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LOW-INTENSITY PULSED ULTRASOUND PROMOTES CHONDROGENIC PROGENITOR CELL MIGRATION VIA FOCAL ADHESION KINASE PATHWAY

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Abstract

Low-intensity pulsed ultrasound (LIPUS) has been frequently studied for its beneficial effects on the repair of injured articular cartilage. Here, we hypothesized that these effects are due to stimulation of chondrogenic progenitor cell (CPC) migration toward injured areas in cartilage through focal adhesion kinase (FAK) activation. CPC chemotaxis in bluntly impacted osteochondral explants was examined by confocal microscopy and migratory activity of cultured CPCs was measured in trans-well and monolayer scratch assays. FAK activation by LIPUS was analyzed in cultured CPCs by western blot. LIPUS effects were compared with the effects of two known chemotactic factors; formylated-methionine peptides (fMLF), and high-mobility group box 1 (HMGB1) protein. LIPUS significantly enhanced CPC migration on explants and in cell culture assays. Phosphorylation of FAK at the kinase domain (Tyr 576/577) was maximized by 5 minute exposure to LIPUS at a dose of 27.5 mW/cm² and at a frequency of 3.5 MHz. Treatment with fMLF, but not HMGB1 enhanced FAK activation to a degree similar to LIPUS, but neither fMLF nor HMGB1 enhanced the LIPUS effect. LIPUS-induced CPC migration was blocked by suppressing FAK phosphorylation with a Src family kinases (SFKs) inhibitor that blocks FAK phosphorylation. Our results imply that LIPUS might be utilized to promote cartilage healing by inducing the migration of CPCs to injured sites, which could delay or prevent the onset of post-traumatic osteoarthritis (PTOA).

Keywords

Low-intensity pulsed ultrasound (LIPUS); Articular Cartilage; Post-traumatic osteoarthritis (PTOA); Focal adhesion kinase (FAK); Cell migration

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INTRODUCTION

Osteoarthritis (OA) is a joint disorder characterized by progressive loss of cartilage, which results in the clinical symptoms of joint pain, stiffness and restricted motion. Although the etiology of OA is unclear, risk factors include aging, obesity and history of joint overuse (Martin and Buckwalter 2002; Martin and Buckwalter 2003; Buckwalter et al. 2005). Joint trauma is also strongly associated with OA, particularly when it leads to extensive cartilage damage and chondrocyte death (Buckwalter and Brown 2004; Buckwalter and Martin 2004; Martin et al. 2009). Diverse approaches for the repair of intra-articular cartilage injury have been implemented including surgical procedures, cell transplantation, and scaffold injection, however, these methods have met with limited success (Buckwalter and Mankin 1998; Buckwalter and Martin 2004).

There have been frequent attempts to use low-intensity pulsed ultrasound (LIPUS) to stimulate cartilage repair. These studies have shown that LIPUS stimulates cartilage anabolism by enhancing the production of matrix molecules including proteoglycan and collagen (Parvizi et al. 1999; Zhang et al. 2002; Zhang et al. 2003; Korstjens et al. 2008; Khanna et al. 2009; Naito et al. 2010). In addition, LIPUS has been proven to attenuate the progression of cartilage degradation *in vivo* and has been proposed as a tool to induce mesenchymal stem cells (MSCs) to differentiate into articular chondrocytes (Ebisawa et al. 2004; Schumann et al. 2006; Cui et al. 2007; Park et al. 2007; Gurkan et al. 2010; Lai et al. 2010). However, progress in understanding the mechanism of these benefits has been slow.

