Optimal Intensity Shock Wave Promotes the Adhesion and Migration of Rat Osteoblasts via Integrin β1-mediated Expression of Phosphorylated Focal Adhesion Kinase

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Running title: Shock Wave-induced Osteoblast Adhesion and Migration

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Background: The effects of extracorporeal shock wave (ESW) on adhesion and migration of osteoblasts have not been reported until now.

Results: Optimal intensity shock wave promotes osteoblast adhesion and migration.

Conclusion: ESW promotes the adhesion and migration of osteoblasts via integrin β1-mediated expression of phosphorylated FAK.

Significance: It provides mechanistic basis for improving the effectiveness of ESWT on fracture healing and tissue engineering.

SUMMARY

To search for factors promoting bone fracture repair, we investigated the effects of ESW on the adhesion, spreading and migration of osteoblasts and its specific underlying cellular mechanisms. After a single period of stimulation by 10KV (500 impulses) of SW, the adhesion rate was increased as compared to the vehicle control. The data from both wound healing and transwell tests confirmed that an acceleration in the migration of osteoblasts by SW treatment. RT-PCR, flow cytometry and western blotting showed that SW rapidly increased the surface expression of α5 and β1 subunit integrins, indicating that integrin β1 acted as an early signal for ESW-induced osteoblasts adhesion and migration. It has also been found that a significant elevation occurred in the expression of phosphorylated β-catenin and focal adhesion kinase (FAK) at tyrosine 397 site in response to SW stimulation after the increasing expression of integrin β1 molecule. When siRNAs of integrin α5 and β1 subunit were added, the level of FAK phosphorylation elevated by SW declined. Interestingly, the adhesion and migration of osteoblasts were decreased when these siRNA reagents as well as the ERK1/2 signaling pathway inhibitor, U0126 and PD98059 were present. Further studies demonstrated that U0126 could inhibit the downstream integrin-dependent signaling pathways, such as FAK signaling pathway, while had no influence on the synthesis of integrin β1 molecule. In conclusion, these data suggest that ESW promotes the adhesion and migration of osteoblasts via integrin β1-mediated expression of phosphorylated FAK at tyr397 site, in addition, Erk1/2 are...
also important for osteoblasts adhesion, spreading, migration and integrin expression.

Extracorporeal shock wave (ESW) is indicated as an alternative, non-invasive but promising method for treatment of bone fractures, effective even in delayed fracture healing or nonunion (1-6). The cure rate of the above disorders after ESW therapy was reported to be 75%-91% (7). ESW used is generated by an electronhydraulic shock wave generator which can cause an explosive evaporation of water and produce high energy acoustic waves (8). The acoustic waves are focused on a fluid-filled head with a silicone-type membrane and therefore can be transmitted into a specific site (8).

Fracture healing is a complex physiologic process that involves the coordinated participation of several cell types. The osteoblasts play a crucial role in this process. For bone formation to occur, osteoblasts precursor cells must migrate from bone marrow compartment to bone surfaces where they adhere, differentiate and deposit the bone matrix. After transient or long-term treatment of ESW, a positive effect on osteogenic activation has been shown according to the data from studies on osteoblasts proliferation and differentiation either in vitro or in vivo (9,10). The promotion of osteoblasts proliferation and differentiation is well documented to be through significant inductions of numerous cellular factors such as bone morphogenetic proteins (BMPs) (11), transforming growth factor (TGF-β1) (12), and vascular endothelial growth factor (VEGF) (12,13), as well as osteocalcin (14). However, no previous reports focused on the effect of shock wave on the adhesion and migration of osteoblasts. It is unknown whether optimal intensity energy of ESW can promote osteoblast adhesion and migration.

