Low intensity pulsed ultrasound promotes the migration of bone marrow-derived mesenchymal stem cells via activating FAK-ERK1/2 signalling pathway

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ABSTRACT
To investigate the promoting effects and mechanisms of low intensity pulsed ultrasound (LIPUS) on the migration of bone marrow-derived mesenchymal stem cells (BMSCs). The BMSCs migration was researched from cell and animal experiments. In the cell experiment, the BMSCs was treated using LIPUS (30 mW/cm², 20 min/day, 2 days), and the wound healing and transwell migration were observed. In the animal experiment, the BMSCs labelled with green fluorescent protein (GFP) were injected into rats with femoral defects via the tail vein (1 × 10⁶/mL). The healing of bone was detected using x-ray and sampled for hematoxylin & eosin (H&E) staining and fluorescence microscopy. About the mechanisms, the cellular F-actin of cytoskeleton was stained with FITC-phalloidin. The changes of BMSCs genes after LIPUS treatment were screened using microarray assay and verified using quantitative real-time polymerase chain reaction (qRT-PCR). The biological processes of those genes were predicted by KEGG analysis. The protein expression levels of FAK, ERK1/2 and myosin II related migration were detected using western blotting. The results showed LIPUS promoted the BMSCs migration (p < .05) without significant temperature changes (p > .05) in vitro and in vivo than control group (p < .05). The cytoskeletal rearrangement was carried out, and the ITGA8 gene related with cell migration was found with high expression after LIPUS treatment (p < .05). FAK inhibitor (PF-573228) and ERK1/2 inhibitor (U0126) were proved, in turn, decreased the BMSCs migration induced using LIPUS (p < .05). LIPUS can promote the BMSCs migration in vitro and in vivo, one mechanism may be related to the activation of FAK-ERK1/2 signalling pathways using LIPUS.

GRAPHICAL ABSTRACT
Experiments in vivo and in vitro were designed to explore the migration and mechanisms of BMSCs treated using LIPUS.

Introduction
Bone marrow-derived mesenchymal stem cells (BMSCs) are considered as ideal seed cells for stem cell transplantation, which are widely used in musculoskeletal, neurological disorders and other diseases [1,2]. However, its clinical application in stem cell transplantation is limited by lack of method to extract high-purity cell and the low efficiency of cell homing in vivo [3,4]. The delivery method of BMSCs administration should be high efficacy, availability and safety to facilitate BMSCs homing to the target tissue. Intravenous infusion is one of the major routes [5]. But, the migration rate may be low and the
cells arriving at the target area may be a small part from the cells infused [6] because BMSCs will be trapped into capillary beds of various tissues, especially the lungs [7,8]. If the quantities of BMSCs infused were enhanced for efficient homing, there may lead to “microvascular occlusions” [9] and trigger for hypoxia and injury of normal tissues. The other route such as injecting the BMSCs directly in the damaged site [10,11] is invasive and may be dangerous to patients.

As we all known, cell migration, an important part of cell homing, is the movement of cells from source to the region where there is a requirement of response or action [12]. The effects on BMSCs homing will be improved when migration rate is increased. Recently, preconditioning strategies have provided new ideas for promoting homing of endogenous and exogenous mesenchymal stem cells [13]. Low intensity pulsed ultrasound (LIPUS) has been reported to promote the proliferation and differentiation of BMSCs and accelerate the repair of tissues [14–16]. We considered LIPUS can improve the BMSCs migration as same as accelerating the proliferation and differentiation of BMSCs. So, we designed the project to research the LIPUS effects on the BMSCs migration in vitro and in vivo, and the roles of functional genes involved in migration, FAK and ERK1/2 signalling pathways related to cell migration.

These findings may provide evidence comprehensively to declare LIPUS can promote the migration rate of BMSCs and the pre-treatment to BMSCs using LIPUS will be an effective, safe and helpful clinical strategy in cell-based therapy in the future.