Dynamic regulation of cell-ECM adhesion is required for cell migration, as well as for cell proliferation, differentiation and survival (Lauffenburger and Horwitz 1996; Parsons et al. 2000). The formation and turnover of integrin-associated focal adhesion complexes is regulated not only by cytoskeleton-linked proteins such as talin, vinculin, α -actinin and paxillin, but also by intracellular signaling proteins such as focal adhesion kinase (FAK), c-Src, protein kinase C (PKC), phosphatidylinositol 3 kinase (PI3K) and Rho kinase (ROCK) (Amano et al. 1997; Lim et al. 2003; Merlot and Firtel 2003; Mofrad et al. 2004; Humphries et al. 2007; Choi et al. 2008; Crichtley and Gingras 2008; Huveneers and Danen 2009; Schaller 2010). Integrins trigger signal transduction *via* tyrosine phosphorylation (Clark and Brugge 1995; Howe et al. 1998; Giancotti and Ruoslahti 1999) and there is substantial evidence showing that FAK is a major player in relaying signals from integrins to downstream factors, which in turn causes cell motility (Hanks et al. 1992; Ilic et al. 1995; Cary et al. 1996; Horwitz and Parsons 1999; Sieg et al. 1999; Petit and Thiery 2000; Sieg et al. 2000; Parsons 2003; Mitra et al. 2005; Zhao and Guan 2011).

Focal adhesion associated protein tyrosine kinases such as FAK and Src family kinases (SFKs) play significant role in cell motility (Cabrita et al. 2011; Sanchez-Bailon et al. 2012). Current understanding suggests that inhibited recruitment of SFKs leads to block FAK phosphorylation at Tyr 576/577 which triggers signaling pathway for cell migration (Calalb et al. 1996; Hauck et al. 2001; Chaturvedi et al. 2008b; Chaturvedi et al. 2008a; Ciccimaro et al. 2009; Green et al. 2009). Directional cell migration is critical instance for living organisms to maintain homeostasis or immune response. N-formyl-methionyl-leucyl-phenylalanine (fMLF) and High-mobility group protein B1 (HMGB1) are well known

chemo-attractants for directional cell migration toward target location (Degryse et al. 2001; Carrigan et al. 2007; Palumbo et al. 2009; Filaferro et al. 2013).

Previously, we reported the discovery and characteristics of a chondrogenic progenitor cell (CPC) response to cartilage injury. We found that mechanically-induced chondrocyte death caused CPCs to migrate from nearby healthy cartilage toward injured cartilage, resulting in repopulation of the matrix within 7–14 days (Seol et al. 2012). Those CPCs showed significantly higher expression of genes involved in cell migration and their migratory activity in response to chemo-attractants was remarkably higher than normal chondrocytes. Thus CPCs could accelerate the repair of injured cartilage by replenishing extracellular matrix (ECM) macromolecules such as proteoglycans, collagen fibers. Therefore, we hypothesized that LIPUS stimulation promotes the migratory activity of CPCs toward injured sites in articular cartilage and this action is mediated by FAK activation.

MATERIALS AND METHODS

Osteochondral explants and CPCs isolation

Mature bovine stifle joints were obtained from a local abattoir (Bud's Custom Meats, Riverside, IA) and approximately $2.5 \times 2.5 \text{ cm}^2$ of osteochondral explants including the central loaded area from tibial plateau were prepared by manually sawing. The explants were gently washed in Hank's Balanced Salt Solution (HBSS) (Invitrogen Life Technologies, Carlsbad, CA, USA) and cultured for 2 days in 45% Dulbecco's modified Eagle medium (DMEM) and 45% Ham's F-12 (F12) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies), 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 2.5 $\mu\text{g/ml}$ Amphotericin B at 37°C , 5% CO_2 and 5% O_2 . To make a traumatized cartilage injury model, the explants were rigidly fixed prior to single blunt impact with 14 J/cm² using customized drop-tower device and the explants were then returned to cultures. At five to seven days of post-impact, the explants were gently washed with HBBS and the cartilage part was incubated in 0.25 % trypsin-EDTA (Invitrogen Life Technologies) for 10 minutes at the same culture condition. The CPCs from trypsin-EDTA treated cartilage were then isolated as previously described (Martin et al. 2009; Goodwin et al. 2010; Sauter et al. 2012; Seol et al. 2012).

