct cell counting (A) and CCK-8 assay (B). Chondrocyte matrix synthesis was analyzed using qPCR assay and western blotting. Aggrecan (C) and type II collagen (D) gene expressions and aggrecan (E) and type II collagen (F) protein expressions were adopted to reflect the levels of matrix synthesis of the chondrocytes. Chondrocyte proliferation and matrix synthesis results are shown in the histogram ($n=5$, $*$, $p<0.05$ for each). Chondrocyte proliferation and matrix synthesis in the NSC23766 pretreatment groups were significantly inhibited compared with those in the control group ($n=5$, $p<0.05$ for each, Student unpaired t -test).

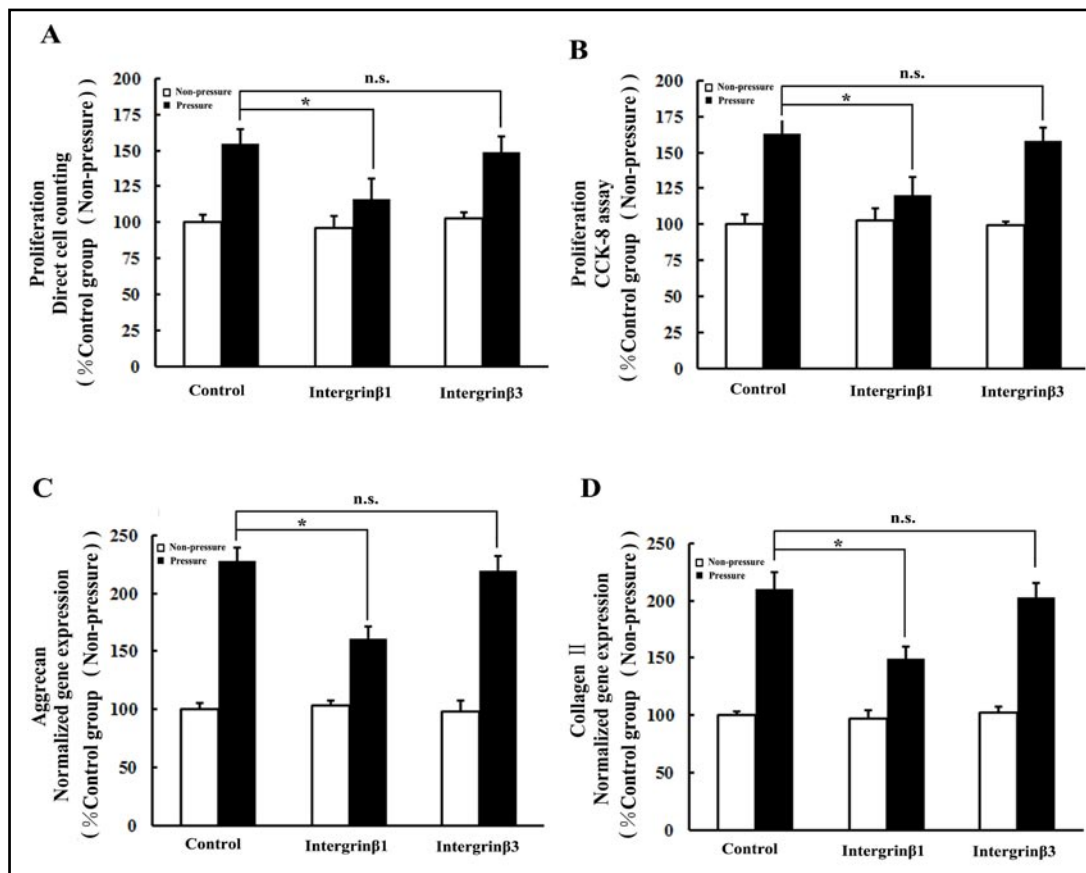


Fig. 4. Effects of integrin β 1 and integrin β 3 on chondrocyte proliferation and matrix synthesis under conditions of periodic mechanical stress. After pretreatment with control vehicle (DMEM) or blocking antibodies against integrin β 1 or integrin β 3, rat chondrocytes were cultured for 3 d under static conditions or with periodic mechanical stress for 8 h per day prior to proliferation studies, or chondrocytes were cultured *in vitro* for 8 h with or without periodic mechanical stress prior to matrix synthesis studies. Chondrocyte proliferation was analyzed using direct cell counting (A) and CCK-8 assay (B). Chondrocyte matrix synthesis was analyzed using qPCR assay. Aggrecan (C) and type II collagen (D) gene expressions were adopted to