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Original Paper

Periodic Mechanical Stress Stimulates the FAK Mitogenic Signal in Rat Chondrocytes Through ERK1/2 Activity

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Key Words
Periodic mechanical stress • Chondrocyte proliferation • Matrix synthesis • FAK • Src • Integrin β1

Abstract
Background/Aims: The biological effects of periodic mechanical stress on chondrocytes have been studied extensively over the past few years. However, the mechanisms underlying chondrocyte mechanosensing and signaling in response to periodic mechanical stress remain to be determined. In the current study, we examined the effects of focal adhesion kinase (FAK) signaling on periodic mechanical stress-induced chondrocyte proliferation and matrix synthesis. ***Methods and Results:*** Periodic mechanical stress significantly induced sustained phosphorylation of FAK at Tyr^{397} and $Tyr^{576/577}$. Reduction of FAK with targeted shRNA via transfection of NH_2-terminal tyrosine phosphorylation-deficient FAK mutant Y397F or Y576F-Y577F abolished periodic mechanical stress-induced chondrocyte proliferation and matrix synthesis, accompanied by attenuated ERK1/2 phosphorylation. However, activation of Src, PLCγ1 and Rac1 was not prevented upon FAK suppression. Furthermore, pretreatment with the Src-selective inhibitor, PP2, and shRNA targeted to Src or suppression of Rac1 with its selective inhibitor, NSC23766, blocked FAK phosphorylation at $Tyr,^{576/577}$ but not $Tyr,^{397}$ under periodic mechanical stress. Interestingly, FAK phosphorylation neither at Tyr^{397} nor at $Tyr^{576/577}$ was affected by PLCγ1 depletion when periodic mechanical stress was applied. In addition, Tyr^{397} and $Tyr^{576/577}$ phosphorylation levels were reduced upon pretreatment with a blocking antibody against integrin β1 under conditions of periodic mechanical stress. ***Conclusion:*** Our findings collectively suggest that periodic mechanical stress promotes chondrocyte proliferation and matrix synthesis through at least two pathways, integrin β1-Src-Rac1-FAK($Tyr^{576/577}$)-ERK1/2 and integrin β1-FAK (Tyr^{397})-ERK1/2.

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Introduction

Periodic mechanical stress promotes chondrocyte proliferation and matrix synthesis via integrin-initiated pathways. However, the signaling proteins downstream of integrin remain to be identified [1]. Focal adhesion kinase (FAK) is known to be tightly linked to integrin-associated mechanotransduction [2-4]. Accumulating evidence supports an important role of FAK in several signal transduction pathways that affect cellular biological processes, including proliferation, migration and survival [5-7]. Several researchers have established that mechanical stimulation activates FAK signaling in a number of cell types [8, 9]. For instance, in an earlier investigation, Chaturvedi et al. demonstrated an association of FAK with cyclic mechanical strain-induced pulmonary epithelial cell proliferation [10]. However, the specific functions of FAK in chondrocytes under conditions of mechanical stress are obscure. In the current study, the effects of FAK on chondrocyte proliferation and matrix synthesis were examined in a periodic mechanical stress setting.

FAK activity depends on its site-specific tyrosine residues. The autophosphorylation site at Tyr^{397} plays a critical role in catalytic activity, while $Tyr^{576/577}$ located in the catalytic domain possibly enhances the kinase activity of FAK [11]. The functions of Tyr^{397} and $Tyr^{576/577}$ residues of FAK in the regulation of signal pathways appear variable and complex. For instance, Saldana and co-workers demonstrated that activation of FAK Tyr^{397} is essential for the growth and function of hMSCs cultured on metallic surfaces under tensile forces [12], while Kim and collaborators reported dependence of compressive stress-induced expression of M-CSF, TNF-α, RANKL and OPG on FAK Tyr^{576} phosphorylation in PDL cells [8]. However, the role of site-specific tyrosine residues of FAK in chondrocytes exposed to mechanical stimuli is currently unknown. Here, we substituted Tyr^{397} and $Tyr^{576/577}$ of FAK with phenylalanine, with a view to exploring the effects of these residues on periodic mechanical stress-induced chondrocyte proliferation and extracellular matrix synthesis.

In a previous study, we reported that periodic mechanical stress induces chondrocyte proliferation and matrix synthesis through integrin β1-initiated Src, PLCγ1, Rac1 and ERK1/2 signals. FAK acts as an upstream regulator of ERK1/2 signaling under conditions of mechanical stimulation in many non-chondrocytic cell types [13, 14]. Various studies have demonstrated a pivotal role of FAK in integrin-mediated signal transduction pathways in different cell types [15-17]. Although interactions of FAK and Src within signal transduction pathways in different non-chondrocytic cell types have been extensively analyzed, the conclusions are controversial [10, 18]. Furthermore, little is known about the associations between FAK, Rac1 and PLCγ1 in chondrocytes subjected to mechanical stimuli. The activation patterns of individual kinases appear to vary among different systems, cell types, and physical forces. Thus, elucidation of the relationships between FAK, ERK1/2, integrin β1, Src, Rac1 and PLCγ1 in chondrocytes are essential in this context.

The main aim of the current study was to determine whether FAK mediates the periodic mechanical stress-induced cellular response in chondrocytes. We disrupted FAK signaling via overexpression of point mutants in which tyrosine residues were replaced with phenylalanine, with a view to highlighting the roles of site-specific Tyr^{397} and $Tyr^{576/577}$ in a periodic mechanical stress setting. Additionally, associations between FAK, ERK1/2, integrin β1, Src, Rac1 and PLCγ1 in this system were analyzed to establish the mechanotransduction pathway linking 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 obtained from Sigma (USA). Cell Counting Kit-8 (CCK-8) was acquired from Beyotime Institute of Biotechnology (China). Anti-FAK, anti-phospho-FAK (Tyr^{397}), anti-phospho-FAK ($Tyr^{576/577}$), anti-Src, anti-phospho-Src (Tyr^{418}), anti-PLCγ1, anti-phospho-PLCγ1 (Tyr^{783}), anti-Rac1-cdc42, anti-phospho-Rac1-cdc42 (Ser^{71}), anti-ERK1/2, anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), and HRP-goat anti-rabbit IgG 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 β1 and NSC23766 were supplied by BD Biosciences (USA) and Calbiochem (USA), respectively. RNAiso Plus, PrimeScript RT Reagent Kit and SYBR Premix Ex Taq II were acquired from TaKaRa (Japan). FAK shRNA Lentiviral Particles, Src shRNA Lentiviral Particles, Control shRNA Lentiviral Particles and Polybrene were supplied by Santa Cruz. The GV287 vector was obtained from GENECHEM (Shanghai). The FAK mutants (Tyr^{397} to Phe^{397} and Tyr^{576}–Tyr^{577} to Phe^{576}–Phe^{577}) were generated via site-directed mutagenesis using overlapping PCR and subcloned into the GV287 vector. Wild-type FAK cDNA was subcloned into GV287 served as the control.

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

Methods

Cell culture. Chondrocytes were harvested using the method described by Séguin and Bernier [19]. Briefly, under sterile conditions, cartilage derived from the limb joints of two--week-old SD rats was harvested and sliced into $1mm^3$ sections. Tissues were digested with 0.25% trypsin at 37 °C for 0.5 h, followed by digestion with 0.2% collagenase II at 37 °C for 4 h. Cells were harvested by filtering with a 200-mesh filter and cultured in DMEM-F12 medium supplemented with 10% FBS at 37 °C with 5% CO_2 in an incubator. Cells were purified via repeated adherence, and morphology was examined under an inverted phase-contrast microscope by staining with collagen type II according to the conventional ABC method. Second-generation cells were seeded on a glass slide (25 mm × 25 mm) coated with type II collagen at a density of 10^5 cells. Experiments were performed at ~ 70-80% cell confluence.

Inhibitors. Blocking antibody against integrin β1, PP2, U73122 and NSC23766 were used as specific inhibitors of integrin β1, Src, PLCγ1 and Rac1, respectively. Antibodies against integrin β1 and Rac1 were dissolved in DMEM, and the other inhibitors in anhydrous dimethylsulfoxide (DMSO) to form 1,000× concentrated solutions. Aliquots of all inhibitor solutions were stored at -20°C. Each concentrated solution was diluted 1000× immediately prior to use. PP2 and U73122 pretreatment groups contained 0.1% (v/v) DMSO, which was the concentration used for the control group. Cells were pretreated with PP2 (10 μM), U73122 (10 μM), NSC23766 (50 μM) 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 β1 (10 μg/mL) or an equivalent amount of DMEM for 5 h.

Construction of a periodic 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 described previously [20]. Earlier, we showed that rabbit chondrocytes subjected to stress varying from 0 to 200 kPa at 0.1 Hz yielded tissue-engineered cartilage of the best quality. Accordingly, a pressure range of 0-200 kPa and 0.1 Hz frequency were used in the current study.