The cell membrane was altered by low intensity of shock waves and is reported to be the most sensitive part of the cell (15). We hypothesized that the influence on the adhesion and migration of osteoblasts may be also the result of the effects of ESW on cell membranes. As previously described, specific transmembrane proteins, called integrins, mediate the interactions between the cells and the extracellular matrix proteins (ECM). These integrins consist of an α-unit and a β–unit (16). Many researchers have stated that integrin β1 is the major subunit in osteoblasts (17-19). For the attachment of fibronectin or collagen type I, osteoblasts express integrin β1 (17). Once cells have attached, the integrin is also involved in passing information from the ECM to the cell, and from the cell towards the ECM (“outside-in-signaling” and “inside-out -signaling”) (20). Blocking the integrin β1 with specific antibody had an inhibitory effect on the initial attachment to the implants, and thus delayed migration, proliferation and differentiation of osteoblasts (21,22). Based on flow cytometric analysis, the expression of integrins α5- and β1–subunits in cell membrane was transiently increased in response to ultrasound stimulation (23). Focal adhesion kinase (FAK) has been established as a key component of the signal transduction pathways triggered by integrins (24).

However, there has been no prior evidence showing that ESW promotes osteoblasts adhesion and migration. In this study we found that an optimal intensity ESW treatment promoted osteoblasts adhesion and migration via the induction of integrins β1 molecule, which mediated the phosphorylation of FAK and cross-talk with other specific signal transduction pathways. We also sought to investigate the effects of ESW on the adhesion, spreading and migration ex vivo in cell culture model, aiming to clarify the underlying specific molecular mechanisms.

EXPERIMENTAL PROCEDURES

Animals—3 day-old Sprague-Dawley rats (male or female) were obtained from the Experimental Animal Center of Shantou University Medical College (Shantou, China). Care of rats in this investigation conformed to The Guide for the Care of Use of Laboratory
Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and followed the rules of the National Animal Protection of China. The study was approved by the Institutional Animal Care and Use Committee of Shantou University Medical College.

Reagents and Antibodies—Dulbecco’s minimal essential medium (DMEM), fetal bovine serum (FBS) were purchased from Hyclone. Collagen type II and fibronectin were purchased from Sigma-Aldrich (St. Louis, MO, USA). 0.25% Trypsin/ 0.02% EDTA, 1% penicillin/streptomycin, Trizol reagent and Lipofectamine™ RNAiMax were purchased from Invitrogen (Carlsbad, CA, USA). Plastic culture dishes, cell culture plates, Transwell inserts with polycarbonate membrane containing 8.0-μm pores were obtained from Costar (Cambridge, MA, USA) or Nunc (Nalge, Denmark) unless stated otherwise. Mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor (PD98059/U0126), p38 MAPK inhibitor (SB203580) and C-Jun N-terminal kinase (JNK) inhibitor (SP600125) were purchased from Promega (Madison, WI, USA). Phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002), Protein kinase A (PKA) inhibitor (H-89), JAK-2 inhibitor (AG490) were products of Calbiochem (La Jolla, CA, USA). The PCR primers for integrin α5/β1 subunits and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were synthesized by Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). The PCRMix kits were from Tiangen (Beijing, China). The effective small interfering RNA of integrin α5 and β1 subunit were designed and synthesized by GenePharma Co., Ltd (Shanghai, China). For flow cytometry, phycoerythrin (PE)-conjugated anti-rat CD29 and CD49e together with PE-conjugated IgG (isotype control) were purchased from E-biosciences (USA). For western blotting, polyclonal primary antibodies against integrin α5 and β1 subunits were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal antibodies against FAK, p-FAK tyr397, p-FAK tyr576/577, p-FAK tyr925, and β-catenin (6B3), p-β-catenin (Ser33/37/Thr41), and Erk1/2, and p-Erk1/2 were purchased from Cell Signaling Technology, Co., Ltd (USA). Primary monoclonal antibody for β-actin and horse radish peroxidase-conjugated secondary antibody were also from CST. The SuperSignal western blotting detection kits was obtained from Pierce Biotech. Co., Ltd (Rockford, IL, USA). Other chemicals and reagents were of molecular biology grade and were purchased from local commercial stores.