Methods

Cell culture

BMSCs extracted from 6 weeks old Sprague–Dawley (SD) rats were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Shanghai, China). BMSCs were cultured in Dulbecco’s Modified Eagle Medium with low-glucose (L-DMEM) (Hyclone, USA) supplemented with 10% fetal bovine serum (BioInd, Israel) and 1% Pen-Strep antibiotics (Beyotime, China) at 37°C under an atmosphere containing 5% CO2. Culture medium was changed every 1–2 days, and the cells were passaged every 3–4 days. Cell growth was observed under an inverted phase contrast microscope (IX-70, Olympus, Japan). For inhibition of signalling pathways, BMSCs in FAK suppressive group and ERK suppressive group were pre-treated with PF-573228 (40 nmol/L, Target Molecule, USA) [17] for 6 h and U0126 (100 μmol/L, Target Molecule, USA) [14] for 1 h, respectively.

Protocol of LIPUS treatment

The LIPUS device was provided from Chongqing Haifu Medical Technology Co., Ltd, China. The parameters involved a frequency of 0.25 MHz, a duty cycle of 20%, a pulse repetition frequency of 1 kHz and spatial-average temporal-average intensity (I_{50%}) of 30 mW/cm². The 6-well plates with cells were placed on the transducers with a layer of ultrasonic coupling agent between them. WK3-1310 thermometer (Baode Instrument Co., Ltd., China) was used to measure the temperature of the medium (5 points per well) of 6-well plate after LIPUS treatment. Cells in LIPUS groups were exposed to LIPUS in 20 min while those in the control group were performed sham treatment (No energy output from transducers).

Transwell migration assay

A cell migration model was constructed by transwell chamber (Corning, USA) composed of a membrane filter with 8 μm pores in a 6-well plate according to instructions for manufacturer. BMSCs were harvested with 0.05% trypsin and suspended in L-DMEM at a concentration of 5 × 10⁵ cells/mL. Per 1 mL BMSCs suspension was loaded into one upper chamber, and 2 mL L-DMEM with 10% FBS was placed into per lower chamber. After 24 h, cells not migrated in the upper room were wiped off by cotton swabs. The membrane of chambers was fixed for 15 min in 4% paraformaldehyde (Beyotime, China). Cells were stained with 0.1% crystal violet staining solution (Beyotime, China) for 15 min and washed three times with Phosphate-Buffered Saline (PBS, Hyclone, USA). The number of BMSCs migrated to the lower surface of the membrane was counted in 5 visual fields randomly chosen.

Wound healing assay

The detections of BMSCs migration were performed in 6-well plates by wound healing assays in vitro. Cells were seeded at a density of 5 × 10⁵ cells per well. L-DMEM was loaded into per well as serum-free medium to starve for 24 h. Wounds were made in the area of BMSCs by scratching with a 200 μL pipette tip (3 wounds per well). The cells were washed three times with PBS to remove any floating cells from wounding. BMSCs were incubated with L-DMEM and exposed to the LIPUS. The scratch of each group was observed using an inverted phase contrast microscope at 0 h, 24 h and 48 h. The area of scratch was measured using Image J v1.42q software (National Institutes of Health, USA).

Animals with bone defect

Female SD rats were provided by the Experimental Animal Center of Chongqing Medical University (Approval number No. SCXK 2017–0001) and were housed in individually ventilated cages under a 12-h light/dark cycle, with free access to standard food and water. Female SD rats with femoral defects were created as uniform bone-fracture models [15]. After 3 days of adaptive feeding, rats’ limbs were first fixed on a plate with the animals in the supine position. The skin and subcutaneous tissue were cut layer by layer (interior longitudinal incision) to reveal the femur after depilation and disinfection. A circular hole (3 mm in diameter) was drilled in the middle of the right femur for all rats after anaesthesia. The 30 injured rats were randomly divided into control and LIPUS group (n = 15 per group). All experimental procedures were approved by the Laboratory Animal Care and Use
Committee at Chongqing Medical University and met requirements described in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

**BMSCs transfection and injection**

To trace exogenous BMSCs into bone defect area, BMSCs were transfected with the adenoviral vector green fluorescent protein (GFP)-mock (Invitrogen, USA). After about 48 h transfection, MSCs-GFP was collected for experiments. GFP expression was observed using a fluorescence microscope (TCS-SP2, LEICA, Germany). A total of 80–90% of BMSCs labelled with green fluorescence was regarded as successful transfection. The BMSCs pretreated using LIPUS were collected and cell density was adjusted to 1 × 10^6/mL. BMSCs were injected into the anaesthetized SD rats with femoral defects via the tail vein (1 mL of cell suspension). The rats were sacrificed after 2 weeks and the tissue containing the bone defect area was taken and then fixed with 4% paraformaldehyde.