Experimental groups. The experiment comprised two steps. In the first step, cells were divided into non-pressure and pressure groups. Cells were maintained under static conditions or periodic mechanical stress for 0, 0.5, 1 or 2 h, prior to Western blot analysis.

In the second step, cells were pretreated with shRNA targeted to FAK or Src or control scrambled shRNA, respectively, or transfected with FAK wild-type or mutant Phe^{397} and $Phe^{576/577}$ plasmid. Moreover, cells were pretreated with blocking antibodies against integrin β1 (10 μg/mL each), PP2 (10 μM), U73122 (10 μM) or NSC23766 (50 μM) and maintained under static conditions or periodic mechanical stress for 1 h prior to western blot analysis, 8 h prior to qPCR analysis (aggrecan and type II collagen gene expression) or 3 days (8 h per day mechanical stress) prior to direct cell counting and CCK-8 assay. All groups of cells were incubated at 37°C under 5% CO_2.

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

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

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

Western blot analysis

Total protein was prepared and western blot analyses were performed as described previously [21]. Total protein was prepared using RIPA buffer, and the Bradford assay was employed to determine protein concentrations. Protein samples were resolved using SDS-PAGE, and transferred to nitrocellulose membranes. Following blocking for 1 h with 5% skimmed milk in TBST, membranes were incubated with antibodies (1:1,000 dilution for antibodies) overnight at 4°C. Blots were incubated with horseradish peroxidase-conjugated secondary antibody at ambient temperature for 1 h, and colors were subsequently developed with ECL. Results were scanned using a Gel Imaging System (UVP Company, USA) and measured using Gel-Pro Analyzer software (Media Cybernetics, USA).

Quantitative real-time PCR (qPCR) analysis

Total RNA was extracted using RNAiso Plus and 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 described previously [22]. The reaction was performed in a 20 μL mixture containing 2 μL cDNA. Each cDNA sample was amplified using specific primers (Table 1, TaKaRa, Japan) under the following cycling conditions: 30 s initial denaturation 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 studies

Proliferation was assessed by two different methods, specifically, direct cell counting and the CCK-8 assay.

Direct cell counting

Cells were trypsinized and counted according to published protocols [23]. Second-generation chondrocytes were seeded on glass slides (25 mm × 25 mm) coated with type II collagen at a density of 10^5 cells and randomly divided into different groups, each including 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 periodic mechanical stress for 8 h per day, prior to direct cell counting. Cells were trypsinized and counted, and the cell number was determined by counting cells from each glass slide independently. Experiments were repeated five times.

CCK-8 assay

Cell proliferation was determined using CCK-8 solution according to the manufacturer's instructions [24]. Cells were added to 10 μL of 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 determined at 450 nm using a microplate reader.

Statistical analysis

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

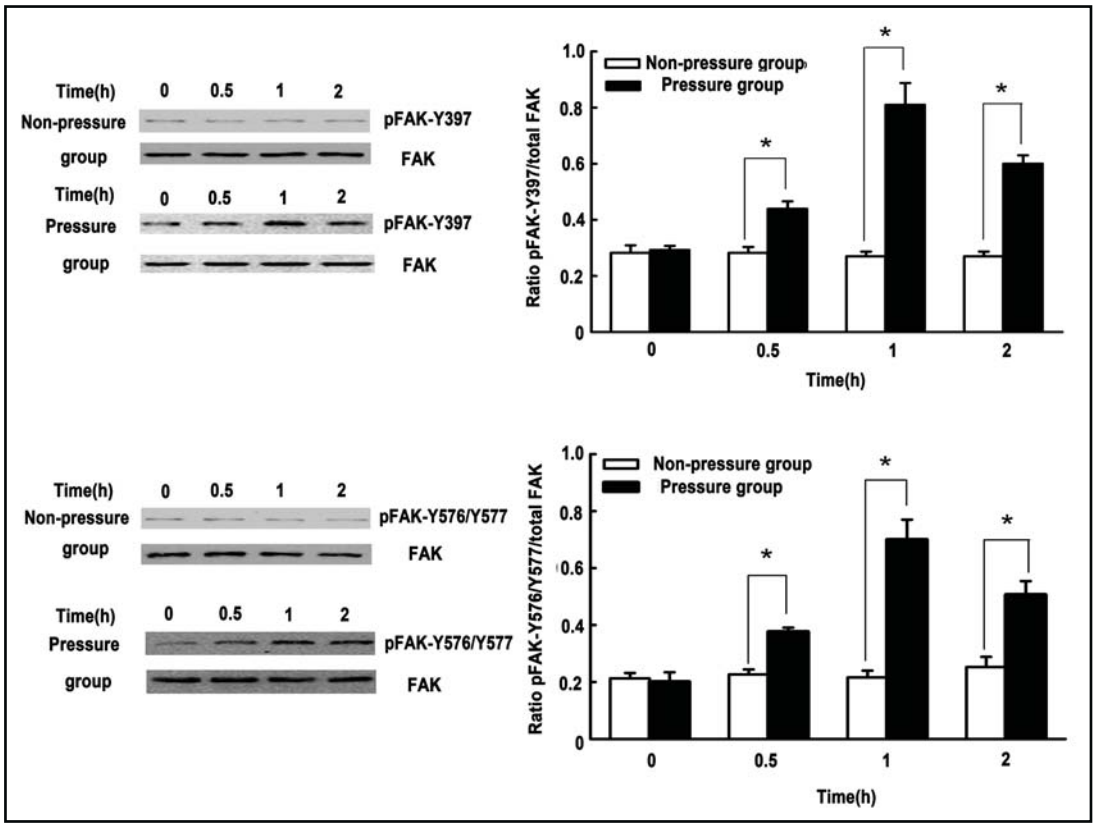


Fig. 1. Effects of periodic mechanical stress on expression and phosphorylation of FAK at Tyr^{397}(pFAK-Y397) and $Tyr^{576/577}$(pFAK-Y576/Y577). Rat chondrocytes were cultured *in vitro* for 0, 0.5, 1 and 2 h with or without periodic mechanical stress. Expression and phosphorylation levels of FAK at Tyr^{397} (pFAK-Y397) and $Tyr^{576/577}$(pFAK-Y576/Y577) were detected via western blotting, and the total quantity of FAK served as the control. Representative western blots of five different experiments are shown, and data averaged in images on the left. The phosphorylation levels of FAK at Tyr^{397} (pFAK-Y397) and $Tyr^{576/577}$ (pFAK-Y576/Y577) in the pressure groups were significantly increased, relative to those in the non-pressure groups (N=5, $P < 0.05$ for each, Student's unpaired *t*-test).

Results

Effects of FAK on periodic mechanical stress-induced chondrocyte proliferation and matrix synthesis

In our experiments, periodic mechanical stress significantly induced phosphorylation of FAK at Tyr^{397} and $Tyr^{576/577}$ at 0.5, 1 and 2 h, compared with the non-pressure group ($P < 0.05$, Fig. 1, N = 5). To determine whether FAK mediates mechanical stress-induced chondrocyte proliferation and matrix synthesis, chondrocytes were transfected with shRNA targeted to FAK or non-targeting (NT) shRNA sequences for 48 h and subsequently assessed for proliferation and matrix synthesis. Periodic mechanical stress-induced chondrocyte proliferation and matrix synthesis were significantly reduced in the targeted shRNA, compared with control shRNA-transfected group ($P < 0.05$, Fig. 2, N = 5).We further determined the effects of FAK-Tyr^{397} and FAK-$Tyr^{576/577}$ sites on periodic mechanical stress-induced chondrocyte proliferation and matrix synthesis. Notably, mechanical stress-induced chondrocyte proliferation and matrix synthesis were significantly reduced in chondrocytes transfected with FAK mutant-expressing plasmids (Phe^{397}, $Phe^{576/577}$), compared with those transfected with FAK wild-type ($P < 0.05$, Figs 3 and 4, N = 5).