LIPUS apparatus

Manually controlled square wave Pulser-Receiver (Panametrics-NDT, Waltham, MA, USA) was used to generate multiple LIPUS doses by adjusting pulse voltage and pulse repetition frequency. A 1, 3.5 or 5 MHz plane water-immersible transducers (NDT System, Huntington Beach, CA, USA) which have effective area with 19 mm and outer diameter with 25 mm were used in our experiments. During LIPUS stimulation, the surface of transducer was directly immersed facing toward cartilage side in osteochondral explants and cultured CPCs in monolayers containing culture media with approximately 1 cm in distance. LIPUS output power levels were evaluated with radiation force balance and the calculated intensity was regarded as spatial averaged and temporal averaged since it measures overall acoustic power without providing spatial and temporal pressure levels (Preston 1986; Zeqiri and Bickley 2000).

CPC migration in injured cartilage with confocal microscopy

Either partial or full thickness cartilage defects were created to examine the effect of LIPUS stimulation on CPC migration. Full thickness cartilage defects were aseptically created in an osteochondral explant using a 2-mm biopsy punch (Miltex Inc, York, PA, USA) and TISSEEL™ fibrin hydrogel (Baxter Healthcare Corp, Westlake Village, CA, USA) was injected into defects. After 24 hours of defects created, 7 consecutive days LIPUS stimulation (1 MHz, 36.7 mW/cm² and 20 min/day) was applied. For the partial thickness cartilage defects, approximately 0.5 mm depth of X-shaped scratches, were created in an osteochondral explant using a 22 gauge sterilized needle as previously described (Seol et al. 2012). After 24 hours, only half in the cartilage was stimulated by LIPUS (3.5 MHz, 27.5 mW/cm² and 5 min/day) for 4 consecutive days, whereas the other half was blocked from LIPUS stimulation by being covered with an acoustic absorber on surface of the cartilage. In order to visualize migrated CPCs into the defects, cartilage of the osteochondral explants were stained with 1 μM Calcein-AM (Invitrogen Life Technologies), a live cell indicator, and 1 μM ethidium homodimer-2 (Invitrogen Life Technologies), a dead cell indicator. The defects in the cartilage were then scanned to an average depth of 240 μm at 20 μm intervals with a Bio-Rad 1024 laser scanning confocal microscope (Bio-Rad Laboratories Inc, Hercules, CA, USA). Z-axis projections of scanned images were stacked using ImageJ (rsb.info.nih.gov/ij).

CPC migration with Cell Invasion kit

Enhanced migratory activity of CPCs by optimized LIPUS (3.5 MHz, 27.5 mW/cm² and 5 min) was confirmed in commercial cell migration assay kit. CytoSelect™ 24-Well Cell Invasion Assay kit (Cell Biolabs Inc., San Diego, CA, USA) was used to quantitate the migratory activity of CPCs induced by LIPUS and its inhibitory activity by a Src family kinases (SFKs) inhibitor (Selleckchem, Houston, TX, USA). Briefly, CPCs were isolated from traumatized osteochondral explants after seven days culture and cell suspension containing 2.5×10^5 CPCs from each treatment groups (untreated control, LIPUS-treated, or 10 μM SFKs inhibitor plus LIPUS-treated) were prepared and added into upper inserts with serum-free medium added into the lower well of the plate by following manufacturer's manual. After 24 hours of incubation, invaded CPCs were lysed and the total amount of DNA was quantitated with M5 SpectraMax (Molecular Devices Inc., Sunnyvale, CA). The ratios of migrated CPCs from the treatment groups were normalized to that of untreated control group. Monolayer scratch assay was performed to confirm the effect of SFKs inhibitor on CPC migration. 1×10^5 CPCs per well were seeded in a polystyrene 6-well culture plate (tissue-culture treated, 9.5 cm² in bottom area, 1.27 mm in well thickness, 16.8 mL in well volume) (Corning Inc., Tewksbury, MA, USA) and cultured for 4 days. At the day of experiment, approximately a 1 mm in width scratch on the culture plate was created using a pipette tip and the scratched line was monitored immediately after creation and after 24 hours to measure the area covered by migrating CPCs. The rate of migrating CPCs was evaluated by calculating an area covered by migrating CPCs over total scratched area.