**X-ray image**

Rats were placed in a prone position on an X-ray machine after anaesthesia, and the limbs were stretched. The healing of bone defect was observed using X-ray imaging (40 kV × 2 mAs) before and after treatment with BMSCs-GFP. The area of the bone defect on the X-ray film was outlined and measured using Image J v1.42q software.

**Hematoxylin & Eosin (H&E) staining**

The affected side femurs of rats were harvested, sectioned and stained by H&E staining before modelling, after modelling. The affected side femurs of rats were harvested, sectioned and stained by H&E staining before modelling, after modelling. The affected side femurs of rats were harvested, sectioned and stained by H&E staining before modelling, after modelling. The affected side femurs of rats were harvested, sectioned and stained by H&E staining before modelling, after modelling.

**Microarray assay**

The total RNA of BMSCs in each group was extracted using TRIzol one-step extraction. NanoDrop Spectrophotometer (Thermo Fisher, USA) was used to evaluate the concentration and purity of RNA. RNA sample was purified using OneArray plus RNA amplification kit (Phalanx Biotech Group, China). After purification of the RNA sample, a hybridization reaction with Phalanx OneArray was performed. The blocking buffer was preheated at 42°C to check the integrity of the chip Rat OneArray Plus (Thermo Fisher ND1000). The chip was slowly placed in a pre-hybridized tube, pre-hybridized in an oven at 42°C for 60 min, centrifuged for 1 min to spin-dry the water on the chip, scanned on a chip placement scanner (Axon GenePix 4000B, USA), and the signal light intensity detected using the scanner. Data after homogenization were analyzed using Gene-Pix pro 3 software (Axon Instruments, USA).

**Western blot**

After treatment, BMSCs were lysed in RIPA lysis buffer (Beyotime, China), and proteins were isolated after centrifugation. The protein concentrations were determined using the Bradford Protein Assay Kit (Beyotime, China). The protein samples were separated using SDS-PAGE and subsequently electro-transferred to PVDF membrane (Millipore, USA). After washing, the membrane was blocked with 5% nonfat milk for 1 h, and then incubated overnight at 4°C with specific primary antibodies for FAK, phospho-FAK (Tyr397) (all from Novus, USA), ERK1/2, phospho-ERK1/2, phospho-myosin light chain 2 (ser19) (all from Cell Signalling Technology, USA). After washing three times, the membrane was incubated for 1 h at room temperature with the secondary antibodies.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted using qRT-PCR kit (Shanghai Dingguo Biotech. Co., Ltd., Shanghai, China) following the manufacturer's instructions. RNA was converted into complementary DNA using an AMV RTase kit (Shanghai Dingguo Biotech). Primer pairs (Shanghai Dingguo Biotech) for target gene amplification are listed in Table 1. Electrophoresis of 10 μL PCR amplification reaction fluid was performed at 150 V for 30 min on 1.5% agarose gel, and images were analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA) using the following function: relative transcript level = target gene expression quantity/β-actin expression quantity. Kurtosis value of each stripe was calculated in terms of their means to represent sample intensity. And, mRNA expression level was semi-quantitatively analyzed using the corresponding index.

**Table 1. Fluorescence quantitative PCR primers.**

| Genes     | Forward (5’-3’)          | Reverse (5’-3’)          |
|-----------|--------------------------|--------------------------|
| ITGA8     | AGACAAGCTCCAGGGTACA      | AGGCCAGGGACAGTAGTAGA     |
| CCL5      | TGAAAGATCCACACGGTCCA     | AATATCCCTCATGGGGGCA      |
| TM7S2F    | CATTGTTTCCCCGAAAGGA     | CCGAGCCAGTGAACACACAG     |
| TUBA3B    | GCTACCATCAAGACCAACGG    | TCAAAATTGTGGCTCCAGGCG    |
| β-ACTIN   | AGTCGGCTGTTGATGGTAGTAC  | GCAAGAGGGCAAGACACACA     |