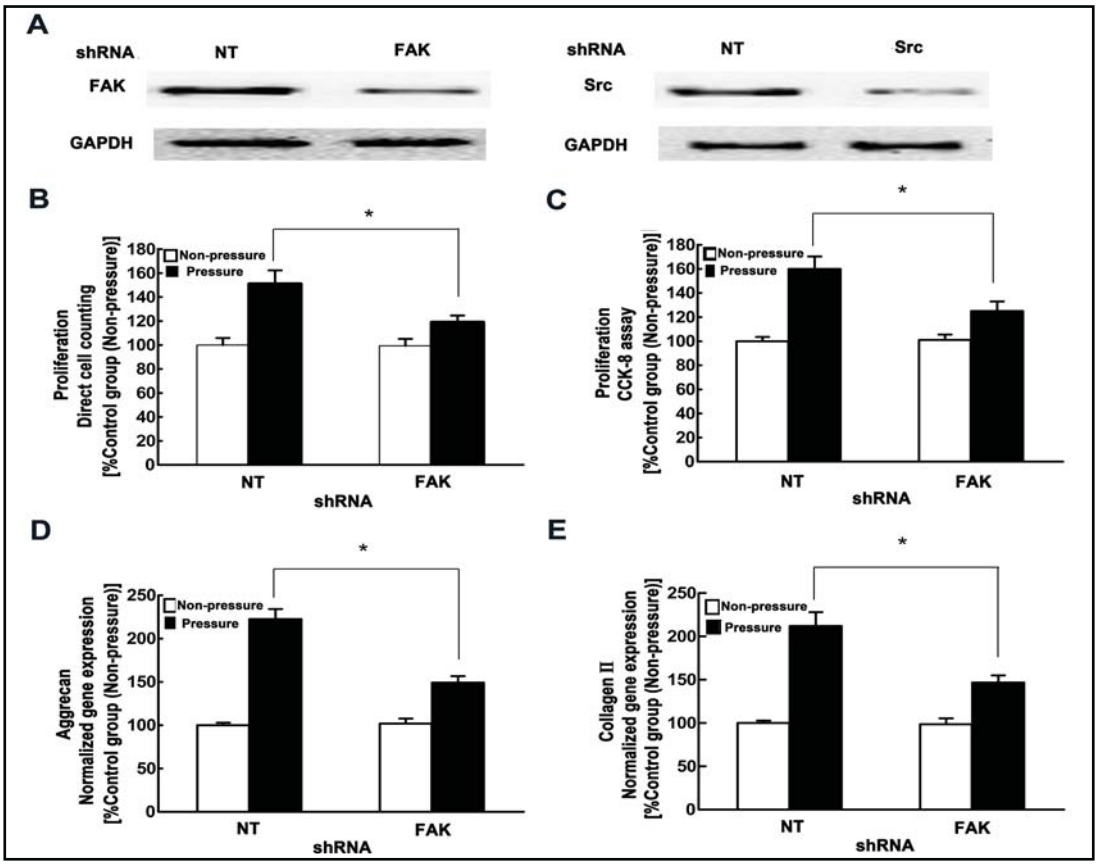


Fig. 2. Effects of FAK on chondrocyte proliferation and matrix synthesis in response to periodic mechanical stress. Chondrocytes were transfected with shRNA targeted to FAK or Src or non-targeting (NT) sequences prior to lysis and Western blot for FAK and Src proteins. Transfection with shRNA for FAK and Src achieved ~50% reduction in FAK and Src protein levels, respectively (A). After pretreatment with control or FAK shRNA, rat chondrocytes were cultured for 3 days under static conditions or periodic mechanical stress for 8 h per day, prior to proliferation studies. Cell proliferation was analyzed via direct cell counting (B) and the CCK-8 assay (C). Rat chondrocytes were cultured *in vitro* for 8 h under static conditions or periodic mechanical stress. Aggrecan (D) and type II collagen (E) gene expression levels were measured using quantitative real-time PCR. In chondrocytes subjected to periodic mechanical stress, cell proliferation and matrix synthesis in FAK shRNA pretreatment groups were significantly decreased, compared with control shRNA groups (N = 5, *P < 0.05, Student's unpaired *t*-test).

Integrin β1 is required for periodic mechanical stress-induced activation of FAK. Src is required for periodic mechanical stress-induced phosphorylation of FAK-Tyr$^{576/577}$, but not FAK-Tyr^{397}

Relative to the control group, both FAK-Tyr^{397} and FAK-Tyr$^{576/577}$ phosphorylation were significantly diminished after pretreatment of chondrocytes with blocking antibody against integrin β1 (10 μg/mL) in response to periodic mechanical stress ($P < 0.05$, Fig. 5, N = 5). Src inhibition with PP2 and targeted shRNA did not suppress periodic mechanical stress-induced FAK-Tyr^{397} phosphorylation, compared with the control groups ($P > 0.05$, Fig. 5, N = 5). However, suppression of Src prevented FAK-Tyr$^{576/577}$ phosphorylation to a significant extent under conditions of periodic mechanical stress ($P < 0.05$, Fig. 5, N = 5).

FAK is not required for periodic mechanical stress-induced Src phosphorylation

Next, we examined the relationship between FAK and Src. FAK induction with targeted shRNA did not inhibit Src phosphorylation, compared with the NT shRNA group, under periodic mechanical stress ($P > 0.05$, Fig. 7, N = 5).

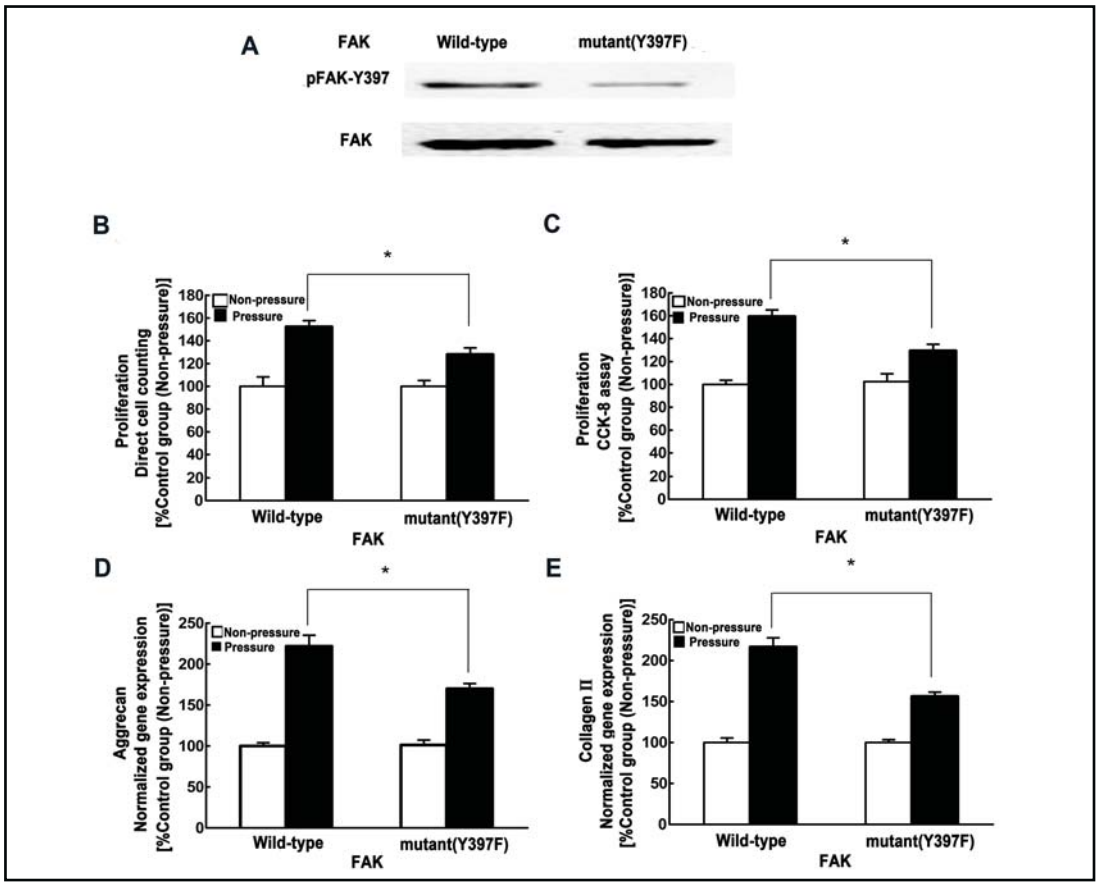


Fig. 3. Effects of inhibition of FAK-Tyr^{397} phosphorylation on chondrocyte proliferation and matrix synthesis under conditions of periodic mechanical stress. Chondrocytes were transfected with a GFP-tagged FAK wild-type GV287 vector or GV287 expressing mutant FAK (Tyr^{397} to Phe^{397}). Phosphorylation of Tyr^{397} in the FAK-Phe^{397} group was significantly abolished, compared with that in the wild-type group (A).Rat chondrocytes were cultured for 3 days under static conditions or periodic mechanical stress for 8 h per day, prior to proliferation studies. Cell proliferation was analyzed using direct cell counting (B) and the CCK-8 assay (C). Rat chondrocytes were cultured *in vitro* for 8 h under static conditions or periodic mechanical stress. Aggrecan (D) and type II collagen (E) gene expression levels were measured using quantitative real-time PCR. Cell proliferation and matrix synthesis in the FAK mutant Phe^{397} groups were significantly decreased, compared with that in FAK wild-type chondrocytes subjected to periodic mechanical stress (N = 5, *P < 0.05, Student's unpaired *t*-test).