Cell Cultures—Primary rat osteoblasts were isolated from calvaria of 3-day-old SD rats and cultured using methods as described previously (25,26). Briefly, the newborn rats were sacrificed by decapitation and immersed in 75% alcohol for 5 min. The calvaria were cleaned of any extraneous tissue and then cut into small pieces. The bone chips were recovered with a bone curette and were digested in Dulbecco’s modified Eagle’s medium (DMEM) with 0.1% collagenase (w/v) for 4 h, containing 10% (v/v) fetal bovine serum (FBS) in a humidified atmosphere of 95% air, 5% CO2 at 37°C. Then the solution with bone chips were pooled and filtered through 70-μm nylon filters (Falcon; BD Biosciences, San Jose, CA, USA). A preplating step was included to reduce the number of contaminating non-osteoblasts. The dispersed cells were plated in DMEM containing 10% heat-inactivated fetal bovine serum (HIFBS) for 30 min to remove the non-osteoblasts, then 2×10⁶ cells/ml (10 ml/dish) were placed in 100-mm culture dishes and maintained in a humidified atmosphere of 95% air, 5% CO2 at 37°C. The medium was replaced every other day. When the cells covered 90% of the bottom of dish, they were detached with 0.25% trypsin and passaged. The purified osteoblasts were identified using alkaline phosphatase assays and cells containing dark brown particles were considered to be osteoblasts and the positive rate of osteoblasts was calculated (Supplemental Fig. 1C, 1D). Alizarin red staining of calcified nodule was also performed (Supplemental Fig. 1B). The cells used in the experiments were of
4th passages while in the siRNAs transfection experiments the 3rd subcultured cells were used.

**ESW Treatment of Rat Osteoblasts in Vitro**—In our experiments, extracorporeal shock wave (ESW) was generated by Huikang type IV shock wave equipment (Huikang, Shengzhen, China) with a focus spot about 25-mm in diameter. Osteoblasts were washed and resuspended with the complete medium. Cell suspensions were subjected to ESW treatment with modification as described before (27,28). Cells were suspended in 15-mL sterile polystyrene tubes at a concentration of 1×10⁶/mL. First of all, to identify what the optimal intensity of ESW was, the ESW treatment with 250, 500, 750, and 1000 impulses at certain energy levels (5, 10, 15, and 20 KV) was applied to the cell suspensions. After ESW treatment, cells were cultured for 24 h and 48 h for cell proliferation assays while stained with trypan blue to assess cell survival at 1 h. Once an optimal intensity of ESW was determined, ESW treatment of osteoblasts suspensions (samples with or without inhibitors/siRNAs) lasted 10 min. Then the cells were placed onto plastic dishes or culture plates for different times as required. Osteoblasts without ESW treatment were run as controls.

**Blocking with Specific Inhibitors of Signal Transduction Pathways**—In studies of the ESW-induced signal transduction of cell adhesion and migration, cells were treated with 50 μM PD98059, a MEK1 inhibitor, 20 μM U0126, a MEK1/2 inhibitor, 25 μM LY294002, a PI3K signal pathway inhibitor, 15 μM SB203580, a p38/MAPK inhibitor, 20 μM SP600125, a C-Jun N-terminal kinase (JNK) inhibitor, 25 μM H-89, a protein kinase A inhibitor, 50 μM AG490, a JAK signal pathway inhibitor, for 60 min prior to ESWT. Cells were washed and resuspended before subjected to optimal intensity of ESW treatment as described above. After the treatment, cells were cultured until the time when a maximum elevation of integrin α5 and β1 subunits would be reached without adding specific inhibitors as indicated. Then we identified the specific signal transduction pathways involved in ESW-induced adhesion and migration by western blotting analysis.

