Original Paper

Periodic Mechanical Stress Activates PKC δ -Dependent EGFR Mitogenic Signals in Rat Chondrocytes via PI3K-Akt and ERK1/2

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Key Words

Chondrocyte proliferation • EGFR • Periodic mechanical stress • PI3K-Akt • PKC δ

Abstract

Background/Aims: The present study aimed to analyze the mechanisms by which periodic mechanical stress is translated into biochemical signals, and to verify the important role of signaling molecules including phosphatidylinositol-3-kinase (PI3K)-Akt, protein kinase C (PKC), and epidermal growth factor receptor (EGFR) in chondrocyte proliferation. 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 need further investigation. **Methods:** Two steps were undertaken in the experiment. In the first step, the cells were pretreated with shRNA targeted to Akt or EGFR or PKC δ or control scrambled shRNA. Moreover, they were pretreated with LY294002, GF109203X, Gö6976, rottlerin, and AG1478. They were maintained under static conditions or periodic mechanical stress for 3 days, 8 h per day, prior to direct cell counting and CCK-8 assay, respectively. In the second step, the cells were pretreated with shRNA targeted to Akt or EGFR or PKC δ or control scrambled shRNA. Moreover, they were pretreated with LY294002, AG1478, and rottlerin. They were maintained under static conditions or periodic mechanical stress for 1 h prior to Western blot analysis. **Results:** Proliferation was inhibited by pretreatment with PKC or PKC δ inhibitor GF109203X or rottlerin and by short hairpin RNA (shRNA) targeted to PKC δ , but not by PKC α inhibitor Gö6976 in chondrocytes in response to periodic mechanical stress. Meantime, rottlerin and shRNA targeted to PKC δ also attenuated EGFR, Akt, and ERK1/2 activation. Furthermore, inhibiting EGFR activity by AG1478 and shRNA targeted to EGFR abrogated chondrocyte proliferation and phosphorylation levels of Akt and extracellular signal-regulated kinase (ERK)1/2 subjected to periodic mechanical stress, while the phosphorylation site of PKC δ was not affected. In addition, pretreatment with the PI3K-Akt-selective inhibitor LY294002 and shRNA targeted to Akt reduced periodic

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mechanical stress-induced chondrocyte proliferation and phosphorylation of ERK1/2, while the phosphorylation levels of EGFR and PKC δ were not inhibited. **Conclusion:** These findings suggested that periodic mechanical stress promoted chondrocyte proliferation through PKC δ -EGFR-PI3K-Akt-ERK1/2. They provide a stronger viewpoint for further investigations into chondrocyte mechanobiology under periodic mechanical stress and the ways to improve the quality of tissue-engineered cartilage.

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Introduction

Periodic mechanical stress is advantageous to simulate *in vivo* physiological conditions for promoting chondrocyte proliferation [1, 2]. However, few studies have addressed the mechanisms underlying the translation of such mechanical inputs into biochemical signaling events within the chondrocyte lineage.

Phosphatidylinositol-3-kinase (PI3K) is a family of enzymes that can generate phosphoinositides that anchor kinases to the cell membrane to form signaling aggregates, activating multiple downstream intracellular signaling cascades [3-5]. PI3K-Akt signals activation has been shown to regulate the mitogenic responses to mechanical stimuli from the cellular peripheral environment [6]. Gayer and colleagues proved that mechanical-shear induced repetitive deformation stimulated the proliferation of intestinal epithelial cells through PI3K-Akt signals [7]. However, no study demonstrated the mitogenic effects of PI3K-Akt signals in chondrocytes in response to mechanical stimulation.