**F-actin staining**

BMSCs grew on slices placed in 6-well plate. Cell culture medium was removed after 24 h. Cells were washed twice with 37°C preheated PBS, fixed for 15 min with 4% paraformaldehyde. For F-actin staining, BMSCs have incubated away from light with Phalloidin staining solution labelled with Fluorescein isothiocyanate (Shanghai Yisheng Biological Technology Co., Ltd., China) for 30 min. Slices were mounted with antifade mounting medium (Beyotime, China). The expression of F-actin was observed using confocal laser scanning microscope (A1¨R, Nikon, Tokyo, Japan). Three fields of view were randomly selected to observe morphological changes of cytoskeleton.
BeyoECL Plus kit (Beyotime, China) was used for colour development according to the manufacturer’s instructions.

**Statistical analysis**

All data were analyzed with SPSS 22.0 (IBM, NY, USA). Quantitative data were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used for multiple-group comparisons. Tukey’s honestly significant difference was used for statistical post-test of ANOVA. The difference was statistically significant at \( p < .05 \).

**Results**

**The LIPUS effects to promoting BMSCs migration in vitro and being decreased using inhibitors of FAK and ERK1/2**

The transwell migration assay and the wound healing assay were used to evaluate the migration ability of BMSCs in vitro. The result of transwell assay showed that the number of BMSCs passing through the membrane in the LIPUS group was higher than that in the control group after 24 h \( (p < .01) \), suggesting that the stimulation of LIPUS could promote the transmembrane migration ability of the BMSCs (Figure 1A).

However, pretreatment of PF-573228 and U0126 significantly reduced the number of cells passing through the membrane after LIPUS treatment \( (p < .01) \) (Figure 1B, C). This indicated that the effect of LIPUS-induced BMSCs migration may be related to the FAK and ERK1/2 signalling pathways. On the other hand, the ability of BMSCs to migrate in two-dimensional plane was explored by wound healing assay (Figure 1D; Table 2). The scratch of 0 h, 24 h and 48 h was measured using ImageJ. After scratching, there was no difference in the size of the scratched area \( (1.37 ± 0.04\text{mm}^2) \) \( (p > .05) \). The scratch in the control group decreased significantly to \( 0.85 ± 0.11\text{mm}^2 \) and \( 0.52 ± 0.12\text{mm}^2 \) at 24 h and 48 h \( (p < .01) \), respectively. However, the healing of scratches between PF-573228 group and U0126 group were significantly inhibited at 24 h and 48 h \( (p < .01) \). Compared with the control group, the scratch of LIPUS group decreased significantly to \( 0.85 ± 0.11\text{mm}^2 \) and \( 0.52 ± 0.12\text{mm}^2 \) at 24 h and 48 h \( (p < .01) \). Similarly, BMSCs responded to LIPUS was inhibited by PF-573228 and U0126.

**LIPUS enhanced BMSCs recruitment in bone defects in vivo**

To trace the recruitment of BMSCs in rats, BMSCs were infected with an adenovirus vector to obtain GFP-labelled BMSCs (BMSCs-GFP) (Figure 2B). The bone defect was scanned using X-ray image. The round defects with uniform size were observed in the femur of the control group \( (666.7 ± 49.40) \) and the LIPUS group \( (714.7 ± 23.29) \), indicating that the bone defect model was successfully established. After 2 weeks of BMSCs injection, round defect was closed in LIPUS group \( (4.7 ± 8.08) \) while bone defects were still visible in control group \( (251.7 ± 93.72) \) (Figure 2C,D). The speed of bone healing in LIPUS group was about 71% higher than that in control group \( (p < .05) \). H&E staining of affected femurs showed that the percentage of non-healing area in LIPUS group \( (19.85 ± 6.29\%) \) was significantly lower than that in control group \( (42.20 ± 7.06\%), \( p < .05 \) (Figure 2E,F). The fluorescence expression of BMSC-GFP in the bone defect site was observed under a fluorescence microscope. In the LIPUS group, the relative fluorescence intensity of BMSCs-GFP in the femoral defect site was higher \( (38.2 ± 0.29) \), while that in the control group was lower \( (12.06 ± 0.23) \) (Figure 2G,H). The fluorescence intensity of BMSCs-GFP in the two groups was statistically different \( (p < .05) \), indicating that the homing ability of exogenously injected BMSCs-GFP pretreated with LIPUS were enhanced.