**Transient Transfection with Small Interfering RNA**—According to the gene sequences of integrin α5 (NM_001108118) and β1 (NM_017022) and the principles of siRNAs design, double strand siRNA oligonucleotides targeting both genes (Sense: 5’-CCGCAUCCUGAGUCUCATT-3’, antisense: 5’-UGAAGACUCAGGAUGCGGT T-3’ for integrin α5 oligonucleotide; sense: 5’-GAUCAGGAGAACCACAGAATT-3’, antisense: 5’-UUCUGUGGUUCUCUGUAUCT T-3’ for integrin β1 oligonucleotide) were synthesized by GenePharma Technology Co., Ltd (Shanghai, China), respectively. A pair of negative control siRNAs were also designed. For transfection, cells were plated onto culture plates of six wells and grown to 70–80% confluency in the complete medium without antibiotics. Then the cells were transfected with integrin α5/β1 siRNAs or a negative control siRNA using Lipofectamine™ RNAiMAX Reagent according to the manufacturer’s recommendations. Briefly, we prepared RNAi duplex-Lipofectamine™ RNAiMAX complexes in following steps for each well of the osteoblast sample: a) dilute 10 μL RNAi duplex in 240 μL Opti-MEM® I reduced serum medium; b) dilute 5 μL Lipofectamine™ RNAiMAX in 245 μL Opti-MEM® I reduced serum medium; c) combine the diluted RNAi duplex with the diluted Lipofectamine™ RNAiMAX and incubate for 10-20 minutes by mixing gently at room temperature. Cells were incubated with the oligonucleotide duplexes in serum-free conditions for 6 h at 37°C. Then the cells were harvested and resuspended with the complete medium. Cell suspensions were given the optimal intensity of ESW treatment as described above. After ESW treatment, cells were incubated for a certain additional period of time. The effects of siRNAs on the expression of integrin α5/β1 and its downstream signal pathway proteins were assessed using RT-PCR or western blotting.

**Cell Survival and Viability Assays**—To
determine the optimal intensity of ESW, we performed cell survival and viability assays. After different doses of ESW treatment, the cell survival was determined with a hemocytometer by staining with 0.4% trypan blue in ammonium chloride. Osteoblasts viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells treated with either vehicle or ESW treatment were cultured in 96-well culture plates (200 μL/well). After 24 h or 48 h, they were supplemented with 10 μL MTT (5 g/L, Fluka Co. Product) and incubated for another 4 h. Then the supernatant was discarded by aspiration and the osteoblast preparation was shaken with 150 μL dimethyl sulfoxide (DMSO) for 10 min, before the OD value was measured at 490 nm by using a microplate reader (Thermo Scientific, Beijing, China).

**Cell Adhesion and Spreading Assays**— Osteoblasts with or without ESW treatment were concentrated by centrifugation (1000 rpm, 5min) and resuspended in the complete medium. Cells were again counted with a hemocytometer and seeded on the plastic culture dishes and incubated for 2 h, 4 h, 6 h, 8 h, 10 h at 37 °C, 95% O₂, 5% CO₂ in a humidified incubator at the same concentration of 1×10⁴ cells per cm². Non-adherent cells were removed by rinsing with PBS and adherent cells were released with 0.25% trypsin/EDTA and counted by independent blinded investigators. Then the adherence rates for the shock wave group and the control group were calculated. Cells plated onto fibronectin-coated culture dishes were run as positive controls. All the samples were routinely observed with an inverted phase contrast microscopy (Nikon, Japan), to confirm the procedure of cell adhesion.

**Cell Migration Assay**—The migration of osteoblasts was evaluated by using a modified transwell insert assay. In brief, osteoblasts with or without ESW treatment in the presence or absence of integrin α5 and β1 subunits were rendered into single-cell suspensions. 2×10⁴ Osteoblasts in 200 μL complete medium were transfered into the upper chamber of transwell inserts (8 μm pore size; Costar). An aliquot of 0.6 ml of complete medium containing with VEGF (50 ng/mL) was placed in the bottom wells. After incubation for 12 h and 24 h at 37 °C, the non-migrated cells on the upper side of membranes were wiped gently with ice-cold PBS-soaked cotton swabs. Then, the migrated cells on the bottom side of membranes were washed with PBS and fixed with methanol and stained with 4′,6-diamidino-2-phenylindole (DAPI) solution. For quantification, cells migrating into the lower chamber were counted manually in 5 even high-power (×100) microscopic fields by using fluorescent microscopy (Nikon, Japan), and the average numbers of cells/ field were determined.