Protein kinase C, also known as PKC, is a family of protein kinases involved in controlling the activation of downstream signaling proteins [8]. PKC signals can be activated by multiple stimuli including mechanical stimulation and these pathways can mediate multiple cellular biological activity and functions [9, 10]. A study by Liu confirmed that intermittent mechanical strain-enhanced fetal lung cell proliferation was dependent on activated PKC [11]. Meanwhile, some previous studies showed that PKC activation modulated cell behavior and functions via enhancing PI3K-Akt activity [12]. However, it is presently unclear whether PKC is involved in modulating mechanical stress-initiated mitogenic effects in chondrocytes, and which specific isoforms of the PKCs are involved in this process. Moreover, the nature of the relationship between PKC and PI3K-Akt remains to be elucidated.

Epidermal growth factor receptor (EGFR) is a member of a larger superfamily of receptor kinases with intrinsic tyrosine kinase activity. EGFR can interact with and activate various potential downstream signaling molecules and intracellular signal transduction pathways, thereby eliciting various cellular responses including proliferation, differentiation, and migration [13, 14]. EGFR can be activated by mechanical stimuli. Yano et al demonstrated that mechanical stretching induced proliferative signals on human keratinocytes via EGFR-extracellular signal-regulated kinase (ERK)1/2 [15, 16]. EGFR has been shown to play important roles in the modulation of chondrocyte activity and functions following chemical stimuli [17, 18]. Moreover, the relationship among EGFR, PKC, and PI3K has been alluded to in some studies concerning signaling transduction of other cells [19, 20]. Following periodic mechanical stress, the role of EGFR in mechanosensing, signaling transduction, and final mitogenic effects in chondrocytes, including the nature of any functional association among EGFR, PKC, and PI3K in this system, is yet to be explored.

This study analyzed the mechanisms by which periodic mechanical stress is translated into biochemical signals and verified the important roles of signaling molecules including PI3K-Akt, PKC, and EGFR in chondrocyte proliferation.

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 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-PKC δ , anti-phospho-PKC δ (Thy⁵⁰⁷), anti-EGFR, anti-phospho-EGFR (Tyr¹⁰⁶⁸), anti-Akt, anti-phospho-Akt (Ser⁴⁷³), anti-ERK1/2, anti-phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴), horseradish peroxidase-goat anti-rabbit IgG, and enhanced chemiluminescence (ECL) assay kit were supplied by CST Co., Ltd. (USA). LY294002 was purchased from Promega (USA). Rottlerin was supplied by EMD Biosciences (USA). GF109203X, Gö6976, and AG1478 were purchased from Calbiochem (USA). Akt short hairpin RNA (shRNA) lentiviral particles, EGFR shRNA lentiviral particles, PKC δ shRNA 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.

Methods

Cell culture. Chondrocytes were harvested using the method described by Séguin and Bernier [21]. 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.

Inhibitors. LY294002, GF109203X, Gö6976, rottlerin, and AG1478 were specific inhibitors against PI3K-Akt, PKC, PKC α , PKC δ , and EGFR, respectively. All of the inhibitors were dissolved in anhydrous dimethylsulfoxide (DMSO) to form 1000 concentrated solution. All inhibitors were aliquoted and stored at -20°C. Each concentrated solution was diluted by 1000 × immediately prior to use; inhibitor pretreatment groups contained 0.1% (v/v) DMSO, which was the concentration used for the control group. The cells were pretreated with LY294002 (25 μM), GF109203X (10 μM), Gö6976 (10 μM), Rottlerin (10 μM), AG1478 (5 μM) or an equivalent amount of DMSO (0.1% v/v) for 1 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 [22]. The reciprocating intensifier pump was mainly composed of a compression power device, a piston, and an air-tight pressure chamber filled with a fluid. The compression power device created a mechanical reciprocating motion by connecting with the piston and squeezing the fluid of the air-tight pressure chamber to generate periodic hydraulic pressure, acting on the pressure transducer through the input terminus. The pressure transducer was composed of a diaphragm and a pressure monitor. The diaphragm was located in the middle of the pressure transducer and separated the bilateral fluids of the input terminus and the output terminus, which transmitted the periodic hydraulic pressure produced by compressive deformation of the reciprocating compression pump to the air-tight cell culture device filled with the cell culture medium. The pressure monitor was located in the output terminus of the pressure transducer, permitting monitoring and regulation of hydraulic pressure in real time to maintain a constant intensity and frequency. Hydraulic pressure periodically acted on chondrocytes of the second generation by inoculating them onto the slide in the air-tight cell culture device.