Periodic mechanical stress-induced FAK-Tyr$^{576/577}$ phosphorylation is dependent on Rac1

We further focused on the role of Rac1 in periodic mechanical stress-induced FAK tyrosine phosphorylation. Upon treatment of chondrocytes with NSC23766 (a selective Rac1 inhibitor), phosphorylation of Tyr$^{576/577}$ was significantly diminished, relative to that in control groups, under conditions of periodic mechanical stress ($P < 0.05$, Fig. 6, N = 5). In contrast, no differences in phosphorylation of Tyr^{397} were evident between the NSC23766-treated and control groups subjected to periodic mechanical stress ($P > 0.05$, Fig. 6, N = 5).

Rac1 is not regulated by FAK in response to periodic mechanical stress

Blockage of FAK with targeted shRNA did not inhibit periodic mechanical stress-induced Rac1 phosphorylation, relative to that in the NT shRNA group, under the same conditions ($P > 0.05$, Fig. 7, N = 5).

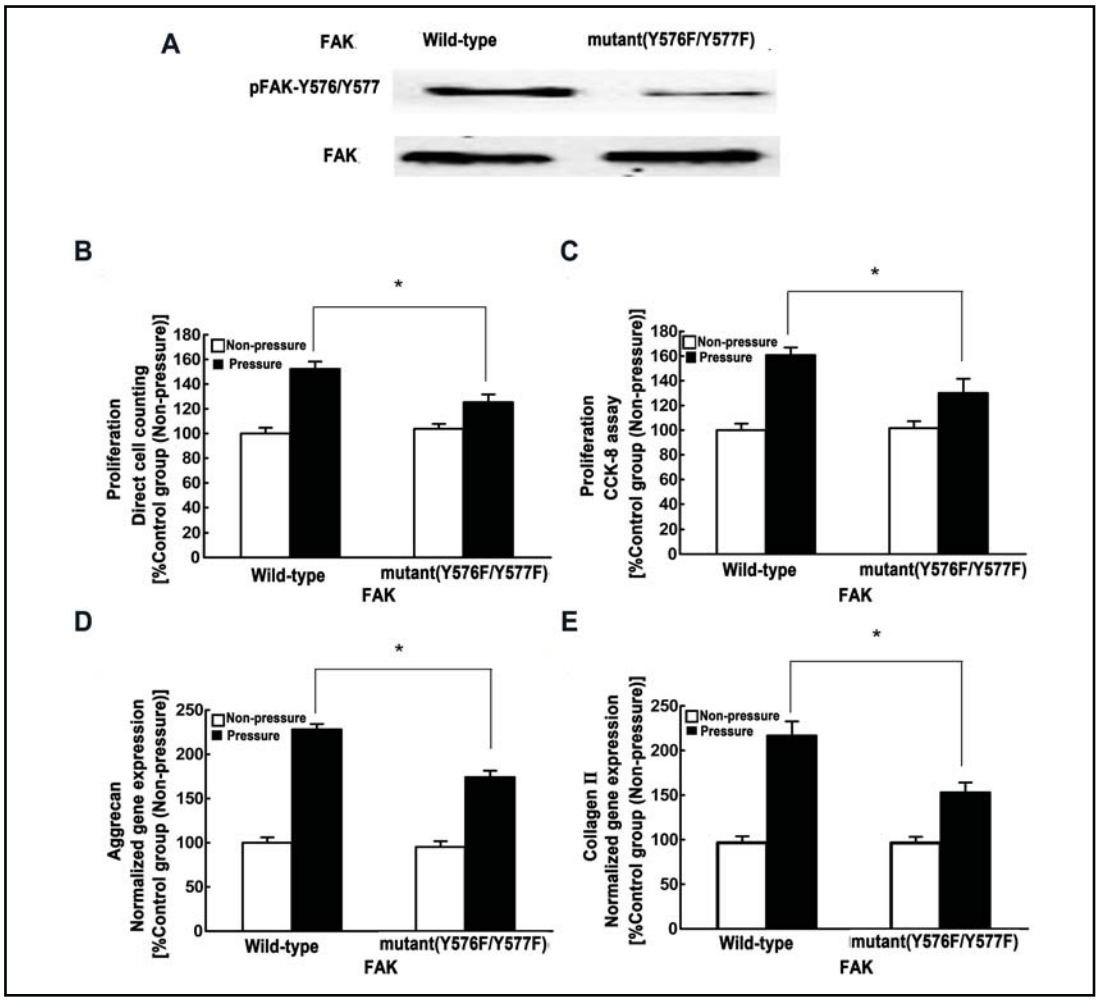


Fig. 4. Effects of blockade of FAK-Tyr$^{576/577}$ phosphorylation on chondrocyte proliferation and matrix synthesis under conditions of periodic mechanical stress. Chondrocytes were transfected with GFP-tagged FAK wild-type GV287 or GV287 expressing mutant FAK (Tyr$^{576/577}$ to Phe$^{576/577}$). Phosphorylation of Tyr$^{576/577}$ in the FAK-Phe$^{576/577}$ group was significantly diminished, relative to that in the wild-type group (A). Rat chondrocytes were cultured for 3 days under static conditions or periodic mechanical stress for 8 h per day, prior to proliferation studies. Cell proliferation was analyzed via direct cell counting (B) and the CCK-8 assay (C). Rat chondrocytes were cultured *in vitro* for 8 h under static conditions or periodic mechanical stress. Aggrecan (D) and type II collagen (E) gene expression were measured with quantitative real-time PCR. Cell proliferation and matrix synthesis in FAK mutant Phe$^{576/577}$ groups were significantly decreased, compared with that of FAK wild-type chondrocytes subjected to periodic mechanical stress (N = 5, *P < 0.05, Student's unpaired *t*-test).

PLCγ1 is not essential for periodic mechanical stress-induced FAK activation

We additionally evaluated the requirement for PLCγ1 for FAK activation under periodic mechanical stress. PLCγ1 inhibition via pretreatment with U73122 (10 μM) did not affect either FAK-Tyr^{397} or FAK-Tyr$^{576/577}$ phosphorylation, compared with levels in DMSO-pretreated chondrocytes grown under similar conditions (P > 0.05, Fig. 6, N = 5).

Role of FAK in periodic mechanical stress-induced PLCγ1 phosphorylation

Reduction of FAK with targeted shRNA did not abolish periodic mechanical stress-induced PLCγ1 phosphorylation, compared with that in the NT shRNA group under similar conditions (P > 0.05, Fig. 7, N = 5).