Wound healing test was also performed to measure osteoblast migration. Briefly, single cell suspensions obtained as described above were inoculated at 1×10⁷/well in 6-well plates. When the cells became fully confluent, a scar was made along a straight line using a 200-μL pipette and the floating cells were rinsed off with PBS. The scar was marked in the plate and the cells were grown in serum-free media. Five viewpoints were photographed at 12 and 24 hours after the scar was made under a microscope at 40× magnification, and the mean migration distance was determined and analyzed using the Image Pro Plus6.0 software.

**Reverse Transcriptase and Polymerase Chain Reaction (RT-PCR)** —The gene expressions of integrin α5 and β1 subunits were examined. Total RNA was isolated from cells using Trizol reagent as instructed by the manufacturer. After the isolation of the RNA, the concentration of RNA was determined based on the optical density of the sample, which was measured at 260 nm. 1-μg RNA was used in the reverse transcriptase (RT) –reaction. The first strand cDNA from the RT-reaction was used as a template in the polymerase chain reaction (PCR). To perform PCR, 12.5 μL PCR Mixture, 1μL cDNA, 9.5 μL RNase-free water, 1 μL reverse primer and 1 μL forward primer were added and vortexed together. The PCR was performed using a thermocycler (Bio-rad, Richmond, CA,
USA). DNA was PCR-amplified under the following conditions: 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 60 seconds for a total of 36 cycles. Primer sequences used were: integrin, alpha 5 (Itga5, NM_001108118) 5'-GATGAGGAACAGTGAACCGAAGG-3' (sense) and 5'-AGCAAAAGCAGGATAGAGGACCA-3' (antisense); integrin, beta 1 (Itgb1, NM_017022) 5'-GGAGGAATGTAACACGACTGC-3' (sense) and 5'-CAGATGAACTGAAGGACCACC-3' (antisense); glyceraldehyde-3-phosphatase (GAPDH, NM_017008) 5'-ACACACAGTCCATGCCATCAC-3' (sense) and 5'-CCACCACCCTGTTGCTGTA-3' (antisense). The reference house-keeping-gene used to normalize the amount of mRNA in the cultures was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All RT-PCR products were electrophoresed in 1.5% agarose gel and visualized under a UV transilluminator after staining with Goldview and photographed. The relative expression was quantified densitometrically by using Quantity One Software (v4.5.2, Bio-Rad, Hercules, CA, USA).

Flow Cytomeric Analysis—To elucidate whether integrin α5- and β1-subunits expressed on the cell membranes were involved in the ESW-enhanced cell adhesion and migration, we determined the quantity of both subunits via flow cytometry, respectively. According to the manufacturer’s protocols, After ESW treatment for 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, osteoblasts were washed with ice-cold PBS, then resuspended in 400 μL of binding buffer at 1 × 10⁶ cells/mL. An aliquot of 390 μL suspensions with single cell were incubated with 10μl phycoerythrin (PE)-conjugated anti-rat CD29 and CD49e (0.2 mg/mL, E-Biosciences) respectively, or PE-conjugated IgG (isotype control, E-biosciences) for 30 min at 4 °C in the dark. Osteoblasts without ESW treatment as control groups were treated according the same procedure. Fluorescence was measured using the FACSort Flow Cytometer (Becton-Dickinson, Franklin Lake, NJ, USA). Approximately 10,000 cells were counted in each sample, and data were analyzed with the use of WinMDI software (v2.9, Bio-Soft Net).

Protein Extraction and Concentration Assays—Osteoblasts were washed twice in ice-cold PBS and allowed to sit on ice for 30 min in 200 μL lysis buffer (25 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1mM NaF, 5mM Na3VO4). Then, the samples were centrifuged at 12,000 ×g for 10 min at 4 °C, and the supernatants of the respective samples were collected and stored at −20 °C. Proteins were denatured by boiling for 5 min after measurement of the concentration in the BCA assays (Kits were from Beyotime, Jiangsu, China). According to the instruction provided with the kit, samples were incubated with BCA reagent for 30 min and absorbance of each was read at 560 nm using a microplate reader. BSA ranging from 0 to 500μg/mL was used as a standard.