reflect the levels of matrix synthesis of the chondrocytes. Chondrocyte proliferation and matrix synthesis results are shown in the histogram (n=5, *, p<0.05 for each). Chondrocyte proliferation and matrix synthesis in the blocking antibody against integrin β 1 pretreatment groups were significantly diminished in comparison with those in the control group in response to periodic mechanical stress (n=5, p<0.05 for each, One-way analysis of variance (ANOVA) followed by post-hoc Fisher's least significance difference (LSD) test). Chondrocyte proliferation and matrix synthesis in the blocking antibody against integrin β 3 pretreatment groups were not appreciably altered in this context (n=5, p>0.05 for each, One-way analysis of variance (ANOVA) followed by post-hoc Fisher's least significance difference (LSD) test).

Src is required for periodic mechanical stress-induced Rac1 phosphorylation and activation

Relative to that in the control groups, Rac1 phosphorylation and activation was significantly diminished after treatment with PP2 (10 μ M) and shRNA targeted to Src in response to periodic mechanical stress (p<0.05 for each, Fig. 6, n=5).

PLC γ 1 inhibition does not prevent periodic mechanical stress-induced Rac1 phosphorylation and activation

We evaluated whether PLC γ 1 was required for the activation of Rac1 stimulated by periodic mechanical stress. PLC γ 1 inhibition by U73122 (10 μ M) pretreatment did not