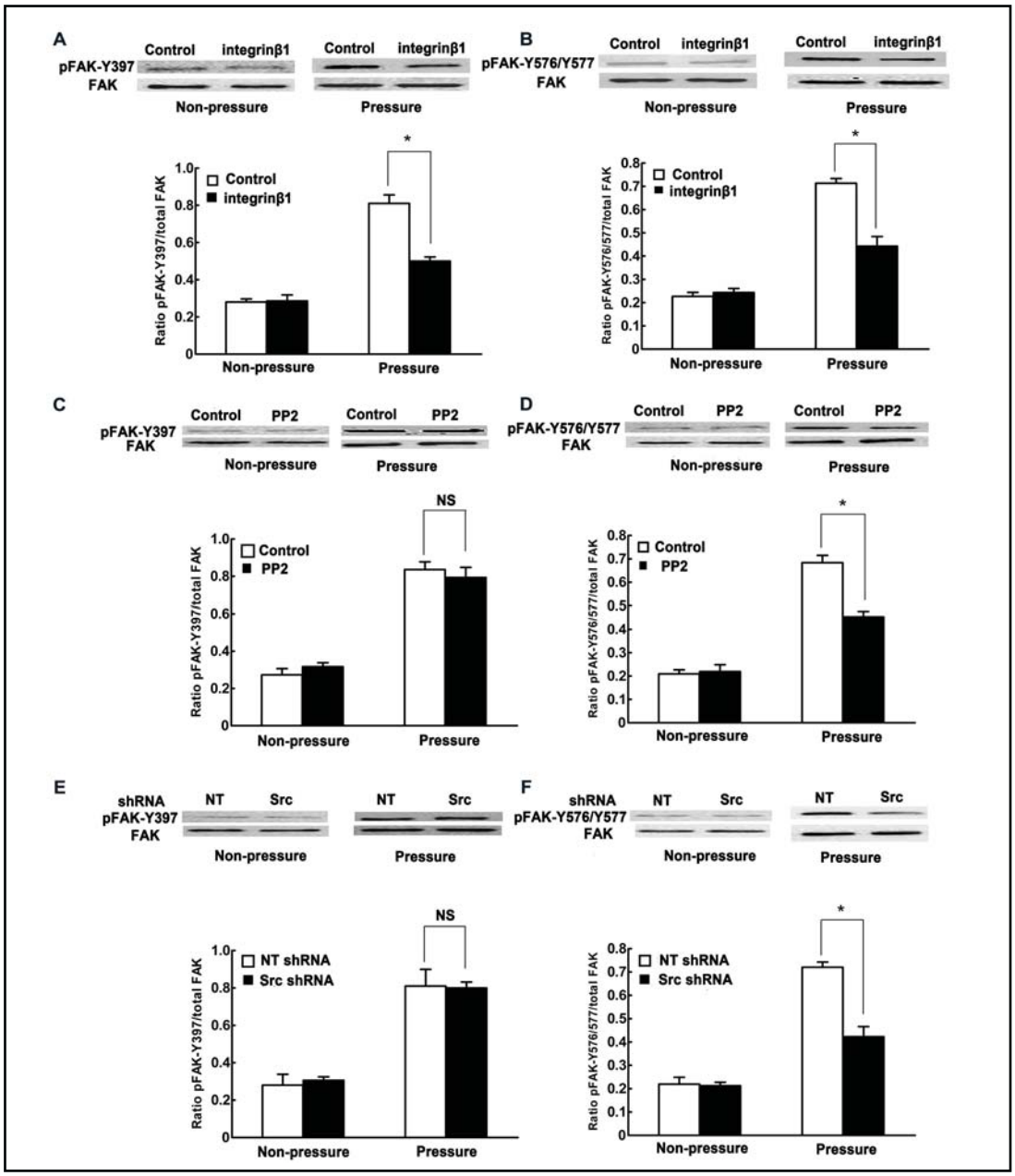


Fig. 5. Effects of integrin β1 and Src on expression and phosphorylation of FAK-Tyr^{397} and FAK-Tyr$^{576/577}$ under conditions of periodic mechanical stress. After pretreatment with control vehicle (DMEM) or blocking antibody against integrin β1, DMSO or Src inhibitor (PP2), and Src shRNA or non-targeting (NT) sequence, rat chondrocytes were cultured *in vitro* for 1 h under static conditions or periodic mechanical stress. Expression and phosphorylation levels of FAK-Tyr^{397} and FAK-Tyr$^{576/577}$ were detected using western blot analysis. Total amounts of FAK served as controls. Representative western blots from five different experiments are shown, and data averaged in a bar graph. The phosphorylation levels of FAK-Tyr^{397} and FAK-Tyr$^{576/577}$ in groups pretreated with a blocking antibody against integrin β1 were significantly diminished, compared with those of the control group, in chondrocytes subjected to periodic mechanical stress (N=5, $P < 0.05$ for each, Student's unpaired *t*-test). The phosphorylation levels of FAK-Tyr$^{576/577}$ in the PP2 and Src shRNA-pretreated groups were significantly reduced, compared to those of the control groups, in chondrocytes subjected to periodic mechanical stress (N=5, $P < 0.05$ for each, Student's unpaired *t*-test). In contrast, phosphorylation levels of FAK-Tyr^{397} in the PP2 and Src shRNA-pretreated groups were not affected (N=5, $P > 0.05$ for each, Student's unpaired *t*-test).

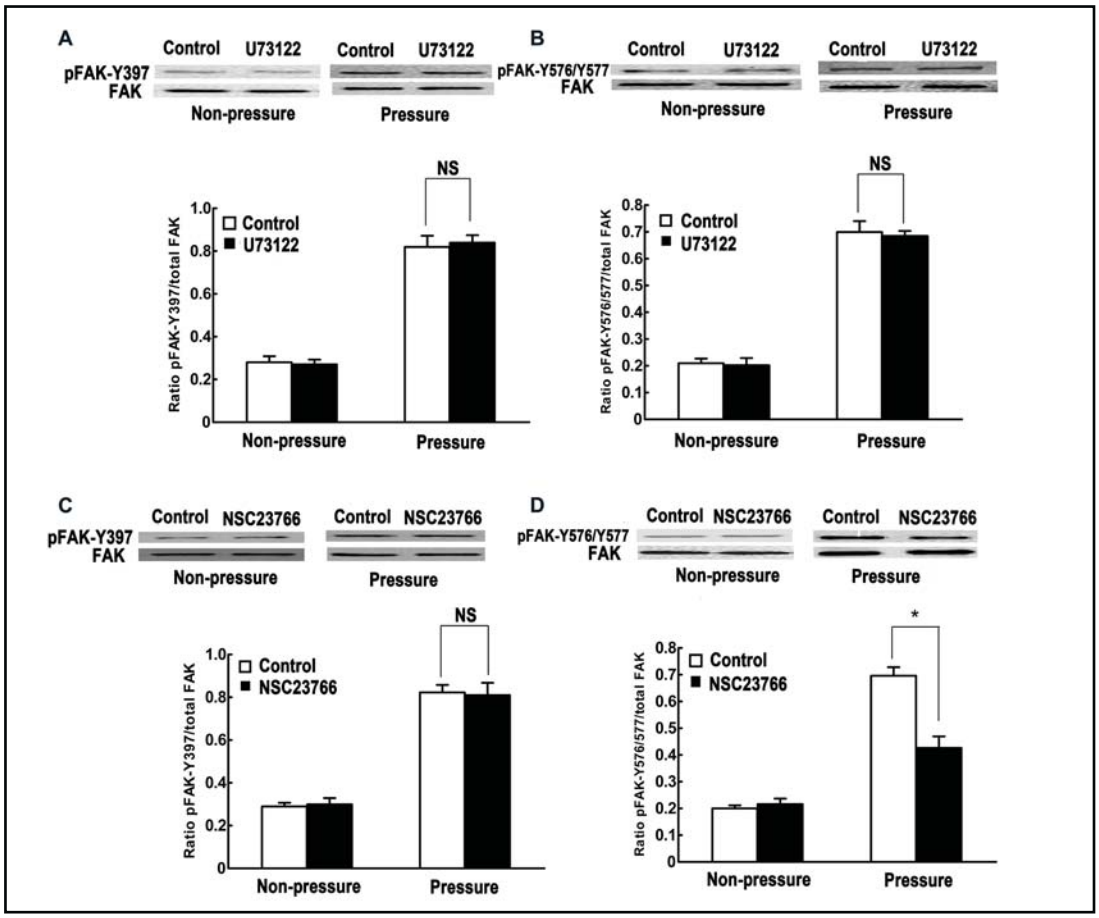


Fig. 6. Effects of PLCγ1 and Rac1 on expression and phosphorylation of FAK-Tyr^{397} and FAK-Tyr$^{576/577}$ under conditions of periodic mechanical stress. After pretreatment with DMSO or the PLCγ1 inhibitor, U73122, or the Rac1 inhibitor, NSC23766, rat chondrocytes were cultured *in vitro* for 1 h under static conditions or periodic mechanical stress. Expression and phosphorylation levels of FAK-Tyr^{397} and FAK-Tyr$^{576/577}$ were detected using western blotting. The total amount of FAK served as the control. Representative western blots from five different experiments are shown, and the data averaged in a bar graph. Phosphorylation levels of FAK-Tyr^{397} and FAK-Tyr$^{576/577}$ in the U73122 pretreatment groups were not altered in chondrocytes in response to periodic mechanical stress (N=5, P > 0.05 for each, Student's unpaired *t*-test). Phosphorylation levels of FAK-Tyr$^{576/577}$ in the NSC23766-pretreated groups were significantly reduced, compared with control chondrocytes subjected to periodic mechanical stress (N=5, P < 0.05 for each, Student's unpaired *t*-test), whereas those of FAK-Tyr^{397}were not affected (N=5, P > 0.05 for each, Student's unpaired *t*-test).

Involvement of FAK in periodic mechanical stress-induced ERK1/2 phosphorylation

The NT shRNA-transfected group displayed increased ERK 1/2 phosphorylation, compared with the FAK shRNA-transfected group, in response to periodic mechanical stress (P < 0.05, Fig. 7, N = 5).To clarify the specific roles of FAK-Tyr^{397} and Tyr$^{576/577}$ in periodic mechanical stress-induced ERK1/2 activation, we transfected chondrocytes with GFP-tagged FAK wild-type or phosphorylation-deficient mutant expression constructs in which Tyr^{397}, Tyr^{576}-Tyr^{577} were mutated to phenylalanine (Phe^{397}, Phe^{576}-Phe^{577}) to prevent tyrosine phosphorylation. ERK1/2 activation was significantly blocked in both FAK mutant Phe^{397} and Phe^{576}-Phe^{577} groups, compared with the FAK wild-type group, under periodic mechanical stress conditions (P < 0.05, Fig. 8, N = 5).