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 [23, 24]. 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 pretreated with shRNA targeted to Akt or EGFR or PKC δ or control scrambled shRNA. Moreover, they were pretreated with LY294002 (25 μM), GF109203X (10 μM), Gö6976 (10 μM), rottlerin (10 μM), and AG1478 (5 μM). They were maintained under static conditions or periodic mechanical stress for 3 days, 8 h per day, prior to direct cell counting and CCK-8 assay, respectively. In the second step, the cells were pretreated with shRNA targeted to

Akt or EGFR or PKC δ or control scrambled shRNA. Moreover, they were pretreated with LY294002 (25 μ M), AG1478 (5 μ M), and rottlerin (10 μ M). They were maintained under static conditions or periodic mechanical stress for 1 h prior to Western blot analysis.

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 using radioimmunoprecipitation assay (RIPA) buffer, and Western blot analyses were performed as previously described [25]. Total protein was prepared using RIPA buffer and 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 [26]. 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 [27]. The cells were kept in five 96-well plates ($n = 5$); 10 μ L 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), and the results were expressed as mean $\pm$ standard deviation. Student's unpaired t tests and one-way analysis of variance followed by post hoc Fisher's least significant difference tests were used to determine the statistical significance. A P value of 0.05 was considered to be significant.

Results

PI3K-Akt and EGFR Are Required for Periodic Mechanical Stress-initiated Chondrocyte Proliferation

PI3K-Akt and EGFR inhibition with their selective inhibitors (LY294002 and AG1478) and targeted shRNA both blocked periodic mechanical stress-induced chondrocyte proliferation compared with chondrocytes in the control groups in response to periodic mechanical stress ($P < 0.05$ for each, Fig. 1 and 3, $n = 5$).

PKC and PKC δ but Not PKC α Are Required for Periodic Mechanical Stress-stimulated Chondrocyte Proliferation

Pretreatment with the selective inhibitors (GF109203X and rottlerin) and shRNA targeted to PKC δ prevented periodic mechanical stress-stimulated chondrocyte proliferation compared with chondrocytes in the control groups ($P < 0.05$ for each, Fig. 2 and 3, $n = 5$). But PKC α inhibition with Gö6976 did not suppress periodic mechanical stress-induced chondrocytes proliferation compared with the control group ($P > 0.05$, Fig. 2, $n = 5$).

Fig. 1. Effects of PI3K-Akt and EGFR on chondrocyte proliferation under conditions of periodic mechanical stress (inhibitors). After pretreatment with DMSO or PI3K-Akt-selective inhibitor LY294002 (25 μ M), and EGFR selective inhibitor AG1478 (5 μ M), rat chondrocytes were cultured for 3 days under static conditions or conditions of periodic mechanical stress 8 h per day prior to proliferation studies. Chondrocyte proliferation was analyzed using direct cell counting (A) and CCK-8 assay (B). Chondrocyte proliferation results are shown in the histogram ($n = 5$, $*P < 0.05$ for each). Chondrocyte proliferation in the selective inhibitor pretreatment groups significantly decreased relative to that in the control groups under conditions of periodic mechanical stress. ($n = 5$, $P < 0.05$ for each; one-way analysis of variance followed by the post hoc Fisher's least significant difference test).