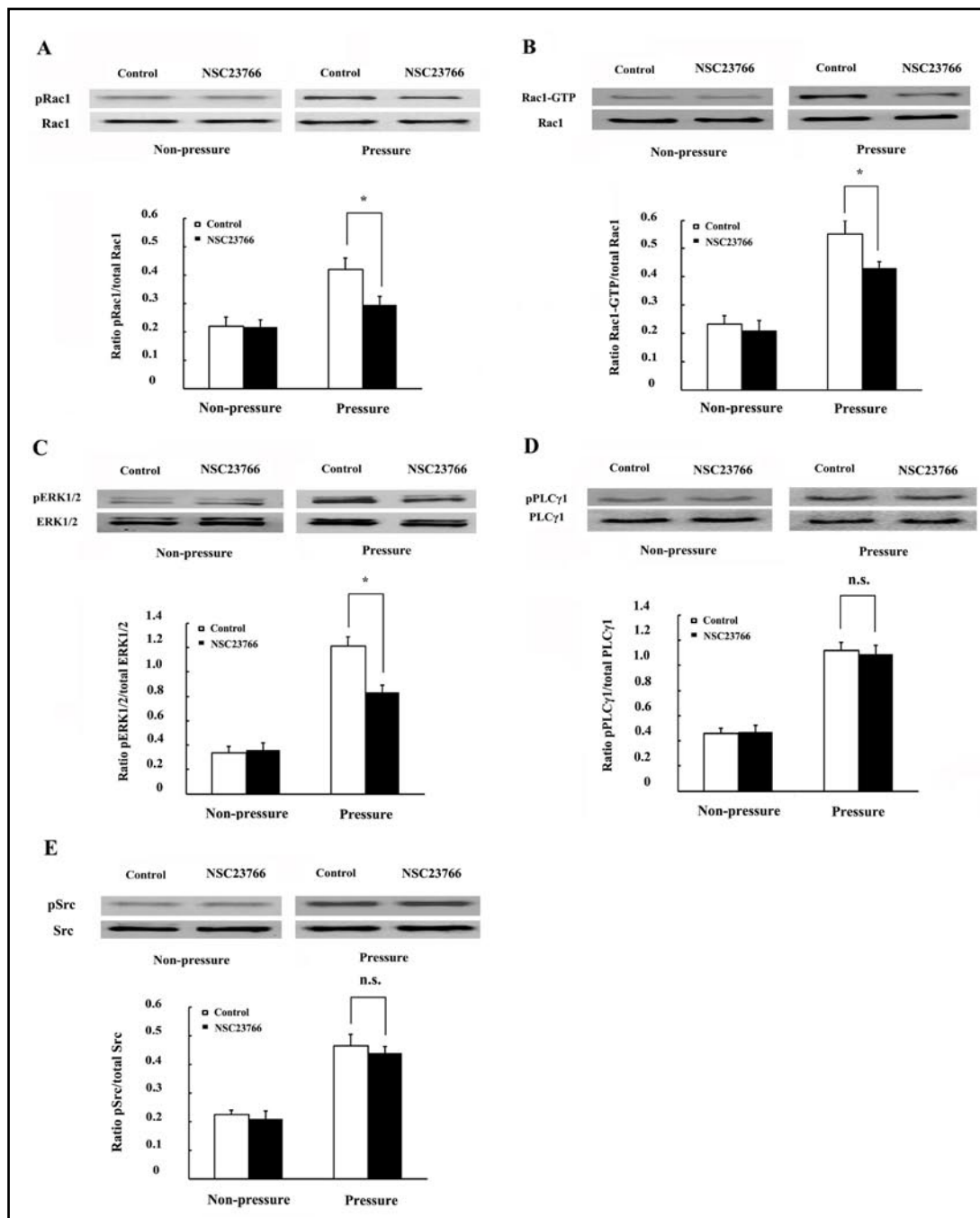


Fig. 5. Effects of Rac1 on the expression and phosphorylation of Src, PLC γ 1, and ERK1/2 under conditions of periodic mechanical stress. After pretreatment with control vehicle (DMEM) or Rac1 inhibitor NSC23766, rat chondrocytes were cultured *in vitro* for 1 h under static conditions or conditions of periodic mechanical stress. The expression and phosphorylation levels of Rac1, Src, PLC γ 1, and ERK1/2 were detected by western blotting, and Rac1 activation was quantified by Rac1 GTPase activity assay. The total amounts of Rac1, Src, PLC γ 1, and ERK1/2 served as controls, respectively. Gray values are represented with a histogram (n=5, *, p<0.05 for each). The images above are representative results of western blotting and Rac1 GTPase activity assay. The phosphorylation levels of ERK1/2-Thr²⁰²/Tyr²⁰⁴ in the NSC23766 pretreatment groups were significantly diminished relative to those of the control group in response to periodic mechanical stress (n=5, p<0.05, Student unpaired *t*-test), whereas the activation of Src-Tyr⁴¹⁸ and PLC γ 1-Tyr⁷⁸³ in the NSC23766 pretreatment groups were not affected (n=5, p>0.05 for each, Student unpaired *t*-test).