Determination of Integrin α5 and β1 subunits—To detect the expression of Integrin α5- and β1- subunit protein, western blotting was taken into consideration. Equal amounts of proteins were separated on 8% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was blocked by 1-h incubation at room temperature in a Tris-buffered saline solution (TBS-T: 20 mM Tris, pH 7.6, 135 mM NaCl, and 0.05% Tween) containing 5% nonfat dry milk. Membranes were probed with anti-integrin α5 and β1 antibodies (1:600 dilution; Santa Cruz) at 4°C overnight. After the primary antibody incubation, the membrane was washed 3 times with TBS-T. The appropriate secondary antibody, horseradish peroxidase-labeled goat-anti-rabbit IgG, was then added to the membrane according to the vendor’s recommendation (1:8000 dilution; CST) and incubated for 1 h at room temperature. The membrane was again washed 3 times with TBS-T. The bound antibodies were detected by use of SuperSignal western blotting kits (Pierce Biotech, Rockford, IL, USA). Densitometric
analysis of western blots involved the use of Quantity One Software (v4.5.2, Bio-Rad, Hercules, CA, USA). The expression of β-actin was shown as a control for equal protein loading.

**Determination of FAK Phosphorylation and β-catenin Activation**—As described above, the procedures of detecting p-FAK and FAK, p-β-catenin and β-catenin were the same as described above except that 10% SDS-PAGE gels were used and the blocking solution was changed to TBST with 5% BSA. The monoclonal primary antibodies (CST products) worked at dilutions of 1:1000.

**Determination of Erk1/2 Phosphorylation**—The data from studies on blocking the signal transduction pathways with specific inhibitors above indicated that the Erk/MAPK pathway was involved in the ESW-induced integrin expression. We did research on how the Erk1/2 phosphorylation level changed with either ESW treatment or not through western blotting detections using 12% SDS-PAGE gels. The procedure was similar to that as noted above.

**Statistical Analysis**—The data presented are from one of three separate sets of experiments, of which yielded comparable results. All data are expressed as mean ± standard error of the mean (S.E.M), and analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Student’s unpaired t-test was used to compare differences between two groups. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls’s test was used to compare the differences among more than two groups. A probability (P) value less than 0.05 was considered statistically significant.

**RESULTS**

**ESW with 10KV for 500 Impulses was Defined as the Optimal Intensity**—The cell survival was determined with a hemocytometer by staining with 0.4% trypan blue in ammonium chloride. We found that the osteoblasts survival rate was not affected by energy lower than 10KV for 500 impulses, while osteoblasts receiving 15 or 20 kV of shock wave showed significantly reduced survival as compared with the control group (p<0.01) (Supplemental Fig. 2). Cell proliferation assays showed that at 24 hours after shock wave treatment, there was no statistically significant differences between osteoblasts receiving 5 or 10 kV and the control cells (p>0.05). Furthermore, at 48 hours, shock wave at 5 kV induced statistically significant differences in the proliferation of osteoblasts compared with the controls (p<0.05), while shock wave at 10 kV for 500 impulses caused a markedly increase in the proliferation of osteoblasts as compared to the control (p < 0.01) (Supplemental Fig. 3). These findings indicate that shock wave caused a dose-dependent effect on the survival and growth of osteoblasts and 10 kV for 500 impulses was an optimal level for shock wave treatment for osteoblasts. Therefore, ESW at 10KV for 500 impulses was used in the subsequent experiments.