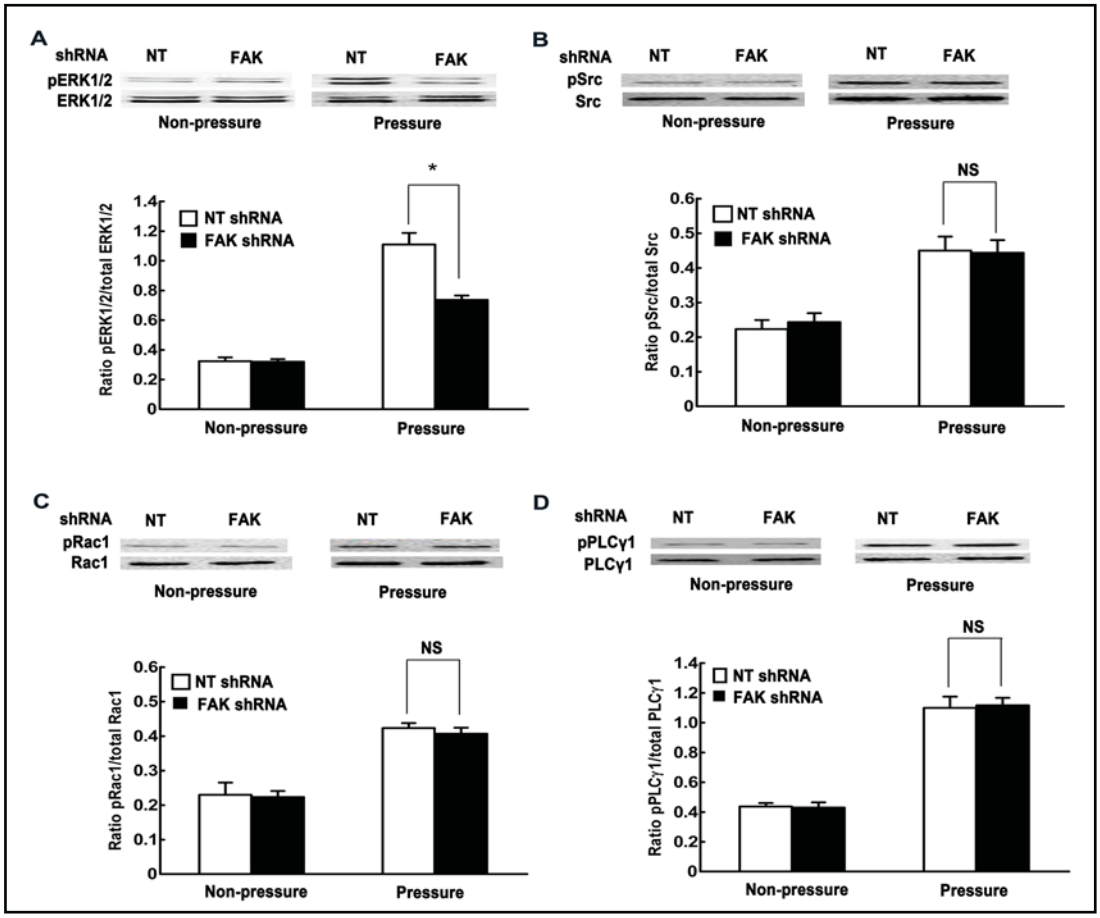


Fig. 7. Effects of FAK on expression and phosphorylation of Src, PLCγ1, Rac1 and ERK1/2 under conditions of periodic mechanical stress. After suppression of FAK with targeted shRNA, rat chondrocytes were cultured *in vitro* for 1 h under static conditions or periodic mechanical stress. Expression and phosphorylation levels of Src, PLCγ1, Rac1 and ERK1/2 were detected with western blot analysis. The total amounts of Src, PLCγ1, Rac1 and ERK1/2 served as controls, respectively. Representative western blots of five different experiments are shown, and data averaged in a bar graph. Phosphorylation levels of ERK1/2-Thr^{202}/Tyr^{204} in FAK shRNA pretreatment groups were significantly diminished relative to those of control shRNA-pretreated groups under periodic mechanical stress (N=5, $P < 0.05$, Student's unpaired *t*-test), while activation of Src-Tyr^{418}, Rac1-Ser^{71} and PLCγ1-Tyr^{783} in the FAK shRNA pretreatment groups remained unaffected (N=5, $P > 0.05$ for each, Student's unpaired *t*-test).

Discussion

In the present study, we showed that the autophosphorylation site of FAK at Tyr^{397} and two other highly conserved tyrosine phosphorylation sites, Tyr$^{576/577}$, are required for periodic mechanical stress-induced integrin β1-ERK1/2 activation, as well as chondrocyte proliferation and matrix synthesis.

The catalytic functions of FAK are dependent on phosphorylation of tyrosine residues. Tyrosine phosphorylation at different sites within FAK is catalyzed in distinct ways and affects various cellular functions. Previous studies regarding the FAK activation state in a variety of non-chondrocytic cell types under mechanical stress have mainly concentrated on the autophosphorylation site [25-27]. In a study on fibroblasts, Tyr$^{576/577}$ residues of FAK were shown to be required for the mechanical stress-induced cellular response [13, 28]. We observed that periodic mechanical stress induces significantly increased phosphorylation of FAK at Tyr^{397} and Tyr$^{576/577}$ in relation to that under static conditions in chondrocytes.

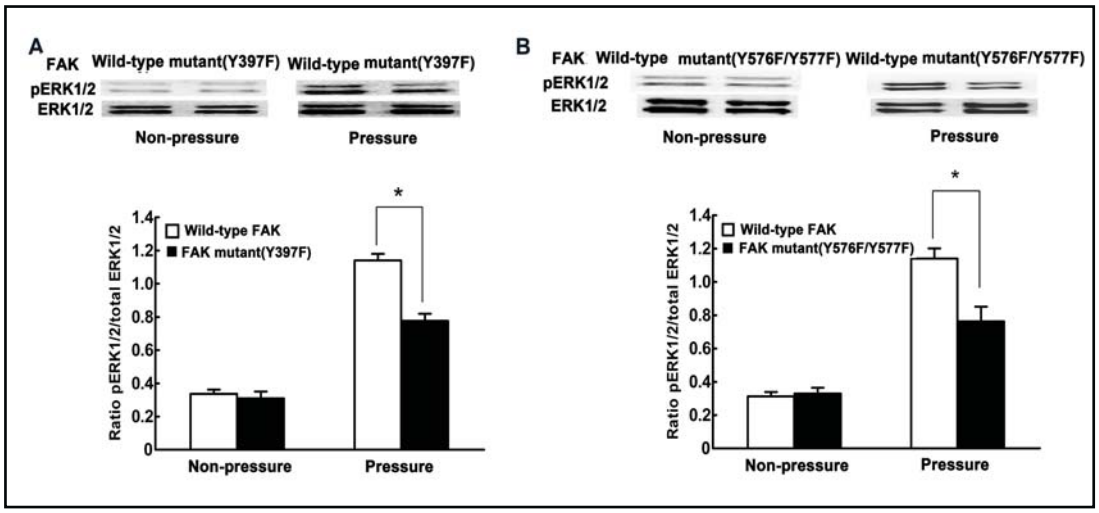
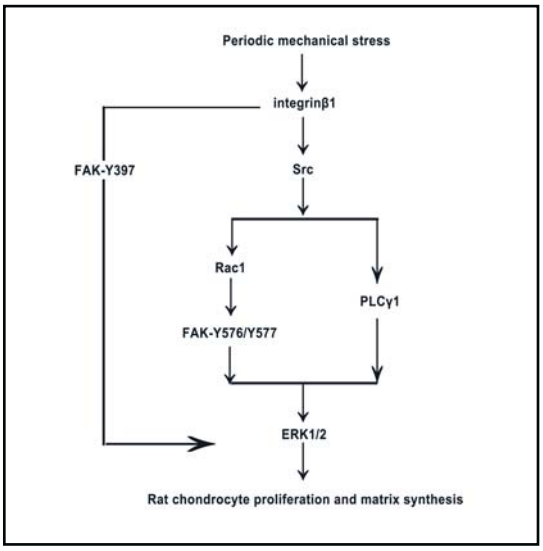


Fig. 8. Effects of prevention of FAK-Tyr^{397} or FAK-Tyr$^{576/577}$ phosphorylation on expression and phosphorylation of ERK1/2 under conditions of periodic mechanical stress. Chondrocytes were transfected with a GFP-tagged FAK wild-type GV287 vector or two GFP-tagged FAK mutant-expressing GV287 (Tyr^{397} to Phe^{397}, Tyr$^{576/577}$ to Phe$^{576/577}$) constructs. Representative western blots of five different experiments are shown, and data averaged in a bar graph. The phosphorylation levels of ERK1/2-Thr^{202}/Tyr^{204} in the FAK mutant Phe^{397} and Phe$^{576/577}$ groups were significantly abolished relative to those of FAK wild-type groups under periodic mechanical stress (N=5, $P < 0.05$, Student's unpaired *t*-test).

