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## Integrin-mediated mechanotransduction pathway of low-intensity continuous ultrasound in human chondrocytes

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### Abstract

Chondrocytes are mechanosensitive cells that require mechanical stimulation for proper growth and function in *in vitro* culture systems. Ultrasound (US) has emerged as a technique to deliver mechanical stress; however, the intracellular signaling components of the mechanotransduction pathways that transmit the extracellular mechanical stimulus to gene regulatory mechanisms are not fully defined. We evaluated a possible integrin/mitogen-activated protein kinase (MAPK) mechanotransduction pathway using Western blotting with antibodies targeting specific phosphorylation sites on intracellular signaling proteins. US stimulation of chondrocytes induced phosphorylation of focal adhesion kinase (FAK), Src, p130 Crk-associated substrate (p130Cas), CrkII, and extracellular-regulated kinase (Erk). Furthermore, pre-incubation with inhibitors of integrin receptors, Src, and MAPK/Erk kinase (MEK) reduced US-induced Erk phosphorylation levels, indicating integrins and Src are upstream of Erk in an US-mediated mechanotransduction pathway. These findings suggest US signals through integrin receptors to the MAPK/Erk pathway via a mechanotransduction pathway involving FAK, Src, p130Cas, and CrkII.

### Keywords

Chondrocyte; Ultrasound; Mechanotransduction; Integrin; focal adhesion kinase (FAK); Src; p130 Crk-associated substrate (p130Cas); CrkII; extracellular-regulated kinase (Erk)

### Introduction

Optimal *in vitro* culture of chondrocytes involves a combination of unique culture conditions, including growth factors and supplements in culture media, three-dimensional culture in scaffolds (Grundmann et al. 1980; Benya and Shaffer 1982; Noriega et al. 2012), and mechanical stimulation (Palmski et al. 1980; Carver and Heath 1999a). Naturally, articular cartilage is subjected to frequent mechanical impacts as the joint performs its normal functions (Wang and Thampatty 2006). Chondrocytes sense these mechanical stresses and convert the mechanical signals into the activation of intracellular signaling molecules that ultimately affect the metabolic activity of chondrocytes (Fitzgerald et al. 2004; Fitzgerald et al. 2006). If cartilage *in vivo* does not receive adequate mechanical stresses, the cartilage tends to atrophy due to decreased metabolic activity of chondrocytes and decreased production of components of the extracellular matrix (ECM). For example,

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immobilization of a joint causes degenerative changes to the cartilage, such as reduced proteoglycan production by chondrocytes (Palmoski et al. 1979; Jortikka et al. 1997). Conversely, the application of mechanical force to cartilage by daily exercise can increase the thickness of cartilage and enhance the glycosaminoglycan content of joint cartilage (Kiviranta et al. 1988) and can enhance cartilage repair of injured joints (Todhunter et al. 1993). This mechanical stress-induced biological response of chondrocytes has been utilized to enhance *in vitro* chondrocyte culture systems, stimulating chondrocyte proliferation and production of components of the ECM. Multiple techniques can be used to apply mechanical stimulation to *in vitro* culture systems, including hydrostatic pressure (Carver and Heath 1999a, 1999b), hydrodynamic shear (Freed et al. 1993), static and dynamic compression (Buschmann et al. 1995; Bonassar et al. 2001; Fitzgerald et al. 2004; Fitzgerald et al. 2006), dynamic shear (Fitzgerald et al. 2006), and application of low-intensity ultrasound (US) (Parvizi et al. 1999; Nishikori et al. 2002; Zhang et al. 2002, 2003; Noriega et al. 2007; Hasanova et al. 2011). Low-intensity US stimulation has emerged as technique to enhance *in vitro* chondrocyte culture systems (Noriega et al. 2007; Hasanova et al. 2011), accelerate fracture healing, and shorten the duration of treatment needed to repair damaged cartilage in patients and in animal models (Heckman et al. 1994; Yang et al. 1996; Rubin et al. 2001; Pounder and Harrison 2008). Our previous work has shown that the expression levels of integrins  $\alpha_5$  and  $\beta_1$ , as well as chondrocytic markers, Sox5, Sox9, collagen II and aggrecan, were increased in chondrocytes exposed to a continuous US signal at 5.0 MHz (0.14 mW/cm<sup>2</sup>) (Hasanova et al. 2011).

Mechanical stimulation of cells can be detected by multiple mechanoreceptors, including stretch activated channels (SAC) (Wright et al. 1996), annexin V (von der Mark and Mollenhauer 1997; Haut Donahue et al. 2004), CD44 (Morris et al. 2010), and integrins (Zhou et al. 2004; Wang and Thampatty 2006). Integrin receptors physically adhere chondrocytes to the ECM (Wang and Thampatty 2006), and when stimulated integrins activate intracellular signaling pathways that promote survival (Coppolino and Dedhar 2000) and mediate ECM component production by chondrocytes (Takeuchi et al. 2008). When integrin receptors are activated they cluster with other integrins, adaptor proteins, and kinases such as focal adhesion kinase (FAK) to form a focal adhesion complex, which then activates intracellular signaling cascades that mediate cellular responses (Vuori 1998; Giancotti and Ruoslahti 1999). The positive effects of US on chondrocytes in culture, including increased proliferation and production of ECM components, are already well documented (Carver and Heath 1999a; Noriega et al. 2007; Hasanova et al. 2011). However, the intracellular signaling components of the mechanotransduction pathways that are responsible for transmitting the extracellular mechanical stimulus to gene regulatory mechanisms are not fully defined and require further research. This study began with multiple hypotheses of potential mechanotransduction pathways responsible for US-mediated effects on chondrocytes based on an extensive literature search of possible signaling molecules and phosphorylation sites. Then each potential component was systematically ruled out until the signaling molecules were narrowed down to the integrin/mitogen-activated protein kinase (MAPK) pathway presented in this manuscript.

## Materials and Methods

### Human chondrocyte culture

Adult human chondrocytes isolated from normal articular cartilage were purchased from Cell Applications Inc. (San Diego, CA, USA). These chondrocytes can be cultured for at least ten doublings according to the manufacturer, and they can produce collagen II protein and deposit extracellular collagen II fibers through passage five (data not shown). Cryopreserved chondrocytes were thawed and cultured in T75 flasks with RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS;

Invitrogen), 1 mM sodium pyruvate (Invitrogen), 23.8 mM sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), and 1% antibiotic-antimycotic (Invitrogen) at 37°C, 5% carbon dioxide, and 99% humidity. To passage chondrocytes, we removed the medium, rinsed cells with 1x trypsin (Invitrogen) in Hank's balanced salt solution (HBSS; Invitrogen), added 2.5 mL 1x trypsin in HBSS to the T75 flask, and incubated at 37°C for 3 minutes, rinsed the flask with RPMI, centrifuged cell suspension for 6 minutes at 200x gravity. After centrifugation, we removed supernatant, resuspended cells in RPMI, counted cells using a hemocytometer, and replated chondrocytes at  $7.5 \times 10^5$  cells/T75 flask. Chondrocytes were passaged at about one week intervals and used between passages two and five.