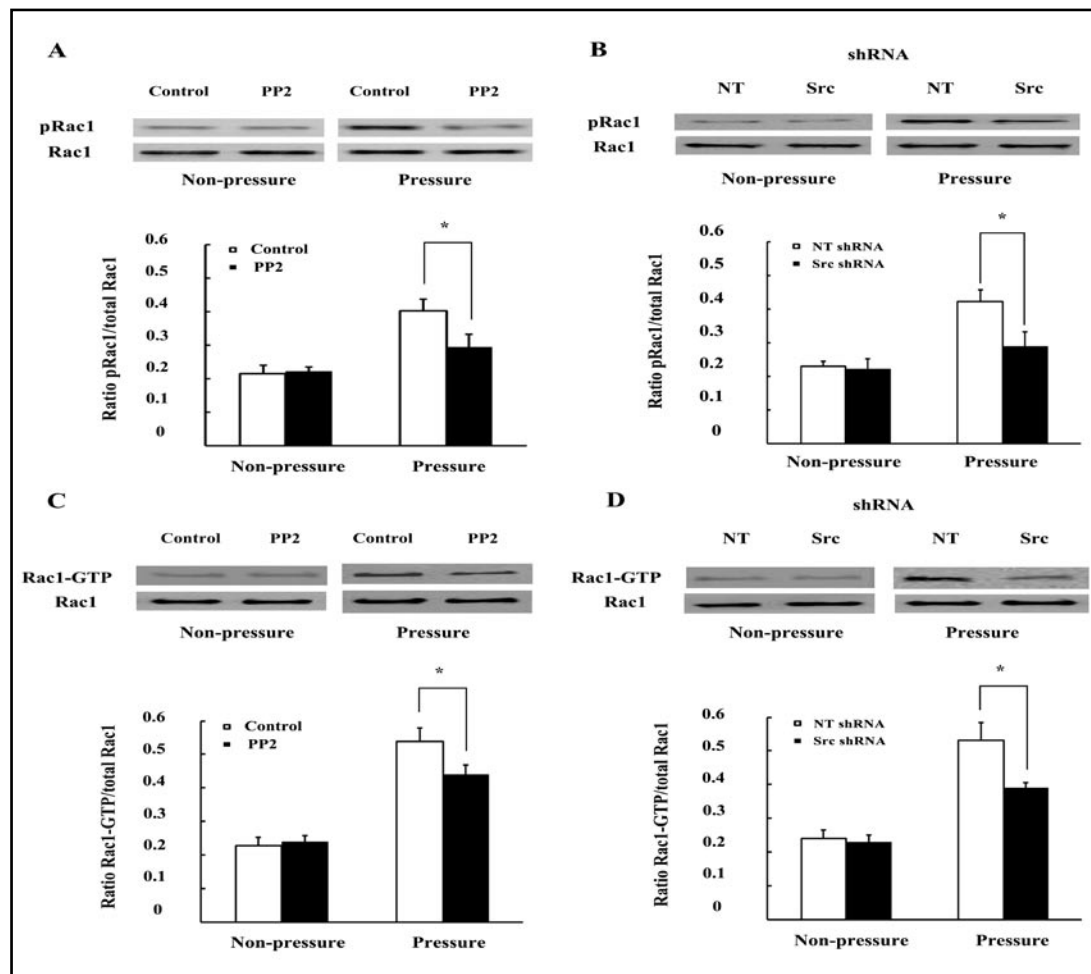


Fig. 6. Effects of Src on the expression, phosphorylation and activation of Rac1 under conditions of periodic mechanical stress. Chondrocytes were transfected with shRNA targeted to Src or with nontargeting NT sequences prior to lysis and Western blotting for Src protein. The Src shRNA sequence achieved about 50% reduction in Src protein level. After pretreatment with DMSO or Src inhibitor PP2, or Control shRNA or Src shRNA, rat chondrocytes were cultured *in vitro* for 1 h under static conditions or with periodic mechanical stress. The expression and phosphorylation levels of Rac1 were detected by western blotting, and Rac1 activation was quantified by Rac1 GTPase activity assay. The total quantity of Rac1 served as the control. Results are represented in the histogram ($n=5$, *, $p<0.05$ for each). The images above are representative results of western blotting and Rac1 GTPase activity assay. The phosphorylation levels of Rac1-Ser⁷¹ and Rac1 activation levels in the PP2 and Src shRNA pretreatment groups were significantly diminished relative to those of the control groups in response to periodic mechanical stress ($n=5$, $p<0.05$ for each, Student unpaired *t*-test).

affect the levels of Rac1 phosphorylation or activation in comparison with DMSO-pretreated chondrocytes growing under the same conditions ($p>0.05$ for each, Fig. 7, $n=5$).