Fig. 2. Effects of PKC, PKC α , and PKC δ on chondrocyte proliferation under conditions of periodic mechanical stress (inhibitors). After pretreatment with DMSO or PKC selective inhibitor GF109203X (10 μ M), PKC α selective inhibitor Gö6976 (10 μ M), and PKC δ selective inhibitor rottlerin (10 μ M), rat chondrocytes were cultured for 3 days under static conditions or conditions of periodic mechanical stress 8 h per day prior to proliferation studies. Chondrocyte proliferation was analyzed using direct cell counting (A) and CCK-8 assay (B). Chondrocyte proliferation results are shown in the histogram ($n = 5$, $*P < 0.05$ for each). Chondrocyte proliferation significantly decreased in the GF109203X and rottlerin pretreatment groups ($n = 5$, $P < 0.05$ for each; one-way analysis of variance followed by the post hoc Fisher's least significant difference test) but not in the Gö6976 pretreatment group ($n = 5$, $P > 0.05$ for each; one-way analysis of variance followed by the post hoc Fisher's least significant difference test) relative to that in the control groups under conditions of periodic mechanical stress.

PI3K-Akt Inhibition Prevents Periodic Mechanical Stress-induced ERK1/2 Phosphorylation

PI3K-Akt inhibition by LY294002 pretreatment and shRNA targeted to Akt both abrogated the levels of ERK1/2 phosphorylation in chondrocytes under conditions of mechanical stress ($P < 0.05$ for each, Fig. 4 and 5, $n = 5$).

PI3K-Akt Is Not Required for Periodic Mechanical Stress-induced EGFR or PKC δ Phosphorylation

Inhibition of PI3K-Akt with the selective inhibitor LY294002 and shRNA targeted to Akt did not reduce periodic mechanical stress-induced EGFR or PKC δ activation in chondrocytes under mechanical conditions ($P > 0.05$ for each, not shown, $n = 5$).

Fig. 3. Effects of PI3K-Akt, EGFR, and PKC δ on chondrocyte proliferation under conditions of periodic mechanical stress (shRNA). Chondrocytes were transfected with shRNA targeted to Akt or EGFR or PKC δ or with nontargeting sequences prior to lysis and Western blotting for Akt, EGFR, and PKC δ proteins. Transfection with shRNA for Akt, EGFR, and PKC δ achieved about a 50% reduction in Akt, EGFR, and PKC δ protein levels, respectively (A). After pretreatment with control or Akt shRNA or EGFR shRNA or PKC δ shRNA, rat chondrocytes were cultured for 3 days under static 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). Chondrocyte proliferation significantly decreased in the shRNA pretreatment groups relative to that in the control groups under conditions of periodic mechanical stress ($n = 5$, $P > 0.05$ for each; one-way analysis of variance followed by the post hoc Fisher's least significant difference test).

Fig. 4. Effects of PI3K-Akt on the phosphorylation of ERK1/2 under conditions of periodic mechanical stress (LY294002). After pretreatment with DMSO or PI3K-Akt inhibitor, rat chondrocytes were cultured *in vitro* for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of Akt and ERK1/2 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. The phosphorylation levels of Akt-Ser⁴⁷³ and ERK1/2-Thr²⁰²/Tyr²⁰⁴ in the PI3K-Akt-selective inhibitor pretreatment group were significantly decreased relative to those of the control group under conditions of periodic mechanical stress ($n = 5$, $P < 0.05$ for each; Student's unpaired *t* test).

EGFR Is Required for Periodic Mechanical Stress-induced PI3K-Akt and ERK1/2 Phosphorylation

Relative to that in the control group, the phosphorylation levels of Akt and ERK1/2 significantly decreased after treatment with AG1478 and shRNA targeted to EGFR in response to periodic mechanical stress ($P < 0.05$ for each, Fig. 6 and 7, $n = 5$).

Fig. 5. Effects of PI3K-Akt on the phosphorylation of ERK1/2 under conditions of periodic mechanical stress (Akt shRNA). After pretreatment with control or Akt shRNA, rat chondrocytes were cultured *in vitro* for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of ERK1/2 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. The phosphorylation levels of ERK1/2-Thr²⁰²/Tyr²⁰⁴ in the Akt shRNA pretreatment group significantly decreased relative to those of the control group under conditions of periodic mechanical stress ($n = 5$, $P < 0.05$ for each; Student's unpaired *t* test).