### Ultrasound stimulation

Human chondrocytes were passaged and cells were seeded in 6-well plates at  $2.0 \times 10^5$  cells/well and allowed to attach and grow for 1–3 days in RPMI-1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 23.8 mM sodium bicarbonate, and 1% antibiotic-antimycotic at 37°C, 5% carbon dioxide, and 99% humidity. The day prior to US stimulation the cells were deprived of serum by removing the RPMI medium with 10% FBS and replacing with 6 mL/well RPMI with 0.1% FBS, to reduce FBS-induced phosphorylation of intracellular signaling molecules that may otherwise mask the US-induced phosphorylation events (Takeuchi et al. 2008).

Serum-deprived chondrocytes were stimulated for 3 minutes with a continuous sinusoidal 5.0 MHz US signal using non-focused immersion transducers (Panametrics, V300, 12.7 mm diameter, 5 MHz center frequency; Waltham, MA, USA) immersed in the media in each well of the 6-well plates 6 mm away from the bottom of the well. The cells were in the near-field of the transducer, and the highly directive acoustic beam is just slightly wider than the transducer diameter at a distance of 6 mm from the transducer face. Wave reflection occurred exclusively at the well bottom, as the well diameter of 35 mm was much greater than that of the beam diameter of 12.7 mm. An air gap below the 6-well plate caused near complete reflection of the incident acoustic wave, resulting in a standing wave system with the cells located roughly 2/3 between a pressure antinode and a node, as predicted using numerical simulation (data not shown). The spatially averaged pressure, quantified using a needle hydrophone (Onda Corporation, CA, USA), was kept constant at 14 kPa.

Following US stimulations, the cells were incubated at 37°C for 5, 15, 30, or 60 minutes prior to protein extraction. One full 6-well plate was used for each condition with protein from all 6 wells combined for each sample. Cells that did not receive US stimulation were used as controls. For assays using inhibitor pre-incubation, 500  $\mu\text{g}/\text{mL}$  Glycine-Arginine-Glycine-Aspartic Acid-Serine-Proline (GRGDSP) (AnaSpec Fremont, CA, USA), 50  $\mu\text{M}$  4-Amino-5-(methylphenyl)-7-(t-butyl)pyrazolo-(3,4-d)pyrimidine (PP1; Sigma-Aldrich), 50  $\mu\text{M}$  PD98059 (Sigma-Aldrich), or vehicles alone for inhibitor-free controls were added to each well of the 6-well plate for four hours prior to US stimulation. All samples, including the non-US-stimulated control, received equal concentrations of water and DMSO vehicles that were used to reconstitute the inhibitors. Following four hours of inhibitor pre-incubation, the chondrocytes were treated with US as described above.

### Protein extraction

Protein was extracted from the chondrocytes using Pierce IP lysis buffer (Thermo Scientific Pierce, Rockford, IL, USA) supplemented with 1x Halt protease and phosphatase inhibitor cocktail (Thermo Scientific Pierce) following a 5, 15, 30, or 60 minute incubation after US stimulation or from control chondrocytes that did not receive US stimulation. Chondrocytes were rinsed twice with cold HBSS, and then 200  $\mu\text{L}$  of IP lysis buffer with Halt inhibitor

was added to each well and incubated for at least 5 minutes prior to combining the lysis buffer from all six wells. The 1.2 mL of protein and lysis buffer was then lyophilized for 3 hours to a volume of around 300  $\mu$ L to increase the concentration of the protein samples. The protein concentration was then analyzed using the bicinchoninic acid assay (Thermo Scientific Pierce) method.

### Western blotting analysis

Protein (20–25  $\mu$ g/well) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis using the NuPAGE system following the manufacturer's instructions (Invitrogen). Proteins were combined with NuPAGE 4x lithium dodecyl sulfate sample buffer and NuPAGE 10x sample reducing agent (Invitrogen), and then heated at 100°C for 10 minutes in a heating block. Denatured and reduced proteins were separated on 10% or 4–12% NuPAGE gels (Invitrogen) and electrophoretically transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using a Pierce fast semi-dry blotter according to the manufacturer's protocol (Thermo Scientific Pierce). Membranes were blocked with 5% dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for one hour, and then incubated with primary antibodies in TBST with 5% bovine serum albumin (BSA; Sigma-Aldrich) overnight at 4°C. Membranes were rinsed with TBST then incubated with secondary horseradish peroxidase (HRP)-linked antibodies for one hour at room temperature and rinsed with TBST. Protein bands were visualized by enhanced chemiluminescence (ECL) Western blotting substrate (Millipore or Thermo Scientific Pierce) and captured with GE Healthcare Amersham Hyperfilm ECL (GE Healthcare, Piscataway, NJ, USA). Primary antibodies used were: phospho-Y397-FAK (1:250, Cell Signaling Technology, Beverly, MA, USA), FAK (1:250, Cell Signaling Technology), phospho-Y416-Src (1:500, Cell Signaling Technology), Src (1:500, Cell Signaling Technology), phospho-Y249-p130 Crk-associated substrate (p130Cas) (1:500, Cell Signaling Technology), p130Cas (1:500, Millipore), phospho-Y221-CrkII (1:250, Cell Signaling Technology), CrkII (1:1000, Cell Signaling Technology), phospho-T202/Y204-extracellular-regulated kinase (Erk) (1:1000, Cell Signaling Technology), and Erk (1:1000, Cell Signaling Technology).  $\beta$ -actin (1:1000, Sigma-Aldrich) was used as a loading control. Secondary antibodies used were: anti-rabbit (1:5000; KPL, Gaithersburg, MD, USA) and anti-mouse (1:10000, KPL). Immunoblots were scanned and images were quantified using the open access software NIH ImageJ (Abramoff 2004).

### Statistical analysis

Data were expressed as means  $\pm$  standard error of the mean (SEM). The data were evaluated statistically by the analysis of variance (ANOVA), followed by the Tukey-test for paired observations. Significance was considered to be  $p < 0.05$ .

## Results

### US-induced FAK phosphorylation

Following US-induced activation of integrin receptors and formation of the focal adhesion complex in chondrocytes, FAK autophosphorylates at tyrosine (Y) 397, which then acts as a binding site for Src family kinases (Schaller et al. 1994; Takeuchi et al. 2008). Human chondrocytes that were serum-deprived overnight were treated with 5.0 MHz US for three minutes, and then the cells were lysed to collect protein 5, 15, 30 and 60 minutes after the US mechanical stress. Western blotting was used to analyze the phosphorylation of FAK at Y397 compared to total FAK. US stimulation induced phosphorylation of FAK Y397 (Fig. 1).