Western blot analysis for FAK phosphorylation

Two chemo-attractants, High-mobility group protein B1 (HMGB1) (Chondrex Inc., Redmond, WA, USA) and N-Formyl-Met-Leu-Phe (fMLF) (R&D Systems, Minneapolis, MN, USA), were tested along with LIPUS to examine if LIPUS synergizes with a chemo-attractant in terms of activating FAK. Antibodies, total endogenous FAK and phosphor-specific FAK at Tyr 576/577, β -actin and horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG, were used in our experiments (Cell Signaling Technology Inc., Danvers, MA, USA). For FAK activation analysis, 1×10^5 CPCs per well were seeded in a 6-well culture plate and cultured in DMEM/F12/10% FBS supplemented with antibiotics and fungicide at 37°C in a humidified atmosphere of 5% CO₂-5% O₂ for 4 days. Media were switched to serum free ones 24 hours prior to the beginning of the experiments. Western blot analysis was performed to examine the optimal LIPUS dose on FAK phosphorylation and its synergistic effect with the chemoattractants. For the study of optimal LIPUS dose on FAK phosphorylation, cells were LIPUS-stimulated with 15.3 mW/cm² or 36.7 mW/cm² at 1 MHz, 15.3 mW/cm² or 27.5 mW/cm² at 3.5 MHz, 15.8 mW/cm² or 31.6 mW/cm² at 5 MHz, with duration either 5 or 20 minutes. Approximately 30 percent of CPCs in 6-well culture plate was stimulated by LIPUS since the transducer's effective area was smaller in size than a 6-well culture plate. Cells were then lysed in cold lysis buffer (L.B) containing protease and phosphatase inhibitor with a 1:100 dilution of proteinase inhibitor cocktail III (CalBiochem, San Diego, CA, USA). To study the synergism of FAK activation by LIPUS in combination with chemo-attractants, cells were treated with LIPUS (3.5 MHz, 27.5 mW/cm² and 5 min) only, 100 nM HMGB1 only, 100 nM HMGB1 followed by LIPUS, 10 nM fMLF only, 10 nM fMLF followed by LIPUS. After 24 hours, cells were lysed in cold L.B and total protein concentration was determined with a BCA Protein Assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Proteins were then denatured with 2× sample buffer and reduced with 0.05 M Dithiothreitol (DTT). Total 2.5 μ g proteins from each lysis were resolved in 10 % SDS-PAGE gels and blotted onto nitrocellulose membranes. The blots were blocked with 5% non-fat dried milk in tris buffered saline (20 mM Tris buffer containing 140 mM NaCl, pH 7.4) containing 0.1% Tween-20 (TBST) for 1 hour at room temperature (RT). The blots were then incubated at 4°C overnight with total, phosphor-FAK, anti-beta actin antibody in a 1:1000 dilution in 5% BSA in TBST. After washing, the blots were incubated with HRP conjugated goat anti-rabbit IgG in a 1:2000 dilution in 5% BSA in TBST for 1 hour at RT followed by reaction with SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). Chemiluminescent signals were detected with Kodak Biomax Xar Film from Sigma-Aldrich® (Rochester, NY, USA). The integrated density (ID) of bands were measured by ImageJ and normalized to that of untreated control. The relative fold increase of integrated density was calculated as $[ID_{pFAK(LIPUS)} / ID_{tFAK(LIPUS)}] / [ID_{pFAK(control)} / ID_{tFAK(control)}]$.