**Effects of LIPUS on the gene expression of BMSCs**

To further study the LIPUS-induced transcriptional regulation mechanism, microarray analysis was performed. After LIPUS treatment, the results of gene expression profiles in LIPUS group were screened compared with the control group according to the standard of up-regulated or down-regulated fold change \( ≥ 1 \) and \( p < .05 \). A total of 237 differentially expressed genes were screened out. In group LIPUS, 39 genes were upregulated, and 198 genes were downregulated. The top five up-regulated genes listed in Table 3. To verify the results of the microarray assay, genes associated with cell migration were screened for qRT-PCR, including ITGA8, CCL5, TM7SF2 and TUBA3B. The results showed that their expression in the LIPUS group was significantly higher than that in the control group (Figure 3A, \( p < .05 \)). The number of up-regulated differentially expressed genes in each item of KEGG was counted on KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/). Regulation of actin cytoskeleton was found in the top 10 KEGG pathway and highly correlated with cell migration (Table 4). To further explore functional genes that regulate cytoskeleton, ITGA8 gene was found to be an important factor in initiating this process. And, heat map of interesting differential gene also showed that ITGA8 gene had a higher expression \( (p < .01) \) in the LIPUS group (Figure 3B). The expression levels of ITGA8 gene were significantly increased, with expression levels being 2.3-fold.

**Morphology and distribution of cytoskeleton microfilament**

Cellular F-actin stained with FITC-phalloidin was examined by confocal laser scanning microscopy (Figure 3C). The microfilaments in the control group were filamentous and arranged along the long axis of the cells. Parallel rows of microfilaments spread throughout the BMSCs. The cell morphology of BMSCs treated with PF-573228 or U0126 showed shrinkage, smaller volume and irregular edge. In the cytoplasm, the microfilaments were shortened and their arrangement was disordered. The distribution of cell microfilaments in the U0126 group was uneven, and the fluorescence signal of F-actin decreased. The results suggested that inhibition of FAK or ERK1/2 signalling pathways can cause cytoskeletal...
regulatory disorders. Interestingly, we found the cytoskeleton of BMSCs reorganized after treatment in a sample of LIPUS group, which further verified the analysis results of KEGG. The striped F-actin aggregates to the direction of the cell membrane where high fluorescence signals were showed. And, a ring-like structure was formed around the nucleus in cytoskeletal network while no similar structure was found in other groups (5 samples per group).

**Figure 1.** (A) The number of BMSCs passing through the membrane in the LIPUS group was higher than that in the control group by transwell assay. Cells crossed porous membrane was counted using optical microscope (100×) at 24 h after LIPUS treatment in the presence or absence of PF-573228 (B) and U0126 (C). *p < .05 and **p < .01. Scale bar = 50 μm. (D) The scratches of wound healing assay were observed under inverted phase contrast microscope (×40). LIPUS significantly reduces the unhealed area of the scratch, which was affected by the PF-573228 and U0126. The area of non-healing area was between red lines.

**Table 2.** Scratched area of each group (mm², x ± s).

| Group         | 0 h  | 24 h  | 48 h  |
|---------------|------|-------|-------|
| Control       | 1.38 ± 0.07 | 1.04 ± 0.07 | 0.78 ± 0.18 |
| PF-573228     | 1.39 ± 0.03 | 1.32 ± 0.03* | 1.18 ± 0.09* |
| U0126         | 1.35 ± 0.05 | 1.3 ± 0.04*  | 1.13 ± 0.08* |
| LIPUS         | 1.34 ± 0.03 | 0.85 ± 0.11* | 0.52 ± 0.12* |
| LIPUS + PF-573228 | 1.36 ± 0.05 | 1.18 ± 0.12Δ | 0.93 ± 0.15Δ |
| LIPUS + U0126 | 1.38 ± 0.03 | 1.17 ± 0.11Δ | 0.89 ± 0.12Δ |

*Compared with control group, p < .01.
ΔCompared with LIPUS group, p < .01.