### US-induced Src phosphorylation

Phosphorylated Y397 of FAK acts as a binding site for Src, which is then activated by autophosphorylation of Y416 in the activation loop of the kinase domain of Src (Cooper and MacAuley 1988; Schaller et al. 1994). Serum-deprived human chondrocytes were treated with 5.0 MHz US for three minutes. After the US mechanical stress, the cells were incubated for 5, 15, 30, and 60 minutes and then the cells were lysed to collect protein. Phosphorylation of Src at Y416 was compared to total Src was determined using Western blotting. US stimulation induced phosphorylation of Src Y416 (Fig. 2).

### US-induced p130Cas phosphorylation

Activated Src kinase phosphorylates a multitude of targets such as p130Cas. We analyzed the phosphorylation of p130Cas at three potential phosphorylation sites Y165, Y249, and Y410 in response to US stimulation. Human chondrocytes were serum-deprived overnight and then treated with 5.0 MHz US for three minutes. Chondrocytes were lysed to collect protein 5, 15, 30, and 60 minutes after the US mechanical stress. Western blotting was used to analyze the phosphorylation of p130Cas at Y165, Y249, and Y410 compared to total p130Cas. US stimulation induced transient phosphorylation of p130Cas Y249, with a statically significant increase at 5 minutes compared to control ( $p < 0.05$ ), and decrease back to basal levels by 60 minutes ( $p < 0.05$ ) (Fig. 3). However, we did not observe US-induced phosphorylation at Y165 and Y410 (data not shown).

### US-induced CrkII phosphorylation

Phosphorylated Y249 of p130Cas creates a binding site for the CrkII adapter protein (Shin et al. 2004; Modzelewska et al. 2006). To examine if CrkII plays a role in integrin-mediated US mechanotransduction, we measured the phosphorylation of CrkII at Y221. Serum-deprived human chondrocytes were treated with 5.0 MHz US for three minutes, then 5, 15, 30, and 60 minutes after the US mechanical stress the cells were lysed to collect protein. Phosphorylation of CrkII at Y221 was compared to total CrkII using Western blotting. US stimulation induced transient phosphorylation of CrkII at Y221, with a statically significant increase at 5 minutes compared to control ( $p < 0.05$ ) (Fig. 4).

### US-induced Erk1/2 phosphorylation

Guanine nucleotide exchange factors, including C3G (Knudsen et al. 1994; Matsuda et al. 1994), Son of Sevenless (SOS) (Matsuda et al. 1994), and dedicator of cytokinesis (DOCK180) (Hasegawa et al. 1996), may bind to CrkII and lead to the activation of the MAPK pathway and ultimately the phosphorylation and activation of Erk. Human chondrocytes that were serum-deprived overnight were treated with 5.0 MHz US for three minutes, and then the cells were lysed to collect protein 5, 15, 30, and 60 minutes after the US mechanical stress. Western blotting was used to analyze the phosphorylation of Erk1/2 at threonine (T) 202/Y204 of Erk1 and T185/Y187 of Erk2 compared to total Erk1/2. US stimulation induced transient phosphorylation of Erk1/2, with a statically significant increase at 5, 15, and 30 minutes compared to control ( $p < 0.01$ ), and decrease to near basal levels by 60 minutes ( $p < 0.05$ ) (Fig. 5).

### Inhibitors of integrins, Src, and MEK reduce US-induced Erk1/2 phosphorylation

We demonstrated that FAK, Src, p130Cas, CrkII, and Erk are all phosphorylated in response to US stimulation; thus, it is plausible that they are involved in an US-mediated mechanotransduction pathway. However, without blocking the downstream phosphorylation of Erk by inhibiting the factors upstream we cannot conclude that these phenomena are a part of an interconnected mechanotransduction pathway. Thus, we pre-treated serum-deprived human chondrocytes with 500  $\mu\text{g}/\text{mL}$  GRGDSP, a peptide inhibitor of integrins,

50  $\mu\text{M}$  PP1, an inhibitor of Src, 50  $\mu\text{M}$  PD98059, an inhibitor of MAPK/Erk kinase (MEK), the kinase immediately upstream of Erk, or vehicles alone for inhibitor-free controls for four hours prior to US stimulation. Following inhibitor pre-incubation, the chondrocytes were treated with 5.0 MHz US for three minutes, and then the cells were lysed to collect protein 15 minutes after the US mechanical stress. Western blotting was used to analyze the phosphorylation of Erk1/2 at T202/Y204 of Erk1 and T185/Y187 of Erk2 compared to total Erk1/2. US stimulation induced phosphorylation of Erk1/2 in cells that received US treatment without inhibitor pre-treatment, and both GRGDSP and PP1 reduced Erk1/2 phosphorylation to varying degrees with PD98059 as a positive control for preventing Erk1/2 phosphorylation (Fig. 6). Similar levels of inhibition of Erk1/2 phosphorylation were also observed using lower concentrations of inhibitors: 100  $\mu\text{g}/\text{mL}$  GRGDSP, 10  $\mu\text{M}$  PP1, and 20  $\mu\text{M}$  PD98059 (data not shown). These findings suggest that integrin receptors and Src are both upstream of US-mediated Erk1/2 phosphorylation.

## Discussion

Mechanotransduction is a process in which mechanical stimuli are sensed by cells and are converted into an alteration of intracellular signaling molecules that induce a cellular response. Mechanical stimulation can be detected by multiple mechanoreceptors, including SAC (Wright et al. 1996), annexin V (von der Mark and Mollenhauer 1997; Haut Donahue et al. 2004), CD44 (Morris et al. 2010), and integrins (Zhou et al. 2004; Wang and Thampatty 2006), and redundant mechanisms for US mechanotransduction in chondrocytes involving multiple mechanoreceptors may exist. However, for this study, we focused only on the role of integrin receptors in the mechanotransduction of low-intensity continuous US in human chondrocytes *in vitro*. Inhibition of integrins using an Arginine-Glycine-Aspartic Acid (RGD)-containing peptide reduced downstream US-induced Erk1/2 phosphorylation (Fig. 6), and an integrin-blocking antibody reduced Erk1/2 phosphorylation in a human chondrocyte cell line (Choi et al. 2007). Furthermore, inhibition of integrins using an integrin-blocking antibody or RGD peptide was reported to reduce US-induced proliferation of fibroblasts (Zhou et al. 2004), demonstrating the role of integrins in US-mediated mechanotransduction. Integrins are transmembrane receptors that adhere cells to the ECM via short amino acid sequences, such as RGD motifs, and bind to various intracellular cytoskeletal-adaptor proteins and signaling molecules (Giancotti and Ruoslahti 1999). Integrins act as mechanotransducers that activate intracellular signaling pathways after binding to the ECM or sensing mechanical stimulation. However, integrins do not possess intrinsic catalytic activity; thus, signals from integrin-ECM interactions or mechanical stimuli are transduced into the cell via interactions with and activation of integrin-associated proteins (Mitra and Schlaepfer 2006).