Periodic mechanical stress-induced activation of Src, PLC γ 1, Rac1, and ERK1/2 depends on Integrin β 1

Activations of ERK1/2, Rac1, PLC γ 1, and Src were significantly diminished in the group pretreated with blocking antibody against integrin β 1 (10 μ g/mL) relative to those in the control group under periodic mechanical stress ($p<0.05$ for each, Fig. 8, $n=5$).

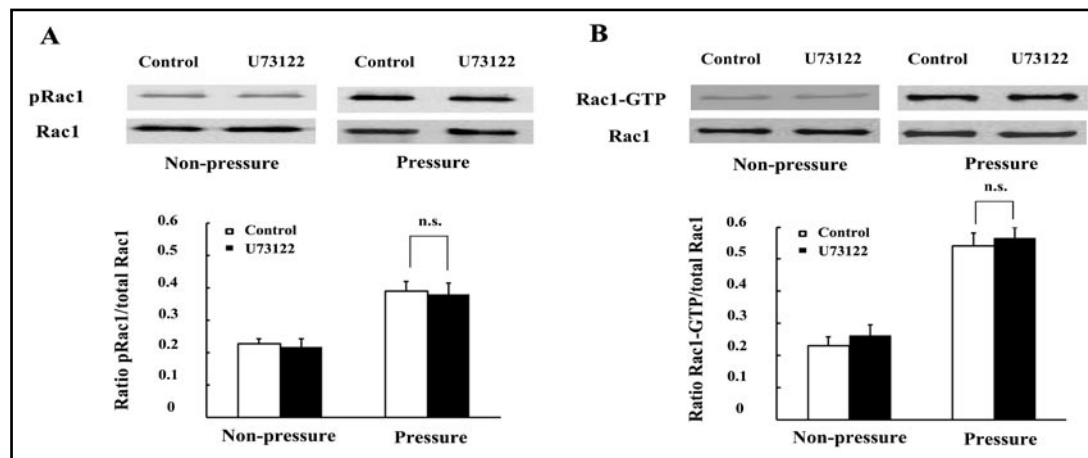


Fig. 7. Effects of PLC γ 1 on the expression, phosphorylation and activation of Rac1 under conditions of periodic mechanical stress. After pretreatment with DMSO or PLC γ 1 inhibitor U73122, rat chondrocytes were cultured *in vitro* for 1 h under static conditions or conditions of periodic mechanical stress. The expression and phosphorylation levels of Rac1 were detected by western blotting, and Rac1 activation was quantified by Rac1 GTPase activity assay. The total quantity of Rac1 served as the control. Results are represented in the histogram ($n=5$, *, $p<0.05$ for each). The images above are representative results of western blotting and Rac1 GTPase activity assay. The phosphorylation levels of Rac1-Ser⁷¹ and Rac1 activation levels in the U73122 pretreatment groups were not appreciably altered in chondrocytes in response to periodic mechanical stress ($n=5$, $p>0.05$ for each, Student unpaired *t*-test).

Discussion

This study was designed to determine the mechanism of signal transduction by which periodic mechanical stress promotes chondrocyte proliferation and matrix synthesis. We demonstrated that periodic mechanical stress activates chondrocyte proliferation and matrix synthesis in part through integrin β 1-Rac1-ERK1/2 signaling.

The small GTP-binding Rho family proteins consist of three components: RhoA, Rac1, and Cdc42. Previous associated studies in chondrocytes mainly focused on the role of RhoA following mechanical stimulation [30, 31]. Haudenschild and colleagues reported that mechanical stimulation activated SOX9 in human chondrocytes and that RhoA signaling plays an important role in that process [30]. The involvement of Rac1 in mechanosensing and signaling transduction in various nonchondrocytic cell types has been demonstrated [32, 33]. Further, one study by Qi et al. demonstrated that cyclic mechanical strain stimulation induces proliferation of the vascular smooth muscle cells by activation via Rac1 signaling [34]. We observed that periodic mechanical stress statistically and significantly enhanced phosphorylation and activation of Rac1, relative to that in chondrocytes under static conditions. We found that blocking Rac1 with NSC23766 prevented the up-regulation of chondrocyte proliferation and matrix synthesis following periodic mechanical stress. Our findings indicate that Rac1 appears to play a key role in chondrocytic adaptation and response to physical periodic mechanical stimulation. To our knowledge, these results are the first to strongly implicate Rac1 signaling in the chondrocytic mitogenic response to periodic mechanical stimulation.