Fig. 6. Effects of EGFR on the expression and phosphorylation of PKC δ , Akt, and ERK1/2 under conditions of periodic mechanical stress (AG1478). After pretreatment with DMSO or EGFR inhibitors AG1478, rat chondrocytes were cultured *in vitro* for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of EGFR, PKC δ , Akt, and ERK1/2 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. The phosphorylation levels of EGFR-Tyr¹⁰⁶⁸, Akt-Ser⁴⁷³, and ERK1/2-Thr²⁰²/Tyr²⁰⁴ in the AG1478 pretreatment group significantly decreased relative to those of the control group under conditions of periodic mechanical stress ($n = 5$, $P < 0.05$ for each; Student's unpaired *t* test), whereas the activation of PKC δ -Thr⁵⁰⁷ in the AG1478 pretreatment group was not affected ($n = 5$, $P > 0.05$ for each; Student's unpaired *t* test).

Fig. 7. Effects of EGFR on the expression and phosphorylation of PKC δ , Akt, and ERK1/2 under conditions of periodic mechanical stress (EGFR shRNA). After pretreatment with control or EGFR shRNA, rat chondrocytes were cultured *in vitro* for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of PKC δ , Akt, and ERK1/2 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. The phosphorylation levels of EGFR-Tyr¹⁰⁶⁸, Akt-Ser⁴⁷³ and ERK1/2-Thr²⁰²/Tyr²⁰⁴ in the EGFR shRNA pretreatment group significantly decreased relative to those of the control group under conditions of periodic mechanical stress ($n = 5$, $P < 0.05$ for each; Student's unpaired *t* test), whereas the activation of PKC δ -Thr⁵⁰⁷ in the EGFR shRNA pretreatment group was not affected ($n = 5$, $P > 0.05$ for each; Student's unpaired *t* test).

ERK1/2 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. The phosphorylation levels of EGFR-Tyr¹⁰⁶⁸, Akt-Ser⁴⁷³ and ERK1/2-Thr²⁰²/Tyr²⁰⁴ in the EGFR shRNA pretreatment group significantly decreased relative to those of the control group under conditions of periodic mechanical stress ($n = 5$, $P < 0.05$ for each; Student's unpaired *t* test), whereas the activation of PKC δ -Thr⁵⁰⁷ in the EGFR shRNA pretreatment group was not affected ($n = 5$, $P > 0.05$ for each; Student's unpaired *t* test).

EGFR Is Not Required for Periodic Mechanical Stress-induced PKC δ Phosphorylation

Inhibition of EGFR with its selective inhibitor AG1478 and shRNA targeted to EGFR did not block periodic mechanical stress-induced PKC δ activation in chondrocytes under mechanical stress ($P > 0.05$ for each, Fig. 6 and 7, $n = 5$).

PKC δ Is Required for Periodic Mechanical Stress-induced EGFR, PI3K-Akt and ERK1/2 Phosphorylation

Pretreatment with PKC δ selective inhibitor rottlerin and shRNA targeted to PKC δ both attenuated periodic mechanical stress-induced EGFR, Akt, and ERK1/2 phosphorylation in chondrocytes under conditions of periodic mechanical stimulation ($P < 0.05$ for each, Fig. 8 and 9, $n = 5$).

Discussion

Periodic mechanical stress induction of chondrocyte proliferation may be mediated through a process of mechanotransduction, converting physical forces into biochemical signals and integrating these signals into cellular responses. The present findings indicated that PKC δ , EGFR, and PI3K-Akt mediate the ERK1/2 mitogenic signaling cascade in chondrocytes in response to periodic mechanical stress.