When integrin receptors are activated, they cluster with other integrins, structural adaptor proteins, and kinases to form a focal adhesion complex which then activates intracellular signaling cascades that mediate cellular responses (Vuori 1998; Giancotti and Ruoslahti 1999). Focal adhesion complexes are formed by the interaction of the cytoplasmic tail of integrins with proteins such as paxillin, talin, vinculin, actin, and FAK (Chen et al. 1995; Ezzell et al. 1997; Giancotti and Ruoslahti 1999; Schaller 2001). Following activation of integrins, FAK autophosphorylates at Y397, generating a high-affinity binding site for the Src homology 2 (SH2) domain of Src family kinases (Schaller et al. 1994). Mutation of Y397 of FAK to phenylalanine abolished cyclic strain-induced phosphorylation of Erk2 in osteoblasts (Boutahar et al. 2004), demonstrating the role of FAK phosphorylation at Y397 in integrin-mediated mechanotransduction.

Multiple lines of evidence suggest that Src, a non-receptor tyrosine kinase that consists of a SH3 domain, SH2 domain, and tyrosine kinase domain, is involved in integrin-mediated

mechanotransduction of US mechanical stimulation of chondrocytes. Src has previously been shown to bind to phosphorylated Y397 of FAK via the SH2 domain (Schaller et al. 1994; Schlaepfer et al. 1994; Boutahar et al. 2004). Furthermore, mutation of Y397 to phenylalanine prevented stable binding of Src to FAK (Schaller et al. 1994) and suppressed mechanical strain-induced Erk2 phosphorylation in osteoblasts (Boutahar et al. 2004), demonstrating that Src-Y397-FAK interaction is a prospective component of integrin-mediated mechanotransduction. US stimulation induced Src autophosphorylation of Y416 of Src as demonstrated in Figure 2; moreover, other studies reported US-induced Src Y416 phosphorylation in fibroblasts (Zhou et al. 2004) and cyclic strain-induced Src Y416 phosphorylation in osteoblasts (Boutahar et al. 2004). Inhibition of Src by PP1 prevented US-induced downstream phosphorylation of Erk1/2 in chondrocytes as demonstrated in Figure 6 as well as in fibroblasts (Zhou et al. 2004). Taken together this evidence suggests that Src plays a role in integrin-mediated mechanotransduction of chondrocytes *in vitro* after US mechanical stimulation.

The p130Cas docking protein has been shown to bind to FAK via a SH3 domain and this interaction has been implicated in integrin-mediated signaling (Polte and Hanks 1995, 1997). A Src binding domain, RPLPSPP, near the C terminus of p130Cas acts as a direct binding site for Src via the SH3 domain (Nakamoto et al. 1996), and p130Cas contains Tyr-X-X-Pro motifs, including Y165, Y249, and Y410, that when phosphorylated may act as binding sites for SH2 domains of numerous proteins, such as Crk (Ruest et al. 2001). FAK acts as a docking protein for both Src and p130Cas, with the SH2 domain of Src binding to phosphorylated Y397 of FAK and the SH3 domain of p130Cas binding to FAK (Ruest et al. 2001). Src may then bind p130Cas through the SH3 domain of Src, enhancing substrate recognition prior to phosphorylation of tyrosine residues on p130Cas (Nakamoto et al. 1996), and possibly strengthening the FAK-Src-p130Cas complex. Following the formation of the FAK-Src-p130Cas complex, Src phosphorylates p130Cas on Tyr-X-X-Pro motifs, such as Y165, Y249, and Y410; however, we were only able to detect phosphorylation of p130Cas at Y249 but not at Y165 or Y410 (Fig. 3; data not shown).

Phosphorylated Y249 of p130Cas creates a binding site for the CrkII adapter protein (Shin et al. 2004; Modzelewska et al. 2006). CrkII is an adapter protein that binds multiple guanine nucleotide exchange factors such as C3G (Knudsen et al. 1994; Matsuda et al. 1994), SOS (Matsuda et al. 1994), and DOCK180 (Hasegawa et al. 1996; Kiyokawa et al. 1998), which can signal to the MAPK/Erk pathway via Rap (Gotoh et al. 1995; Peyssonnaud and Eychene 2001), Ras (Schlaepfer et al. 1998; Giancotti and Ruoslahti 1999), and Rac (Eblen et al. 2002) GTP-binding proteins, respectively. To examine the activation of CrkII, we measured the phosphorylation of CrkII at Y221, which when phosphorylated causes intramolecular binding of Y221 with the SH2 domain of CrkII (Feller et al. 1994; Rosen et al. 1995), disrupting CrkII binding to p130Cas (Kain and Klemke 2001) and downstream guanine nucleotide exchange factors such as C3G (Huang et al. 2008). Even though phosphorylation of Y221 is considered to be a negative regulator of CrkII, phosphorylation of Y221 demonstrates CrkII may be involved in an US-mediated mechanotransduction pathway.

The MAPK/Erk pathway is a well-documented signaling pathway that influences numerous cellular processes including proliferation, survival, migration, and a wide range of gene regulation that control events such as integrin expression in osteoblasts and cartilage ECM remodeling, via alterations in the activity of transcription factors (Cobb 1999; Lai et al. 2001; Pearson et al. 2001; Beier and Loeser 2010). Our results along with other reports indicate that Erk is phosphorylated in response to US mechanical stimulation via an integrin-mediated mechanotransduction pathway (Zhou et al. 2004; Choi et al. 2007; Pounder and Harrison 2008). Erk1/2 is phosphorylated in response to mechanical stimulation by US as demonstrated in Figure 5. US-induced Erk1/2 phosphorylation is downstream of integrin

receptors, as demonstrated by the reduction of Erk1/2 phosphorylation in chondrocytes by an RGD-containing peptide (Fig. 6) and an integrin-blocking antibody (Choi et al. 2007). Furthermore, mechanical strain-induced Erk phosphorylation was reduced by the mutation of Y397 of FAK to phenylalanine in osteoblasts (Boutahar et al. 2004), demonstrating the role of FAK phosphorylation at Y397 in integrin-mediated mechanotransduction. Src has also been shown to be upstream of US-induced Erk1/2 phosphorylation in chondrocytes (Fig. 6) and fibroblasts (Zhou et al. 2004) by inhibition with PP1.

In this study, we present a possible mechanotransduction pathway for US mechanical stimulation of chondrocytes *in vitro*. US stimulation of chondrocytes induced phosphorylation of FAK, Src, p130Cas, CrkII, and Erk; thus, these intracellular signaling molecules may be involved in an US-mediated mechanotransduction pathway. Pre-incubation of chondrocytes with inhibitors of integrin receptors, Src and MEK reduced US-induced Erk phosphorylation levels, indicating integrins and Src are upstream of Erk in a mechanotransduction pathway of US-stimulated mechanical stress of chondrocytes. However, dominant-negative expression or site-specific mutation of the phosphorylation sites of FAK, Src, p130Cas, CrkII, and Erk are needed to further verify the signaling pathway proposed in this study. Furthermore, the results presented in this study remain to be confirmed in primary chondrocytes or cartilage tissue explants.