Statistical analysis

Comparison between untreated control and LIPUS treated groups was done using statistical analysis software package, SPSS (IBM Corporation, Armonk, New York, USA). One way ANOVA with Tukey post-hoc test was used to directly test the all possible pairwise comparisons. The level of significance was set at $p < 0.05$.

RESULTS

Compared to untreated controls (n=3), 7 consecutive days of LIPUS stimulation (1 MHz, 36.7 mW/cm², 20 min/day) remarkably increased the number of CPCs into fibrin-filled defects (n=3) (Fig 1).

LIPUS intensities were measured using radiation force balance. Intensities of approximately 15 and 30 mW/cm² were selected to test the effects of different frequencies (1, 3.5 or 5 MHz) on FAK activation. The results showed that FAK phosphorylation at 27.5 mW/cm² at 3.5 MHz was significantly increased by 3-fold higher (p=0.045) than control among the examine combinations of LIPUS frequencies and intensities (n=4). However, there was no different LIPUS duration dependent FAK activation between 5 and 20 minutes. Hence, 3.5 MHz, 27.5 mW/cm² and 5 min was selected as optimal LIPUS dose for this study.

Time course of FAK activation in response to optimized LIPUS was examined. Immediately after LIPUS stimulation, no distinguishable difference in FAK activation was observed compared to control group, however, a difference became apparent at 5 minutes post-treatment. After 30 minutes, FAK phosphorylation with LIPUS stimulation was significantly higher than control group (Fig 3A). Enhanced FAK phosphorylation in response to LIPUS and chemo-attractants was also detected after 24 hours. LIPUS alone (p=0.004, n=9) and 10 nM fMLF with LIPUS (p=0.003, n=4) or without LIPUS (p=0.005, n=4) significantly enhanced FAK phosphorylation when compared to untreated controls (n=8), however, 100nM HMGB1 showed no effect with (n=4) or without LIPUS (n=4). No synergistic effect was observed when LIPUS was paired with either fMLF or HMGB1 (Fig 3B and C).

CPCs responded to partial-thickness cartilage injuries, which caused massive cell death was (Fig 4A & B). There were significantly more CPCs on the half of the defects exposed to LIPUS stimulation (3.5 MHz, 27.5 mW/cm², 5 min/day) than little change was observed on the half covered by an acoustic absorber (n=4) (Fig 4 C&D).

The effect of SFKs inhibitor on diminishing FAK activation at Tyr 576/577 was confirmed (Fig 5A) and CPC migration was dose-dependently reduced by SFKs inhibitor after overnight incubation in monolayer scratch assays (Fig 5 B&C). The effect of LIPUS and SFKs inhibitor on CPC migration was confirmed again using a cell migration assay kit. Compared to controls (n=5), the percentage of migrated cells was 1.5 fold higher (p=0.004, n=4) in the group treated with LIPUS (3.5 MHz, 27.5 mW/cm², 5 min) and this enhanced migratory activity was abolished by 10 μ M SFKs inhibitor (p=0.001, n=3) (Fig. 5D).

DISCUSSION

Previously we reported CPC chemotaxis in response to alarmins, which resulted in repopulation of areas of the cartilage matrix where chondrocytes had been killed by injury (Seol et al. 2012). In the present study, we found that the chemotactic activity of CPCs was significantly stimulated by LIPUS treatment. This effect could speed the return to normal cellularity in injured cartilage, which is expected to accelerate matrix repair and oppose the progression of OA (Cook et al. 2001; Ebisawa et al. 2004; Schumann et al. 2006; Korstjens et al. 2008; Khanna et al. 2009; Gurkan et al. 2010; Naito et al. 2010).