PI3K-Akt signals could respond to mechanical stimuli and modulate proliferation in several nonchondrocytic cell types [28, 29]. This study investigated and considered PI3K-Akt signals to be candidate signals for inducing chondrocyte proliferation by periodic

Fig. 8. Effects of PKC δ on the expression and phosphorylation of EGFR, Akt, and ERK1/2 under conditions of periodic mechanical stress (roscovetrol). After pretreatment with DMSO or PKC δ inhibitor roscovetrol, rat chondrocytes were cultured *in vitro* for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of PKC δ , EGFR, Akt, and ERK1/2 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. The phosphorylation levels of PKC δ -Thr⁵⁰⁷, EGFR-Tyr¹⁰⁶⁸, Akt-Ser⁴⁷³, and ERK1/2-Thr²⁰²/Tyr²⁰⁴ in the PKC δ selective inhibitor pretreatment group significantly decreased relative to those of the control group under conditions of periodic mechanical stress ($n = 5$, $P < 0.05$ for each; Student's unpaired *t* test).

mechanical stress. Pretreatment with the selective inhibitors of PI3K-Akt LY294002 and shRNA targeted to Akt significantly reduced chondrocyte proliferation under mechanical stress. The present results clearly indicated that the mitogenic effects induced by cyclic mechanical stimulation were at least partly mediated by the PI3K-Akt signals. This was the first study on the mechanosensing and signal transduction role of PI3K-Akt in chondrocytes. The findings are consistent with the results of some other studies and one report by Adam et al. that demonstrated that mechanical stretch stimulation of DNA synthesis in bladder smooth muscle cells was induced by activation via PI3K-Akt signaling [30, 31].

PI3K-Akt could modulate the activity and functions of multiple downstream effectors [32, 33]. In the present study, the activation of ERK1/2 was found to be attenuated as well when the activity of PI3K-Akt was inhibited by LY294002 or shRNA targeted to Akt, suggesting that the PI3K-Akt signal was required for the activation of ERK1/2 under conditions of mechanical stimulation. This finding was consistent with the work of Lee and colleagues that oscillatory shear stress induced osteoblast proliferation through ERK1/2, and the latter depended on

Fig. 9. Effects of PKC δ on the expression and phosphorylation of EGFR, Akt, and ERK1/2 under conditions of periodic mechanical stress (PKC δ shRNA). After pretreatment with control or PKC δ shRNA, rat chondrocytes were cultured *in vitro* for 1 h with or without periodic mechanical stress. The expression and phosphorylation levels of EGFR, Akt, and ERK1/2 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. The phosphorylation levels of EGFR-Tyr¹⁰⁶⁸, Akt-Ser⁴⁷³ and ERK1/2-Thr²⁰²/Tyr²⁰⁴ in the PKC δ shRNA pretreatment group were significantly decreased relative to those of the control group under conditions of periodic mechanical stress ($n = 5$, $P < 0.05$ for each; Student's unpaired t test).

the PI3K-Akt pathway [6]. Meanwhile, the activation of EGFR and PKC δ was not found to be affected after the inhibition of PI3K-Akt under the same conditions, suggesting that PI3K-Akt signals might not be required for the activity of EGFR and PKC δ . The present data supported that the PI3K-Akt signal, which was located upstream of ERK1/2, was responsible for the up-regulation of chondrocyte proliferation by periodic mechanical stress.

EGFR, as a nonintegrin mechanosensor, could mediate mechanical responses, and the reports that EGF-EGFR induced chondrocyte proliferation prompted investigation to examine the involvement of EGFR in the periodic mechanical stress-induced chondrocytic mitogenic effects [34, 35]. The present study showed that the specific EGFR inhibitor AG1478 and shRNA targeted to EGFR blocked chondrocyte proliferation in response to periodic mechanical stress. The results strongly implicated for the first time EGFR kinase signaling in the chondrocytic mitogenic response to periodic mechanical stress. This was consistent with other observations that EGFR mediated the mechanotransduced mitogenic effects in human bronchial epithelial cells, as well as in vascular smooth muscle cells [36, 37]. Despite the importance of EGFR in mediating chondrocyte proliferation, it is possible that other growth factor receptors may also play a role under conditions of mechanical stimuli.