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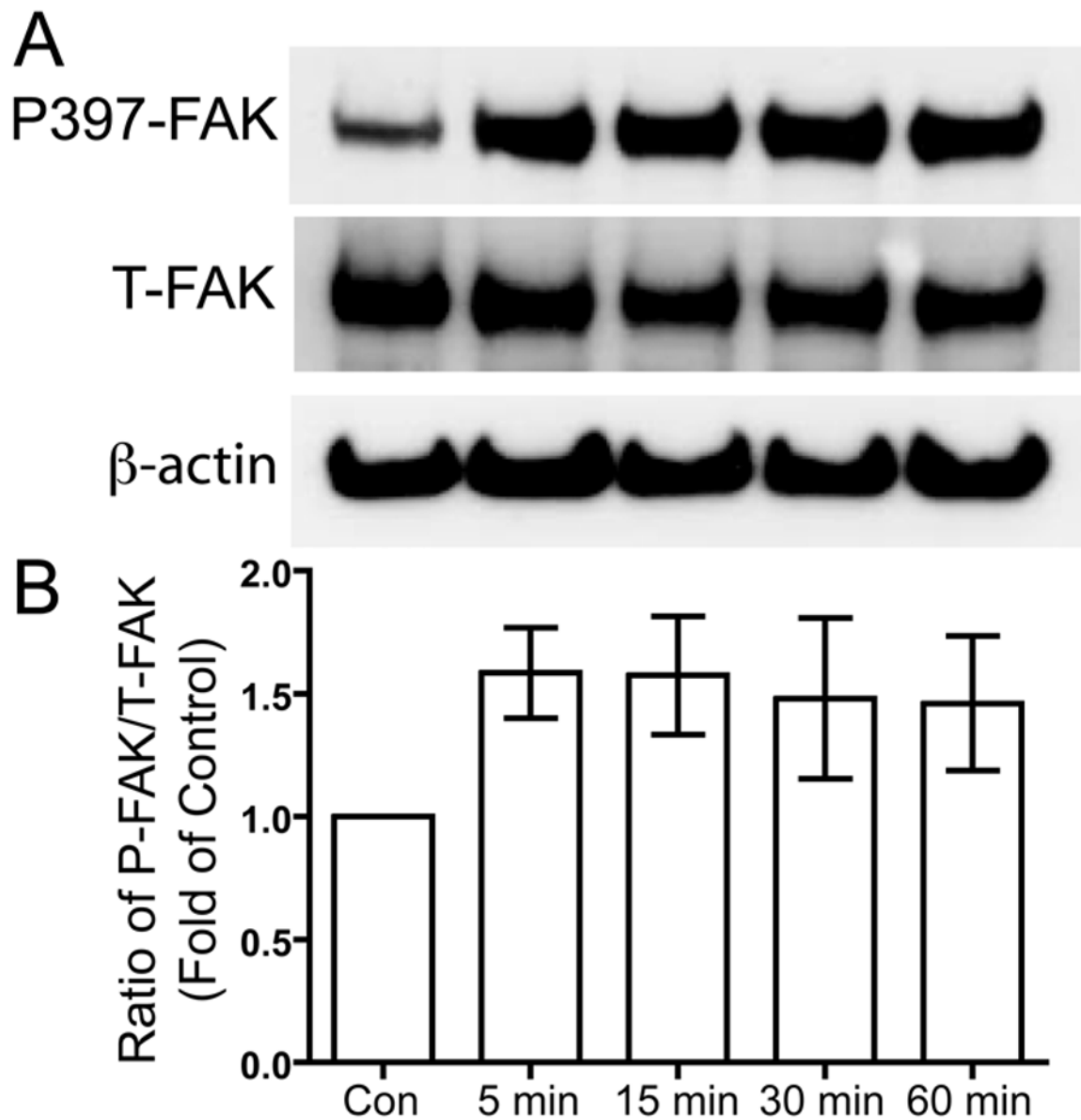
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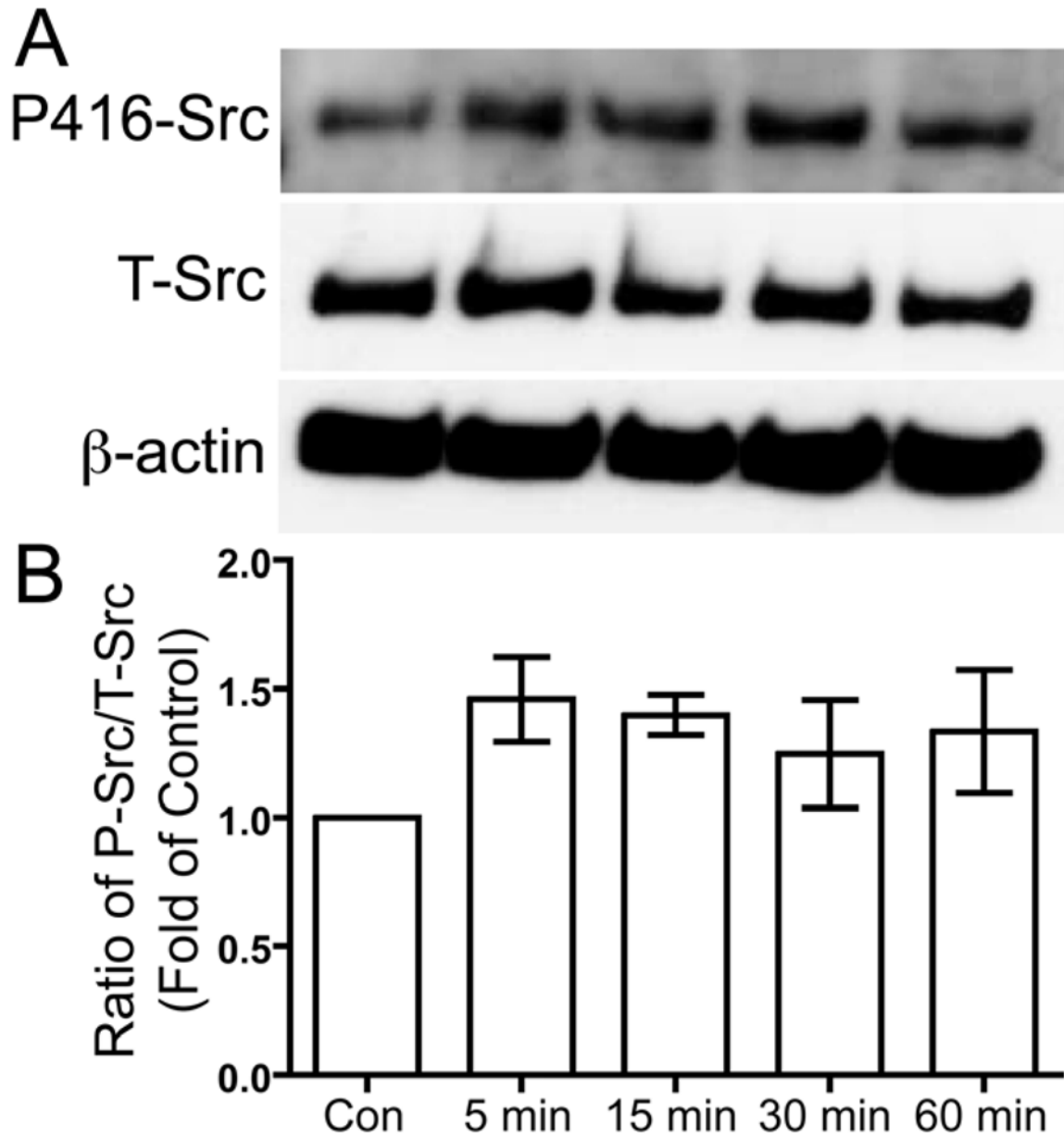