Various kinases can transduce mechanical signals into final mitogenic effects through the ERK1/2 MAPK signaling cascades. The activation of ERK1/2 by Rac1 has recently been demonstrated to be a signaling transduction mechanism in cardiomyocytes and smooth muscle cells for the detection and response to cyclic mechanical stretching [35, 36]. We found that the up-regulated phosphorylation level of ERK1/2 initiated by periodic mechanical stimulation was attenuated when the activity of Rac1 was inhibited, which suggests that

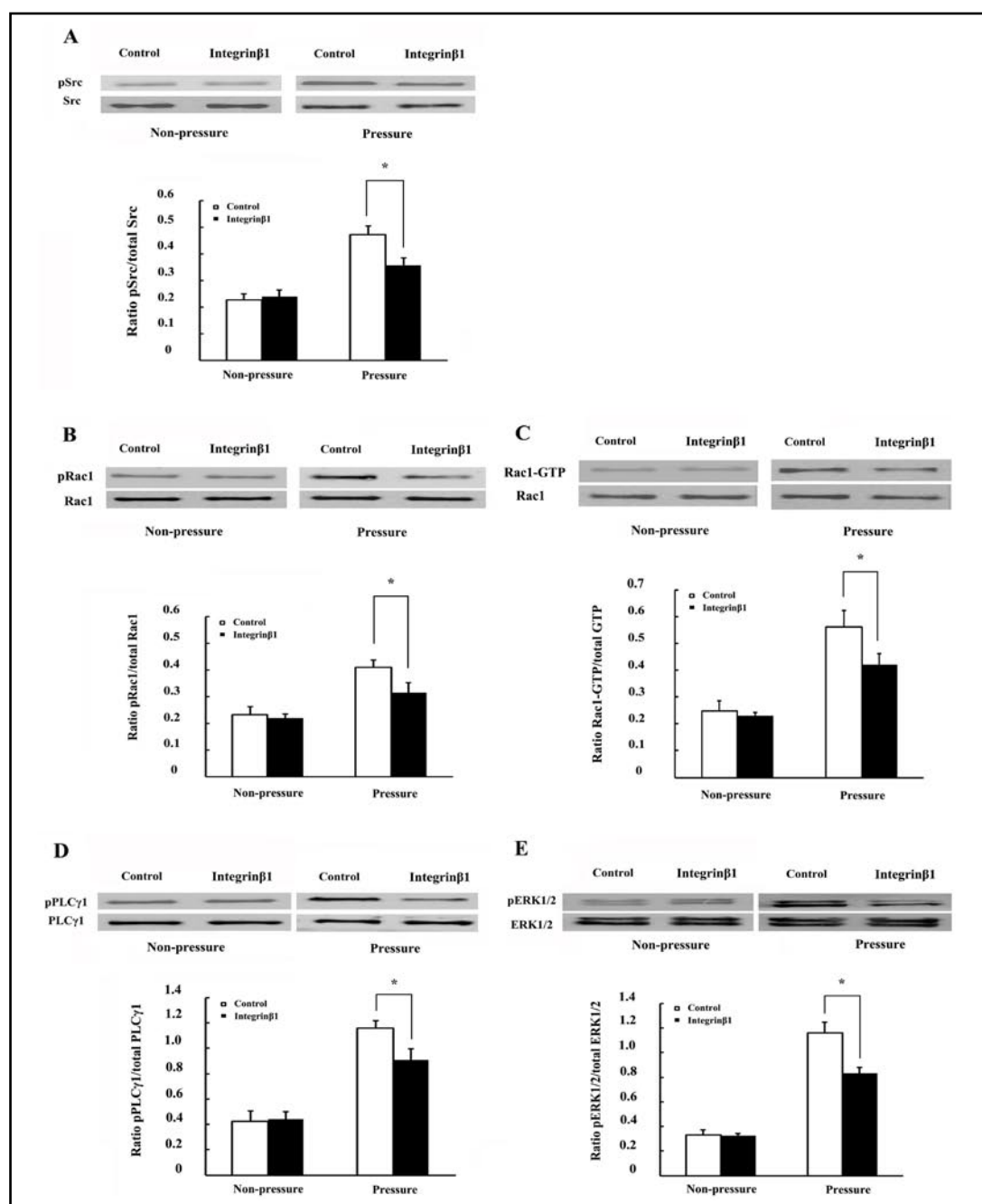


Fig. 8. Effects of integrin β 1 on the expression and phosphorylation of Src, Rac1, PLC γ 1, ERK1/2 and Rac1 activation under conditions of periodic mechanical stress. After pretreatment with control vehicle (DMEM) or blocking antibody against integrin β 1, rat chondrocytes were cultured *in vitro* for 1 h under static conditions or conditions of periodic mechanical stress. The expression and phosphorylation levels of Src, Rac1, PLC γ 1 and ERK1/2 were detected by western blotting, and Rac1 activation was quantified by Rac1 GTPase activity assay. The total amounts of Src, PLC γ 1, Rac1, and ERK1/2 served as controls, respectively. Gray values are represented with a histogram (n=5, *, $p < 0.05$ for each). The images above are representative results of western blotting and Rac1 GTPase activity assay. The phosphorylation levels of Src-Tyr⁴¹⁸, PLC γ 1-Tyr⁷⁸³, Rac1-Ser⁷¹, ERK1/2-Thr²⁰²/Tyr²⁰⁴ and Rac1 activation levels in the blocking antibody against integrin β 1 pretreatment groups were significantly diminished in comparison with those of the control group in chondrocytes in response to periodic mechanical stress (n=5, $p < 0.05$ for each, Student unpaired *t*-test).

Rac1 locates upstream of ERK1/2 in this context. Although our results did not exclude a role for other small GTP-binding Rho family proteins, the activation of Rac1 seems to contribute to at least some mechanical stress-induced ERK1/2 mitogenic mechanotransduction signals in chondrocytes.