Fig. 9. Schematic diagram including present and previous observations regarding the signal transduction pathway of chondrocyte proliferation and matrix synthesis stimulated by periodic mechanical stress. Specifically, two signaling pathways converge on FAK to induce ERK1/2 mitogenic effects in chondrocytes mediated by periodic mechanical stress. Periodic mechanical stress activates Src , leading to activation of Rac1 and PLCγ1 . Rac1 (but not PLCγ1), activates FAK at Tyr$^{576/577}$, while separate phosphorylation at Tyr^{397} stimulated by periodic mechanical stress occurs independently of Src, Rac1 and PLCγ1. Both pathways are initiated by integrin β1.



Moreover, depletion of FAK with shRNA resulted in blockage of periodic mechanical stress-induced up-regulation of chondrocyte proliferation and matrix synthesis. These results strongly indicate that FAK plays a critical role in chondrocyte sensing and response to periodic mechanical stress. Meanwhile, FAK Y397F and Y576F/Y577F mutants abolished proliferation and matrix synthesis in chondrocytes exposed to periodic mechanical stress, compared to control groups, further supporting the involvement of these phosphorylation sites of FAK. To the best of our knowledge, this is the first study to highlight the significance of site-specific tyrosine residues within FAK in chondrocyte mitogenic signaling under periodic mechanical stress.

FAK activation is essential for several downstream mechanotransduction signal events, including ERK/MAPKS [3, 29]. An earlier study by Ward et al. demonstrated that cyclic tensile strain-induced FAK activation modulates human mesenchymal stem cell

behavior and function via enhancing ERK1/2 phosphorylation levels [30]. In the present investigation, pretreatment of chondrocytes with shRNA targeted to FAK significantly abolished ERK1/2 phosphorylation, compared with control groups, under conditions of periodic mechanical stress, suggesting that FAK is located upstream of ERK1/2. Moreover, both FAK Y397F and Y576F/Y577F mutant groups attenuated ERK1/2 phosphorylation induced by periodic mechanical stress. Our results suggest that mechanical stress-induced ERK1/2 phosphorylation in chondrocytes is at least partly regulated by activation of FAK at Tyr^{397} and $Tyr^{576/577}$, and do not exclude a possible role for other signal proteins downstream of FAK.

The relationship between Src and FAK is complex and controversial. Increasing evidence has confirmed that activated Tyr^{397} of FAK serves as a high-affinity binding site to recruit Src kinase. Activated Src phosphorylates FAK at $Tyr^{576/577}$ to form a FAK-Src signaling complex [31, 32]. In our experiments, FAK reduction with shRNA did not prevent Src activation in chondrocytes under periodic mechanical stress, while Src inhibition attenuated periodic mechanical stress-induced FAK phosphorylation at $Tyr^{576/577}$, but not Tyr^{397}. These findings indicate that Src activation of FAK at $Tyr^{576/577}$, but not Tyr^{397}, contributes to the mechanism by which chondrocytes sense and respond to periodic mechanical stress. Our data are consistent with a study showing that cyclic mechanical strain-induced Tyr^{576} activation of FAK is dependent on activation of Src in human pulmonary epithelial H441 cells [10]. However, contradictory findings have also been documented. In an investigation on vascular smooth muscle cell mechanotransduction, Rice et al. reported that Src activation leads to FAK-Tyr^{397} activity to mediate the cellular growth response [18]. A study by Chaturvedi et al. showed that in human Caco-2 intestinal epithelial cells, repetitive strain induced activation of both FAK-Tyr^{397} and FAK-Tyr^{576} located downstream of Src [33]. In addition, FAK has been implicated upstream of Src in several cell types in response to different stimuli [34, 35]. These differences may be attributable to the activation of FAK by multiple inputs and variations in cell types and settings.

Previous studies have revealed variable relationships between FAK and Rac1. Rac1 has been shown to be required for activation of FAK in many non-chondrocyte cell types subjected to various stimuli [36, 37]. We observed that pretreatment of chondrocytes with the Rac1 inhibitor, NSC23766, significantly abolishes periodic mechanical stress-induced FAK-$Tyr^{576/577}$ phosphorylation in chondrocytes, but does not affect FAK-Tyr^{397} phosphorylation. In addition, FAK inhibition with shRNA did not prevent Rac1 phosphorylation in chondrocytes subjected to periodic mechanical stress. Therefore, Rac1 phosphorylation at Ser71 appears to play an indispensable role in activation of FAK-$Tyr^{576/577}$, but not FAK-Tyr^{397}, under conditions of periodic mechanical stress. The observation is consistent with data from a study focusing on the mechanotransduction pathways of human Caco-2 intestinal epithelial cells in response to cyclic mechanical deformation [23].

Next, we examined the interactions between PLCγ1 and FAK. Pretreatment with the PLCγ1 inhibitor, U73122, did not block periodic mechanical stress-stimulated activation of FAK at Tyr^{397} or $Tyr^{576/577}$. Moreover, FAK inhibition with shRNA did not attenuate the PLCγ1 phosphorylation levels under conditions of periodic mechanical stress. Based on the results, we suggest that periodic mechanical stress-initiated PLCγ1 and FAK-$Tyr^{576/577}$ signals act in parallel.

FAK is generally viewed as an important integrin signal-associated protein that transmits mechanical signals from the extracellular matrix into intracellular biochemical signals [4, 28]. In chondrocytes pretreated with a functional blocking antibody against integrin β1, Tyr^{397} and $Tyr^{576/577}$ phosphorylation levels of FAK were significantly attenuated under periodic mechanical stress conditions. The data clearly suggest that integrin β1 activation is required for periodic mechanical stress-induced FAK-Tyr^{397} and FAK-$Tyr^{576/577}$ activation in chondrocytes. However, this may not be universally true, since mechanosensors other than integrins also transmit mechanical signals [38, 39]. For instance, a study on pulmonary artery-derived smooth muscle cells disclosed that mechanical stretch-induced FAK phosphorylation is mediated by activation of cell surface PDGFR [39].

In conclusion, FAK is a crucial decision-making protein participating in chondrocyte ERK1/2 mitogenic effects mediated by periodic mechanical stress. Specifically, two signaling pathways converge upon FAK, one requiring activation of Src and Rac1, but not PLCγ1, which, in turn, stimulates activation of FAK at $Tyr^{576/577}$, and another whereby separate phosphorylation at Tyr^{397} occurs independently of Src, Rac1 and PLCγ1, although both pathways are initiated by integrin β1 (Fig. 9). Briefly, periodic mechanical stress promotes chondrocyte proliferation and matrix synthesis, at least in part, through separate integrin β1-Src-Rac1-FAK ($Tyr^{576/577}$)-ERK1/2 and integrin β1-FAK (Tyr^{397})-ERK1/2 pathways.

Abbreviations

FAK (focal adhesion kinase); ERK (extracellular signal-regulated kinase); DMSO (Dimethyl sulfoxide); HRP (Horseradish Peroxidase); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); PDGFR (platelet-derived growth factor receptor).

Conflict of Interest

The authors declare no competing interests.

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References

1 Ren K, Liu F, Huang Y, Liang W, Cui W, Wang Q, Fan W: Periodic mechanical stress activates integrinbeta1-dependent src-dependent plcgamma1-independent rac1 mitogenic signal in rat chondrocytes through erk1/2. Cell Physiol Biochem 2012;30:827-842.

2 Galli D, Benedetti L, Bongio M, Maliardi V, Silvani G, Ceccarelli G, Ronzoni F, Conte S, Benazzo F, Graziano A, Papaccio G, Sampaolesi M, De Angelis MG: In vitro osteoblastic differentiation of human mesenchymal stem cells and human dental pulp stem cells on poly-l-lysine-treated titanium-6-aluminium-4-vanadium. J Biomed Mater Res A 2011;97:118-126.

3 Shih YR, Tseng KF, Lai HY, Lin CH, Lee OK: Matrix stiffness regulation of integrin-mediated mechanotransduction during osteogenic differentiation of human mesenchymal stem cells. J Bone Miner Res 2011;26:730-738.

4 Lee DY, Li YS, Chang SF, Zhou J, Ho HM, Chiu JJ, Chien S: Oscillatory flow-induced proliferation of osteoblast-like cells is mediated by alphavbeta3 and beta1 integrins through synergistic interactions of focal adhesion kinase and shc with phosphatidylinositol 3-kinase and the akt/mtor/p70s6k pathway. J Biol Chem 2010;285:30-42.

5 Dekkers BG, Spanjer AI, van der Schuyt RD, Kuik WJ, Zaagsma J, Meurs H: Focal adhesion kinase regulates collagen i-induced airway smooth muscle phenotype switching. J Pharmacol Exp Ther 2013;346:86-95.