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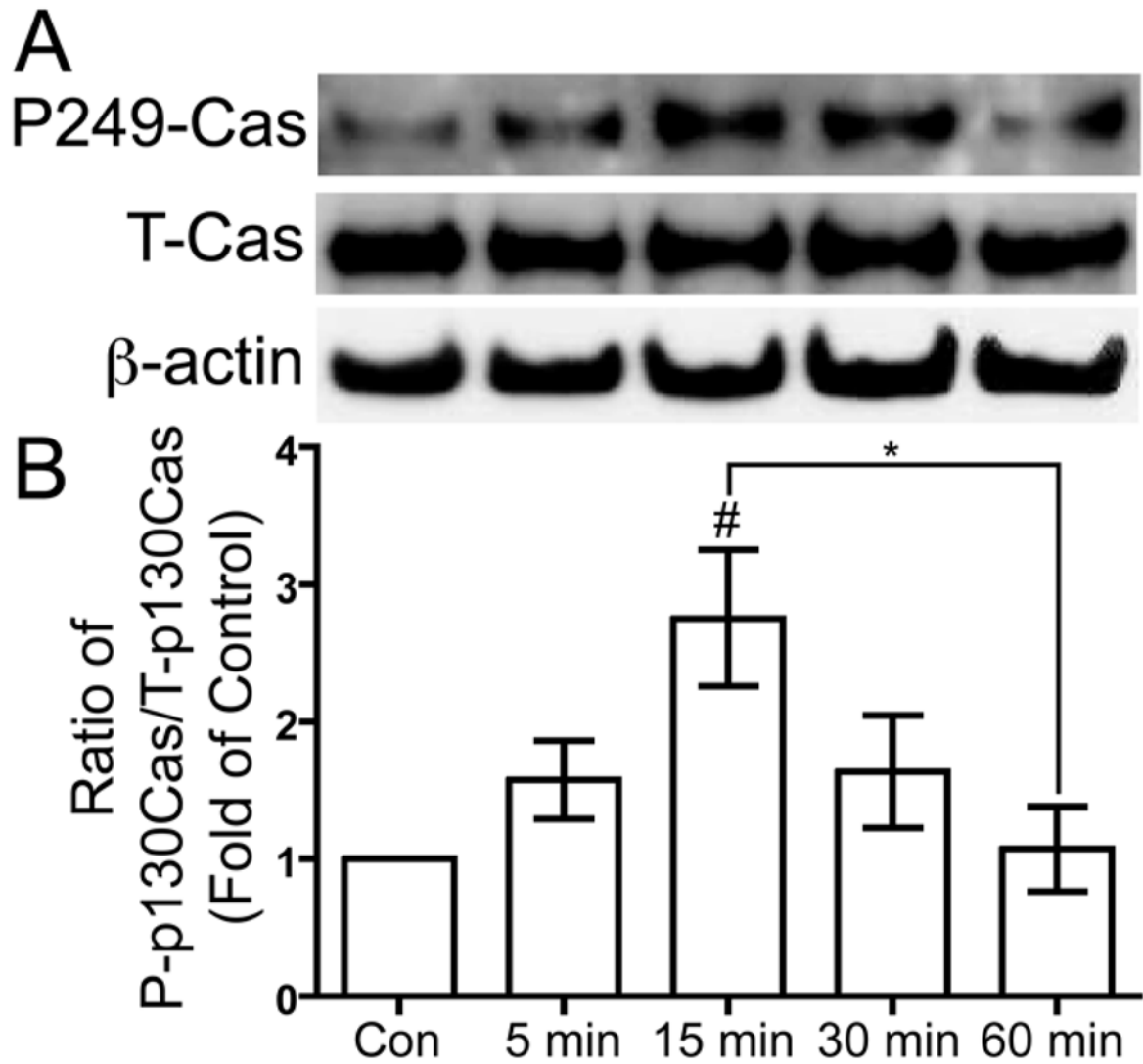
**Figure 1. US-induced phosphorylation of FAK at tyrosine 397**