Then, the question arises as to how Rac1 is being regulated in response to cyclic mechanical stress in chondrocytes. The coincidence of Src and PLC γ 1 with Rac1, as upstream mediators of ERK1/2 modulation of chondrocytic mitogenic effects, raises the possibility that there may be some causal relationship between the activation of Rac1 and the activation of Src and PLC γ 1. Moreover, Src has often been reported to be an upstream mediator of Rac1 in many other systems [11, 14]. Zhang et al. demonstrated that mechanical strain stimulation of ROS generation in glomerular mesangial cells is induced by Rac1 activation via an Src signaling pathway [13]. In response to other non-mechanical stimuli, Rac1 is also recognized as a downstream signaling molecule of Src signals [37-41]. In our study, pretreatment of cells with the Src inhibitor PP2 and shRNA targeted to Src blocked periodic mechanical stress-induced Rac1 phosphorylation and activation, which suggests that Src activation of Rac1 at Ser71 is responsible for the signaling transduction mechanisms in chondrocytes under conditions of cyclic mechanical stimulation. Undoubtedly, Rac1 can be activated not only by Src but also by other mechanosensors and signaling proteins following mechanical stimulation [10, 36]. The nature of the relationship between PLC γ 1 and Rac1 is complex and remains controversial. PLC γ 1 is required for Rac1 activation in the development and progression of breast cancer metastasis [15]. Similarly, in a study of keratinocyte cell-cell adhesion and differentiation, Bourguignon concluded that activated Rac1 signal mediates the phosphorylation level of PLC γ 1 [42]. We found the selective inhibitor of PLC γ 1, U73122, did not attenuate the phosphorylation or the activity of Rac1 in chondrocytes in response to cyclic mechanical stress. Our results strongly imply that PLC γ 1 is not required for the activation of Rac1 that is stimulated by periodic mechanical stress. NSC23766 pretreatment also did not affect the levels of PLC γ 1 phosphorylation in comparison with DMSO-pretreated chondrocytes growing under the same conditions. Therefore, periodic mechanical stress-initiated PLC γ 1 and Rac1 signals may act in parallel with each other. These data support the concept that Src may be a proximal kinase in the signal transduction pathways and that two periodic mechanical stress-activated signaling pathways converge upon ERK1/2, one being Rac1-dependent and the other being PLC γ 1-dependent, and both of which depend on the activity of Src.