A number of autophosphorylation sites have been identified in the intracellular domain of EGFR, which allow for specific binding and activation of downstream signaling molecules [38, 39]. The activation of ERK1/2 and PI3K by EGFR has recently been demonstrated to be a signaling transduction mechanism in SNU-407 colon cancer cells and MH1C1 cells [40, 41]. The present study found that inhibition of EGFR with AG1478 and shRNA targeted to EGFR prevented the activation of Akt and ERK1/2, suggesting functional links between EGFR and PI3K-Akt/ERK1/2 signaling. EGFR may be a key upstream regulator of these cyclic mechanical stimuli-induced signals in this model. This study also observed that the activation of PKCδ was not reduced after pretreatment with AG1478 under the same conditions, suggesting that EGFR might not be located upstream of PKCδ. Hence, it could be concluded that EGFR mediated at least some mitogenic mechanotransduced signals through PI3K-Akt and ERK1/2 in chondrocytes.

Increasing evidence showed that PKC played an important role in the signal transduction cascades in response to mechanical stimulation through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins [9, 42]. The present study found that inhibition of PKC and PKCδ abrogated chondrocyte proliferation in chondrocytes in response to mechanical stress. Also, the mitogenic effects initiated by mechanical stress were not affected after pretreatment with PKCα inhibitor Gö6976. These results clearly showed that PKCδ played a key role in the regulation of the mitogenic responses to periodic mechanical stimuli in chondrocytes. Coincidentally, the study by Han demonstrated a similar phenomenon that cyclically mechanical strain induced Caco-2 intestinal epithelial proliferation via the PKC signal [43]. Hence, it could be concluded that PKCδ played a key role in chondrocytic adaption to physical mechanical stimulation.

Some studies showed that EGFR was required for PKC activation in other cell types in response to various stimuli [44, 45]. Interestingly, instead of being activated by an EGFR-dependent mechanism, the inhibition of cyclic mechanical stress-stimulated EGFR by rottlerin or shRNA targeted to PKCδ suggested that PKCδ might be responsible for the initial EGFR activation in the present study. PKC has also been reported to be located upstream of EGFR in response to several stimuli in different cell culture systems in diverse complex signaling pathways [46, 47]. These differences likely reflect differences in not only cell type but also the nature of the stimulus. A further observation that PKCδ is required for mechanotransduced PI3K-Akt and ERK1/2 activation seems consistent with the reports of PKC interactions with PI3K-Akt and ERK1/2 in other cell types [48, 49]. However, this relationship has not been investigated in chondrocytes under mechanical stimulation. These data suggest that PKCδ might be a proximal kinase in the signaling pathway that leads, through EGFR and PI3K-Akt, to the increased ERK1/2 activation and chondrocyte proliferation in response to periodic mechanical stress.

In conclusion, the present study found that periodic mechanical stress could stimulate the phosphorylation of PKCδ, which in turn activated EGFR. Activated EGFR led to ERK1/2 mitogenic signaling cascade via regulating the activity of PI3K-Akt in chondrocytes. Thus, PKCδ-EGFR-PI3K-Akt-ERK1/2 constituted at least one critical signal transduction pathway for chondrocyte proliferation induced by periodic mechanical stress. These results of our previous studies and the present study provide a stronger viewpoint for further investigations into chondrocyte mechanobiology under periodic mechanical stress and the way to improve the quality of tissue-engineered cartilage (Fig. 10).

Fig. 10. Schematic diagram of the signal transduction pathway of rat chondrocyte proliferation induced by periodic mechanical stress.

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Disclosure Statement

The authors declare that they have no competing interests.

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