Serum-deprived primary human chondrocytes were treated with US for three minutes, then total cell lysates were collected 5, 15, 30, and 60 minutes after US treatment. Total cell lysate for control was collected from chondrocytes that did not receive US treatment. Phosphorylation of FAK at tyrosine 397, total FAK and  $\beta$ -actin loading control were demonstrated by Western blotting (A). Western blotting data from six separate experiments were quantified using Image J and averages are shown as the ratio of phosphorylated to total FAK (B).



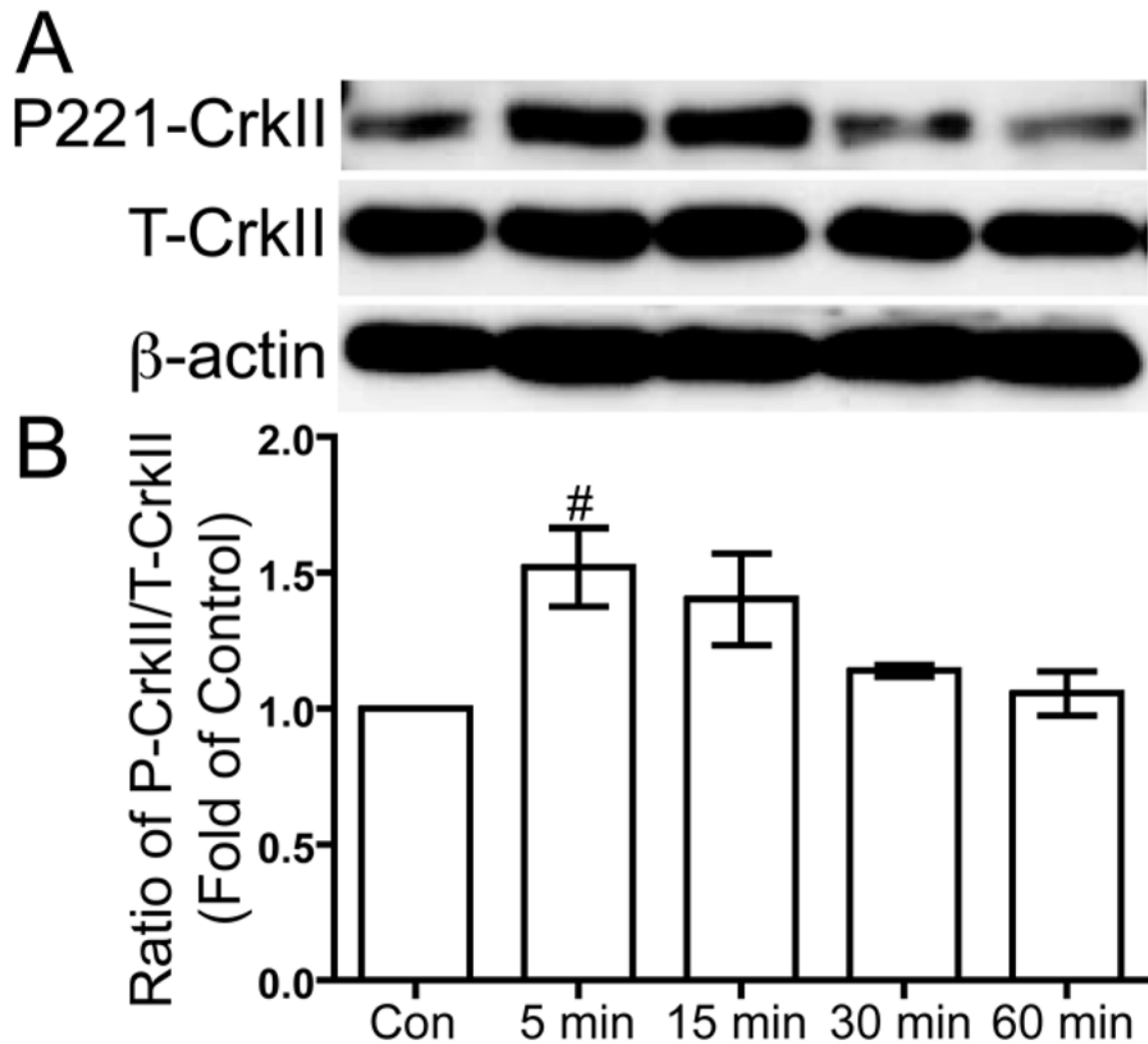
**Figure 2. US-induced phosphorylation of Src at tyrosine 416**

Serum-deprived primary human chondrocytes were treated with US for three minutes, then total cell lysates were collected 5, 15, 30, and 60 minutes after US treatment. Total cell lysate for control was collected from chondrocytes that did not receive US treatment. Phosphorylation of Src at tyrosine 416 and total Src were demonstrated by Western blotting (A). Western blotting data from four separate experiments were quantified using Image J and averages are shown as the ratio of phosphorylated to total Src (B).



**Figure 3. US-induced phosphorylation of p130Cas at tyrosine 249**

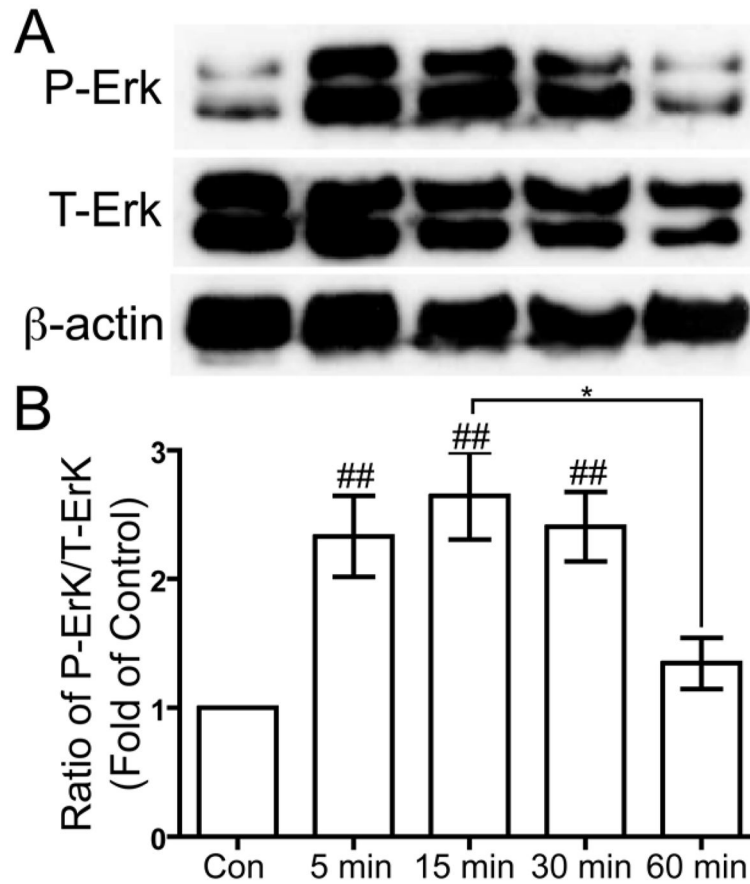
Serum-deprived primary human chondrocytes treated with US for three minutes, then total cell lysates were collected 5, 15, 30, and 60 minutes after US treatment. Total cell lysate for control was collected from chondrocytes that did not receive US treatment. Phosphorylation of p130Cas at tyrosine 249, total p130Cas and  $\beta$ -actin loading control were demonstrated by Western blotting (A). Western blotting data from four separate experiments were quantified using Image J and averages are shown as the ratio of phosphorylated to total p130Cas (B). \*  $p < 0.05$ . #  $p < 0.05$  vs. control.



**Figure 4. US-induced phosphorylation of CrkII at tyrosine 221**

Serum-deprived primary human chondrocytes were treated with US for three minutes, then total cell lysates were collected 5, 15, 30 and 60 minutes after US treatment. Total cell lysate for control was collected from chondrocytes that did not receive US treatment.

Phosphorylation of CrkII at tyrosine 221, total CrkII and  $\beta$ -actin loading control were demonstrated by Western blotting (A). Western blotting data from three separate experiments were quantified using Image J and averages are shown as the ratio of phosphorylated to total CrkII (B). #  $p < 0.05$  vs. control.