CPCs harvested from injured cartilage showed activation of FAK as early as 5-minutes post-LIPUS stimulation. Among the examined combinations of intensity and frequency, LIPUS at 3.5 MHz and 27.5 mW/cm² was optimal in terms of inducing FAK activation. Considered that only 30 % of CPCs was within the field of LIPUS stimulation due to the different dimensions between the effective area of transducers and the area of 6-well cell culture plate, we expect that FAK activation in CPCs by LIPUS would be greater if all CPCs were LIPUS-stimulated. FAK was also significantly activated by treatment with fMLF, a mitochondrial alarmin (p=0.003) (Degryse et al. 2001; Carrigan et al. 2007; Palumbo et al. 2009). In the explant model, fMLF and other factors released from dead chondrocytes appear to initiate CPC migration in the immediate aftermath of impact; however, this effect is unlikely to be sustained for more than 48 hours due to spontaneous degradation of the factors (Martin et al. 2009). In contrast, LIPUS was repeated daily throughout the experiment. Thus, it seems probable that the greater number migrating CPCs found on LIPUS-treated explants was due to continued stimulation of FAK phosphorylation after peptide chemotactic factors had cleared.

In partial thickness cartilage injuries, increased numbers of CPCs in the LIPUS stimulated half of cartilage tissue were observed, while few CPCs migrated in the LIPUS blocked half. This result suggested that optimized LIPUS stimulation enhanced FAK phosphorylation of CPCs in cartilage, resulting in increased migratory activity. Cell migration assay result confirmed that LIPUS stimulation significantly enhanced CPC migration compared to LIPUS untreated control group (p=0.004). SFKs inhibitor has been well studied for its inhibitory effect on phosphorylation and activation of FAK at Tyr 576/577 (Calalb et al. 1996; Hauck et al. 2001; Chaturvedi et al. 2008b; Ciccimaro et al. 2009; Green et al. 2009). SFKs inhibitor also blocked LIPUS-induced increases in chemotaxis in transwell assays where migrating cells were required to invade a 3-dimensional collagen matrix, and in monolayer scratch assays where migration was unhindered by matrix.

We measured LIPUS power by radiation force balance which provides approximate LIPUS intensity levels without specific spatial and temporal pressure levels. Based on the measured intensity levels, we assumed that the cavitation was not generated during LIPUS stimulation in our system since the intensity levels are presumably below the threshold of cavitation (Ogurtan et al. 2002; Dalecki 2004; Fowlkes et al. 2008; Santos 2009). However, in our experimental setup, CPCs in monolayer in a cell culture incubator were LIPUS stimulated without a backing block such as water tank and an acoustic absorber at the bottom of the monolayer culture plate which could minimize the ultrasonic reflection or scattering when ultrasound penetrates into monolayer culture plate (Kinoshita and Hynynen 2006). Therefore, in this format, inconsistent LIPUS intensity due to unstable constructive and destructive interference waves could not be avoided.

Although recent evidences suggest that triggering integrin mediated mechanotransduction pathways is regarded as one of the mechanism of therapeutic ultrasound (Zhou et al. 2004; Lee et al. 2006; ter Haar 2010), our data suggest that enhanced migratory activity of CPCs *via* FAK activation in response to LIPUS is closely associated with not only mechanotransduction pathways, but also the effect of thermal, cavitation and standing waves. Future studies are required to clarify the precise mechanism of LIPUS on CPC migration *via* FAK

activation by measuring LIPUS pressure values using hydrophone and also by measuring FAK activation in response to LIPUS in monolayer with minimized LIPUS reflection.

In conclusion, our results in an in vitro model suggest that some of the well-established beneficial effects of LIPUS on cartilage repair may be mediated by increased FAK activation in CPCs, which greatly enhance the repopulation of hypocellular cartilage after an injury. (Parvizi et al. 1999; Zhang et al. 2002; Zhang et al. 2003; Ebisawa et al. 2004; Schumann et al. 2006; Cui et al. 2007; Park et al. 2007; Korstjens et al. 2008; Khanna et al. 2009; Gurkan et al. 2010; Lai et al. 2010; Naito et al. 2010). Whether repopulation improves cartilage repair depends on the ability of CPCs to re-establish a functional cartilage matrix *in situ*, which remains unproven. It may be advantageous to follow pro-migratory LIPUS treatment with treatments designed to encourage chondrogenic differentiation and discourage chondrolytic activity. In any event, the findings described here justify further enquiry to determine the potential for LIPUS to oppose the initiation and progression of OA.