**Activation of FAK and ERK1/2 in LIPUS-induced BMSCs**

Western blot was performed to monitor the change of FAK and phosphorylation of FAK in BMSCs. The result showed that significant phosphorylation of FAK was rapidly induced using LIPUS. The levels of phospho-FAK significantly increased and reached its peak at 30 min after the LIPUS treatment (Figure 4A). Therefore, 30 min was selected as time
point for the following western blot analysis. ERK1/2 and myosin II, as downstream effectors of FAK, were also detected using western blot analysis. When FAK was activated or inhibited, the activity of ERK1/2 and myosin II also changed accordingly. The result demonstrated that LIPUS promoted phosphorylation and activation of FAK, ERK1/2, and myosin II. The levels of phosphor-FAK, phosphor-ERK1/2 and phosphor-myosin II decreased significantly after pretreatment with PF-573228 and U0126, respectively (Figure 4B-D, p < .01). However, activation of FAK, ERK1/2, and myosin II-induced using LIPUS were significantly inhibited by PF-573228 and U0126, respectively.

Discussion
Cellular migration is an integrated and multistep process, including tethering and rolling in the capillary, adhesion on the surface of the endothelium and transmigrating across it,
Table 4. Top 10 of KEGG pathway analysis.

| Term                                | Input number | Background number | p-Value |
|-------------------------------------|--------------|-------------------|---------|
| Regulation of autophagy             | 2            | 34                | .0011   |
| Olfactory transduction              | 6            | 1252              | .0063   |
| Oocyte meiosis                      | 2            | 113               | .0105   |
| FoxO signalling pathway             | 2            | 136               | .0148   |
| Steroid biosynthesis                | 1            | 20                | .0278   |
| Regulation of actin cytoskeleton    | 2            | 221               | .0361   |
| Maturity onset diabetes of the young| 1            | 27                | .0369   |
| Prion diseases                      | 1            | 34                | .0460   |
| Aldosterone-regulated sodium Reabsorption | 1       | 41                | .0549   |
| Fatty acid degradation              | 1            | 47                | .0625   |

Input number and background number refer to the number of differential genes and all genes annotated to the pathway, respectively.

Figure 3. (A) Verification of the microarray results with qRT-PCR (*p < .05). (B) Heat map of interesting differential gene. Red indicates high expression, and green indicates low expression. The depth of colour is positively correlated with the level of expression. Cellular F-actin stained with FITC-phalloidin was examined using confocal laser scanning microscope (×400). (C) (a) The microfilaments of BMSCs in the control group were arranged in parallel. (b,c) BMSCs pretreated with PF-573228 or U0126 showed changes in cell morphology and cytoskeletal structure. (d) The white arrow indicates that a ring-like structure was formed around the nucleus after LIPUS treatment. The red arrow indicates that F-actin aggregates into the cell membrane. [e,f] Treatment of LIPUS-induced BMSCs with PF-573228 or U0126 caused morphological changes and cytoskeletal structure disorder. Scale bar = 50 µm.
then extravasation through extracellular matrix [18]. Although local transplantation or injection of BMSCs represents potential approaches that may be useful in certain settings, a significant barrier to the effective implementation of BMSCs therapy is still the inability to target these cells to tissues of interest with high efficiency and engraftment [19]. LIPUS is distinguished from other engineering methods in that it generates mechanical waves with periodic changes in sound pressure which can be transmitted to living cells and affect the basic functions such as cell proliferation, differentiation, and migration. However, there was still few literature investigating the effect and mechanism of LIPUS on cell migration. In previous studies, LIPUS has been proven to elevate the production of interleukin, chemokine and matrix metalloproteinase, which was able to induce angiogenesis and macrophages migration [20]. This in vitro study reported that LIPUS significantly promoted BMSCs migration at an intensity of 30 mW/cm² and exposure time of 20 min. The transwell chamber assay was conducted to examine the ability of BMSCs migration across the endothelium, while the wound healing evaluated the ability to sense and migrate to scratched area. Both these two migration capabilities, compared with the control group, were promoted significantly using LIPUS.