Integrins have been regarded as the main mechanosensors in various cell types that are involved in sensing and response to mechanical stimulation [18, 20]. In the study of embryonic stem cells, mechanical stimulation activates mitogenic effects mainly through integrin β 1 [24]. There are contrasting findings, however, and in one report integrin β 3 was demonstrated to participate in a main mechanosensor and signaling transduction mechanism in endothelial cells for detection and response to mechanical shear stress [43]. Lee and colleagues confirmed that proliferation of osteoblast-like cells induced by oscillatory shear stress is mediated by both integrin β 1 and integrin β 3 [23]. We blocked integrin β 1 or integrin β 3 with their functional blocking antibodies and found that up-regulated cell proliferation, aggrecan and type II collagen gene expression were abrogated after inhibition of integrin β 1 but not of integrin β 3 in chondrocytes subjected to cyclic mechanical stimuli. Our findings illustrated that integrin β 1, but not integrin β 3, appears to play a key role in chondrocytic adaptation to physical mechanical stimulation. It is a novel discovery that integrin β 1 modulates periodic mechanical stimuli-initiated chondrocyte proliferation and matrix synthesis.

Integrins have no kinase activity and their transmission of mechanical signals into biochemical signals through integrin-associated signaling kinases. Src is one such kinase and that integrins could play a role through the recruitment and activation of Src kinase. However, the dependence of Src on integrins signaling is not necessarily universal and is still obscure in chondrocytes under mechanical stimulation. And it is possible that Src can be activated by other mechanosensors and that integrins can transmit mechanical signals

through other integrin-associated kinases [23, 44]. ERK1/2 and other MAPKs are also common integrins downstream signals in response to various stimuli [45]. In our present study, we observed the phosphorylation levels of Src, PLC γ 1, Rac1, ERK1/2, and Rac1 activation were all attenuated after pretreatment with functional blocking antibody against integrin β 1 in this setting. Therefore, periodic mechanical stress-activated Src, PLC γ 1, Rac1 and ERK1/2 mitogenic signals depend, at least in part, on integrin β 1. However, we could not exclude the possibility that integrin β 1 transmit mechanical signals through other integrin-associated kinases and MAPKs in our system. Above of all, our present data supports that periodic mechanical stress activates ERK1/2 mitogenic signals through integrin β 1-Src-PLC γ 1/Rac1-ERK1/2 in chondrocytes.

It is well known that periodic physical mechanical stimulation function as major determinants directly affecting the quality of tissue-engineered cartilage. Therefore, it is important to determine the signaling transduction pathways in chondrocytes under periodic mechanical stress. The results of our studies provide a strong framework for further investigations regarding chondrocyte mechanobiology under periodic mechanical stress and how to improve the quality of tissue-engineered cartilage.

In conclusion, our findings have shown that periodic mechanical stress activates two signaling pathways converging on ERK1/2: one is Rac1-dependent and the other is PLC γ 1-dependent, and both depend on the activation of Src. Integrin β 1 links periodic mechanical stimulation with Src-ERK1/2 signaling so that they converge into mitogenic cascades in chondrocytes. Briefly, periodic mechanical stress enhances chondrocyte proliferation and matrix synthesis at least in part through the integrin β 1-Src-PLC γ 1/Rac1-ERK1/2 pathway.

Competing Interests

The authors declare that they have no competing interests.

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