6 Gillory LA, Stewart JE, Megison ML, Nabers HC, Mroczek-Musulman E, Beierle EA: Fak inhibition decreases hepatoblastoma survival both in vitro and in vivo. Transl Oncol 2013;6:206-215.

7 Bryant PW, Zheng Q, Pumiglia KM: Focal adhesion kinase is a phospho-regulated repressor of rac and proliferation in human endothelial cells. Biol Open 2012;1:723-730.

8 Kim SJ, Park KH, Park YG, Lee SW, Kang YG: Compressive stress induced the up-regulation of m-csf, rankl, tnf-alpha expression and the down-regulation of opg expression in pdl cells via the integrin-fak pathway. Arch Oral Biol 2013;58:707-716.

9 Wong VW, Rustad KC, Akaishi S, Sorkin M, Glotzbach JP, Januszyk M, Nelson ER, Levi K, Paterno J, Vial IN, Kuang AA, Longaker MT, Gurtner GC: Focal adhesion kinase links mechanical force to skin fibrosis via inflammatory signaling. Nat Med 2012;18:148-152.
10 Chaturvedi LS, Marsh HM, Basson MD: Src and focal adhesion kinase mediate mechanical strain-induced proliferation and erk1/2 phosphorylation in human h441 pulmonary epithelial cells. Am J Physiol Cell Physiol 2007;292:C1701-1713.
11 Yu T, Junger WG, Yuan C, Jin A, Zhao Y, Zheng X, Zeng Y, Liu J: Shockwaves increase t-cell proliferation and il-2 expression through atp release, p2x7 receptors, and fak activation. Am J Physiol Cell Physiol 2010;298:C457-464.
12 Saldana L, Crespo L, Bensiamar F, Arruebo M, Vilaboa N: Mechanical forces regulate stem cell response to surface topography. J Biomed Mater Res A DOI: 10.1002/jbm.a.34674.
13 Diercke K, Kohl A, Lux CJ, Erber R: Strain-dependent up-regulation of ephrin-b2 protein in periodontal ligament fibroblasts contributes to osteogenesis during tooth movement. J Biol Chem 2011;286:37651-37664.
14 Hong SY, Jeon YM, Lee HJ, Kim JG, Baek JA, Lee JC: Activation of rhoa and fak induces erk-mediated osteopontin expression in mechanical force-subjected periodontal ligament fibroblasts. Mol Cell Biochem 2010;335:263-272.
15 Luo DY, Wazir R, Tian Y, Yue X, Wei TQ, Wang KJ: Integrin alphav mediates contractility whereas integrin alpha4 regulates proliferation of human bladder smooth muscle cells via fak pathway under physiological stretch. J Urol 2013;190:1421-1429.
16 Ju L, Zhou C: Association of integrin beta1 and c-met in mediating egfr tki gefitinib resistance in non-small cell lung cancer. Cancer Cell Int 2013;13:15.
17 Luu NT, Glen KE, Egginton S, Rainger GE, Nash GB: Integrin-substrate interactions underlying shear-induced inhibition of the inflammatory response of endothelial cells. Thromb Haemost 2013;109:298-308.
18 Rice DC, Dobrian AD, Schriver SD, Prewitt RL: Src autophosphorylation is an early event in pressure-mediated signaling pathways in isolated resistance arteries. Hypertension 2002;39:502-507.
19 Seguin CA, Bernier SM: Tnfalpha suppresses link protein and type ii collagen expression in chondrocytes: Role of mek1/2 and nf-kappab signaling pathways. J Cell Physiol 2003;197:356-369.
20 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.
21 Wang P, Cao X, Nagel DJ, Yin G: Activation of ask1 during reperfusion of ischemic spinal cord. Neurosci Lett 2007;415:248-252.
22 Kloesch B, Liszt M, Steiner G, Broll J: Inhibitors of p38 and erk1/2 mapkinase and hydrogen sulphide block constitutive and il-1beta-induced il-6 and il-8 expression in the human chondrocyte cell line c-28/i2. Rheumatol Int 2012;32:729-736.
23 Chaturvedi LS, Marsh HM, Shang X, Zheng Y, Basson MD: Repetitive deformation activates focal adhesion kinase and erk mitogenic signals in human caco-2 intestinal epithelial cells through src and rac1. J Biol Chem 2007;282:14-28.
24 Bae JY, Han DW, Wakitani S, Nawata M, Hyon SH: Biological and biomechanical evaluations of osteochondral allografts preserved in cold storage solution containing epigallocatechin gallate. Cell Transplant 2010;19:681-689.
25 Tsai SW, Liou HM, Lin CJ, Kuo KL, Hung YS, Weng RC, Hsu FY: Mg63 osteoblast-like cells exhibit different behavior when grown on electrospun collagen matrix versus electrospun gelatin matrix. PloS one 2012;7:e31200.
26 Xu B, Song G, Ju Y, Li X, Song Y, Watanabe S: Rhoa/rock, cytoskeletal dynamics, and focal adhesion kinase are required for mechanical stretch-induced tenogenic differentiation of human mesenchymal stem cells. J Cell Physiol 2012;227:2722-2729.
27 Boccafoschi F, Mosca C, Bosetti M, Cannas M: The role of mechanical stretching in the activation and localization of adhesion proteins and related intracellular molecules. J Cell Biochem 2011;112:1403-1409.
28 Wen H, Blume PA, Sumpio BE: Role of integrins and focal adhesion kinase in the orientation of dermal fibroblasts exposed to cyclic strain. Int Wound J 2009;6:149-158.
29 Lal H, Verma SK, Smith M, Guleria RS, Lu G, Foster DM, Dostal DE: Stretch-induced map kinase activation in cardiac myocytes: Differential regulation through beta1-integrin and focal adhesion kinase. J Mol Cell Cardiol 2007;43:137-147.

30 Ward DF Jr, Williams WA, Schapiro NE, Weber GL, Christy SR, Salt M, Klees RF, Boskey A, Plopper GE: Focal adhesion kinase signaling controls cyclic tensile strain enhanced collagen i-induced osteogenic differentiation of human mesenchymal stem cells. Mol Cell Biomech 2007;4:177-188.
31 Skalski M, Sharma N, Williams K, Kruspe A, Coppolino MG: Snare-mediated membrane traffic is required for focal adhesion kinase signaling and src-regulated focal adhesion turnover. Biochim Biophys Acta 2011;1813:148-158.
32 Van Slambrouck S, Grijelmo C, De Wever O, Bruyneel E, Emami S, Gespach C, Steelant WF: Activation of the fak-src molecular scaffolds and p130cas-jnk signaling cascades by alpha1-integrins during colon cancer cell invasion. Int J Oncol 2007;31:1501-1508.
33 Chaturvedi LS, Gayer CP, Marsh HM, Basson MD: Repetitive deformation activates src-independent fak-dependent erk motogenic signals in human caco-2 intestinal epithelial cells. Am J Physiol Cell Physiol 2008;294:C1350-1361.
34 Chiarugi P, Pani G, Giannoni E, Taddei L, Colavitti R, Raugei G, Symons M, Borrello S, Galeotti T, Ramponi G: Reactive oxygen species as essential mediators of cell adhesion: The oxidative inhibition of a fak tyrosine phosphatase is required for cell adhesion. J Cell Biol 2003;161:933-944.
35 Liao CH, Sang S, Ho CT, Lin JK: Garcinol modulates tyrosine phosphorylation of fak and subsequently induces apoptosis through down-regulation of src, erk, and akt survival signaling in human colon cancer cells. J Cell Biochem 2005;96:155-169.
36 Jung ID, Lee J, Lee KB, Park CG, Kim YK, Seo DW, Park D, Lee HW, Han JW, Lee HY: Activation of p21-activated kinase 1 is required for lysophosphatidic acid-induced focal adhesion kinase phosphorylation and cell motility in human melanoma A2058 cells. Eur J Biochem 2004;271:1557-1565.
37 Hwang SY, Jung JW, Jeong JS, Kim YJ, Oh ES, Kim TH, Kim JY, Cho KH, Han IO: Dominant-negative rac increases both inherent and ionizing radiation-induced cell migration in c6 rat glioma cells. Int J Cancer 2006;118:2056-2063.
38 Li X, Wang X, Ye H, Peng A, Chen L: Barbigerone, an isoflavone, inhibits tumor angiogenesis and human non-small-cell lung cancer xenografts growth through vegfr2 signaling pathways. Cancer Chemother Pharmacol 2012;70:425-437.
39 Tanabe Y, Saito M, Ueno A, Nakamura M, Takeishi K, Nakayama K: Mechanical stretch augments pdgf receptor beta expression and protein tyrosine phosphorylation in pulmonary artery tissue and smooth muscle cells. Mol Cell Biochem 2000;215:103-113.