LIPUS is a kind of mechanical wave, and mechanical and thermal effects are therapeutic effects on living organisms. To investigate whether the migration of BMSCs in vitro was mediated by the thermal effect of LIPUS, the temperature curve of cell culture medium (bottom of cell adherence) during LIPUS treatment was plotted (Figure 2A). The results

Figure 4. Western blot analysis was in BMSCs induced using LIPUS. (A) BMSCs were stimulated using LIPUS and collected for Western blot analysis at the indicated time point after LIPUS treatment. (B–D) Phosphorylation of FAK, ERK1/2 and MLC induced using LIPUS were analyzed using Western blot in the presence of the specific inhibitors of FAK (PF-573228) and ERK1/2 (U0126).
showed that the temperature changes of the LIPUS group and the control group were all within 0.3°C during the whole treatment process. Any biological effect of ultrasound that is accompanied by temperature increments less than 1°C above normal physiologic levels is called a mechanical effect [21]. Hence, we further confirmed that the biological effects of LIPUS were mainly mediated by mechanical effects rather than thermal effects.

Previous studies have shown that a series of specific molecules were released into the damaged area and enter the circulatory system when bones are damaged, and then the BMSCs from the injured tissue and the circulatory system migrated to the damaged site and differentiate into specific tissues [22]. BMSCs are directly involved in fracture healing. BMSCs from the injured tissue and the circulatory system when bones are damaged, and then the progenitor cells were released into the damaged area and enter the circulatory system when bones are damaged, and then the BMSCs from the injured tissue and the circulatory system migrated to the damaged site and differentiate into specific tissues [22]. BMSCs are directly involved in fracture healing. [21]. Hence, we further confirmed that the biological effects of LIPUS were mainly mediated by mechanical effects rather than thermal effects.

An important issue in cell biology is how transmembrane receptors transmit extracellular signals to the interior of cells and then regulate cell migration. Cell migration requires the participation of adhesion proteins such as integrins and activation of FAK. Recent study showed that ERK1/2, one of the downstream of FAK, was found to be activated using LIPUS [14]. Therefore, we hypothesized that LIPUS can promote cell migration by activating the FAK-ERK signalling pathway.

In this study, we found that LIPUS-activated FAK and ERK1/2 signalling pathways and enhanced the migration of BMSCs. FAK is present at focal contacts, becomes phosphorylated after integrin-mediated cell adhesion and plays a role as an adapter protein for integrin-mediated cell signalling [33]. Phosphorylation of FAK at tyrosine 397 is critical for the induction of downstream effects [34]. The activated FAK produces a site that binds with the protein-containing SH2 region and serves as a scaffold protein to recruit proteins such as paxillin and Src, passing signals to MAPK and RhoGTPase, and eventually linking FAK and the dynamic changes of cytoskeleton and the dissociation of the focal contact [35]. Recent research showed that phosphorylation of FAK at Tyr-397 increased specifically the time-residency of FAK at focal adhesions but not in cytosol. This, in turn, induced disassembly of focal adhesions at the cell tail and promotes cell motility [36]. ERK1/2 activated using LIPUS mediates the process of extracellular signal transduction into cells. ERK1/2 has been shown to phosphorylate myosin light chain kinase (MLCK), which phosphorylates myosin light chain (MLC) at Thr18/Ser19 and thereby stimulates MLC activity [37,38]. Activated MLC promoted microfilament contraction and accelerates cell migration [39]. In this study, FAK and ERK1/2 activated LIPUS enhanced MLC activation, thereby facilitating cell migration, and that this process was inhibited by specific FAK inhibitor (PF-773228) and ERK inhibitor (U0126), the amounts of phospho-FAK, phospho-ERK1/2, phospho-MLC significantly decreased, which was highly possible that LIPUS promotes the BMSCs migration via the phosphorylation and activation of FAK-ERK signalling pathway.
However, the exact mechanism by which LIPUS activates the FAK-ERK signalling pathway remains unclear. In addition, we have also observed that the above-mentioned cytoskeletal structure that was beneficial for migration returned to its original state after a certain period of time. The application of LIPUS to facilitate migration may have an optimal time window. These inferences should be studied further.

Conclusions

In conclusion, LIPUS can promote the migratory ability of BMSCs in vivo and in vitro, one mechanism of which may be related to the activation of FAK-ERK1/2 signalling pathways using LIPUS. These findings provide evidence for clinical application of stem cell transplantation and selection of preconditioning strategies.

Disclosure statement

The authors report no conflicts of interest and are responsible for the content and writing of the manuscript.

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