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Figure 1. Schematic diagram of experimental setup

(A), LIPUS transducer was immersed in monolayer culture plate containing culture media and LIPUS-stimulation was induced approximately 1 cm in distance. (B), LIPUS transducer was immersed in sterilized specimen container containing culture media and LIPUS-stimulation was induced approximately 1 cm in distance with an osteochondral explant. (C), schematic diagram of experimental procedure explains represents that LIPUS stimulation enhanced CPC migration and it was involved with FAK activation. LIPUS was optimized (3.5 MHz, 27.5 mW/cm², 5 min) in terms of FAK activation and it was confirmed with migration assays.

Figure 2. Fibrin hydrogel embedded full thickness cartilage defects

Confocal microscopy images showing live cells (green) and dead cells (red) in full thickness cartilage defects. (A and C), No difference of CPC migration observed in untreated control group at day 0 and 7. (B and D), significantly higher migrated CPCs into fibrin-injected cartilage defects observed in 7 consecutive LIPUS stimulated group at day 0 and 7. Bars = 500 μm .

Figure 3. FAK phosphorylation dependent on LIPUS dose

Western blot and its quantitated integrated density showing that FAK is phosphorylated dependent on LIPUS dose. Comparing integrated density dependent on LIPUS dose, frequencies at 1, 3.5 and 5 MHz and intensities at approximately 15 or 30 mW/cm², FAK phosphorylation was maximized at 3.5 MHz and 27.5 mW/cm² ($p=0.045$). Row at the bottom represents LIPUS frequency [MHz]/intensity [mW/cm²]. Asterisk represents statistically significant ($*p< 0.05$). Error bars represent sample variations ($n=4$).

Figure 4. FAK phosphorylation in response to LIPUS and chemo-attractants

(A), FAK activation examined immediately after optimized LIPUS stimulation showing that the difference of FAK phosphorylation became apparent after 5 minutes and significant after 30 minutes, compared to control group. (B and C), Enhanced FAK phosphorylation after 24 hours of LIPUS stimulation, 100 nM HMGB1 and 10 nM fMLF and its relative fold increase. LIPUS alone ($p=0.004$) and 10 nM fMLF with ($p=0.003$) and without LIPUS ($p=0.005$) significantly enhanced phosphorylation of FAK respectively, while HMGB1 showed no difference. (–) and (+) represent with or without LIPUS respectively and asterisk represents statistical significant ($**p<0.01$) Error bars represent sample variations ($n=4-9$).

Figure 5. Scratches induced partial thickness cartilage defects

Confocal microscopy images showing live cells (green) and dead cells (red) in partial thickness cartilage defects. (A and C), Acoustic absorber covered region at day 0 and 4 showing that apparently few migrated CPCs into injured areas were observed. (B and D), 4 consecutive days of LIPUS stimulated cartilage region showing that significantly higher number of CPCs were observed in defects compared at day 0. Bars = 500 μm .

Figure 6. The effect of LIPUS stimulation and 10 μ M Src family kinases (SFks) inhibitor on CPC migration

(A), Western blot result showing that SFks inhibitor reduced FAK phosphorylation by dose dependent manner. (B and C), Monolayer wound healing assay showing that migratory activity of CPCs was reduced by SFks inhibitor dose dependent manner. (D), Cell migration assay confirming that migratory effect of CPCs was enhanced by optimized LIPUS stimulation ($p=0.004$) and abolished by 10 μ M SFks inhibitor ($p=0.001$). The column indicates the relative fold increase of CPC migration and the data are normalized to LIPUS-untreated control group. (-) and (+) represent with or without LIPUS respectively and asterisk represents statistical significant (** $p<0.01$, *** $p<0.001$) Error bars represent sample variations ($n=3-5$). Bars = 500 μ m.