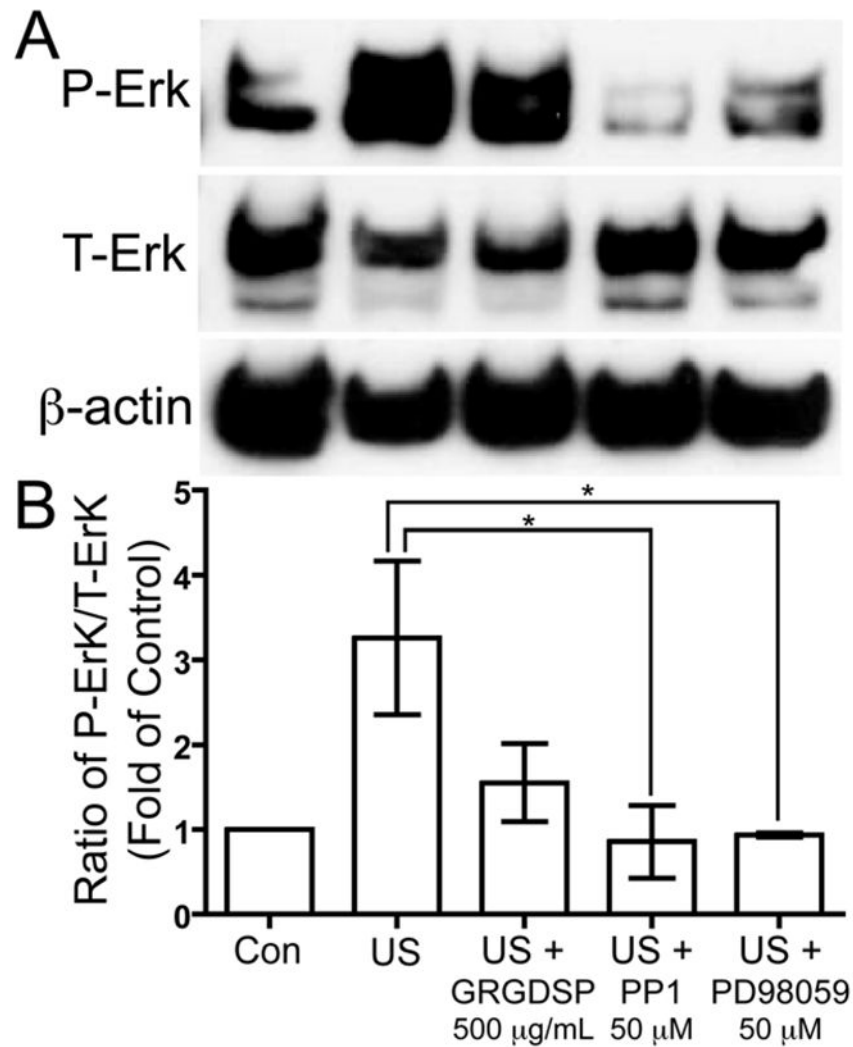


**Figure 5. US-induced phosphorylation of Erk at threonine 202 and tyrosine 204**

Serum-deprived primary human chondrocytes were treated with US for three minutes, then total cell lysates were collected 5, 15, 30 and 60 minutes after US treatment. Total cell lysate for control was collected from chondrocytes that did not receive US treatment.

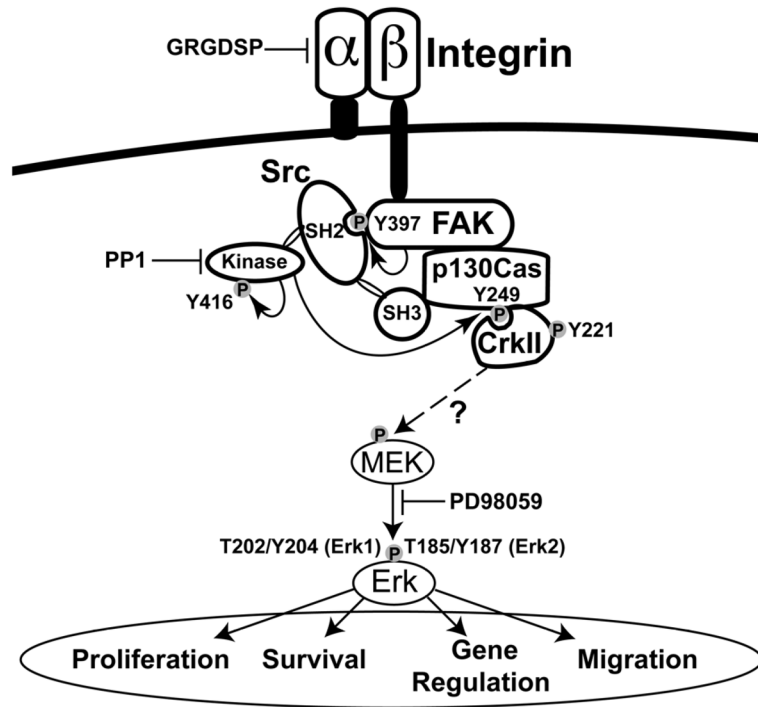
Phosphorylation of Erk1/2 at threonine 202 and tyrosine 204, total Erk1/2 and β-actin loading control were demonstrated by Western blotting (A). Western blotting data from six separate experiments were quantified using Image J and averages are shown as the ratio of phosphorylated to total Erk (B). \*  $p < 0.05$ . ##  $p < 0.01$  vs. control.





**Figure 6. Inhibitors of integrins, Src and MEK reduced levels of US-induced phosphorylation of Erk at threonine 202 and tyrosine 204**

Serum-deprived primary human chondrocytes were pre-treated for 4 hours with 500  $\mu$ g/mL GRGDSP, a peptide inhibitor of integrins, 50  $\mu$ M PP1, an inhibitor of Src, and 50  $\mu$ M PD98059, a MEK inhibitor, then cells were treated with US for three minutes. Total cell lysates were collected 15 minutes after US treatment, and total cell lysate for control was collected from chondrocytes that did not receive US treatment. Phosphorylation of Erk1/2 at threonine 202 and tyrosine 204, total Erk1/2 and  $\beta$ -actin loading control were demonstrated by Western blotting (A). Western blotting data from three separate experiments were quantified using Image J and averages are shown as the ratio of phosphorylated to total Erk (B). \*  $p < 0.05$ .



**Figure 7. Schematic illustration of the proposed signaling pathway for US activation of chondrocytes via integrin receptors**

Following US-induced activation of integrin receptors, FAK autophosphorylates at Y397, which is a binding site for Src (Schaller et al. 1994; Takeuchi et al. 2008). Src binds to FAK and is activated by autophosphorylation of Y416 in the activation loop of the kinase domain of Src (Cooper and MacAuley 1988; Schaller et al. 1994). The p130Cas docking protein binds to FAK and Src, forming an FAK-Src-p130Cas complex, and then Src phosphorylates p130Cas on Y249 (Nakamoto et al. 1996; Ruest et al. 2001), which is a binding site for the CrkII adapter protein (Shin et al. 2004; Modzelewska et al. 2006). We propose that the binding and activation of CrkII leads to the phosphorylation and activation of Erk, which influences numerous cellular processes including proliferation, survival, migration, and a wide range of gene regulation; however, the pathway through which CrkII signals to Erk has not been elucidated in this study.