**ESW Promotes Osteoblast Adhesion and Migration Which Were Mediated by Integrins**—Adhesion, spreading property, migratory capacity of cells, were also important aspects for bone cells behaviors while there was no report focusing on the effect of ESW on the adhesion and migration of osteoblasts. In the present study, the effect of ESW on osteoblasts adhesion was evaluated in an adhesion assay. The SW at 10KV for 500 impulses enhanced osteoblast adhesion significantly. The adhesion rates of osteoblasts were significant higher at 2 h, 4 h, 6 h, 8 h, 10 h after ESWT (Fig. 1). Dynamic observation under an inverted microscope shown that osteoblasts more fully spreading after ESWT (data no shown). The migration of osteoblasts was analyzed in a modified transwell test and wound healing assay. Results from the wound healing assays were consistent with the transwell tests. ESW accelerated osteoblast migration. After ESWT for 24h, the average migration distance was increased by ~3-fold and the migrated cell number was increased by ~4-fold (Fig. 2). In addition, the adhesion and migration of osteoblasts after ESW were impaired in the
absence of α5 and β1 integrins (Fig. 1 and Fig. 2). We found that cell adhesion to matrices was primarily mediated by integrins under condition of ESWT. These results prompted us to investigate whether shock wave could induce expression of integrins or not (shown in Fig. 3 and Fig. 4). Adding signal transduction pathway inhibitors, U0126 could inhibit the ESW-augmented adhesion and migration of osteoblasts while no influences were observed from the other several inhibitors (data not shown). This encouraged us to perform more experiments to evaluate if the Erk1/2 signaling pathway could be activated by ESW (results are shown in Fig. 7).

**ESW Promotes Integrin α5- and β1- Subunit mRNA Expression**—Primary osteoblasts were treated with ESW at an intensity of 10KV for 500 impulses intensity. There were no significant differences in cell viability between the ESW and control groups. Results from RT-PCR indicated that both integrin α5- and β1-subunit mRNAs were significantly elevated, peaking at 1 h after ESW, by ~5-fold, and then returning to the base level after 12 h (Fig. 3A and 3B). Though multiple signaling pathway inhibitors of working concentrations were applied to pretreat the osteoblasts prior to ESWT respectively, our result indicated that ESW-augmented integrin α5 and β1 subunit expressions were not attenuated (Fig. 3C and 3D). Moreover, ESW and inhibitors used in this study did not affect the mRNA expressions of the housekeeping gene GAPDH (Fig. 3).

**ESW Promotes Integrin α5 and β1 Protein Expression**—The background expression of integrin α5- and β1- subunit protein was determined in osteoblasts (Fig. 4). During the period from 30 min to 12 h after ESWT, flow cytometry analysis showed that synchronous elevation of both integrin α5 and β1 protein, peaking at 2 h, as compared to the control groups (Fig. 4A). Western blotting results also demonstrated that osteoblasts subjected to ESWT significantly increased integrin α5 and β1 protein levels at 2 h by ~3-fold and ~5-fold, respectively (Fig. 4B and 4C). What’s more, the effects of ESW on integrin α5 and β1 expressions were abrogated by small interfering RNA pretreatment (Fig. 4B and 4C). These findings suggest that integrin α5 and β1 expressions were promoted in an early period after ESWT.

**ESW Treatment Induces Phosphorylation of FAK which is mediated by integrin α5 and β1**—To determine the change of phosphorylation level of FAK at different sites caused by ESW at 10KV for 500 impulses, osteoblasts were harvested at 0.5 h, 1 h, 2 h, 4 h, 8 h after ESWT. It was found that a marked elevation of FAK phosphorylation at Tyr397 in 2 h, peaking at 4 h, by ~5-fold, and also a slight increase of phosphorylation FAK at Tyr925 in 2 h after ESWT, by ~2-fold, while ESW could not affect the expression of FAK with phosphorylation at Tyr576/577 (Fig. 5A). Moreover, to explore whether the FAK activation was associated with the elevated integrin α5 and β1 proteins, 4 h after the optimal dose of ESWT, the expression of phosphorylation FAK at Tyr397 declined in cells pretreated with integrin siRNAs (Fig. 5B). As shown in Fig. 5B, decline was the most significant when both siItga5 and siItgb1 were present. This indicated that integrin-FAK signaling (interactions with integrin α5β1 and FAK activation) played an important role in ESW-induced adhesion and migration of osteoblasts.

**Erk1/2 Up-regulates Integrin-mediated FAK phosphorylation**—We sought to investigate whether certain mediators were involved in the ESW promotion of integrin-FAK signal pathway. After adding specific signal pathway inhibitors to osteoblasts for 60 min prior to ESWT, we found that both 50 μM PD98059 and 20 μM U0126 (MEK1/2 inhibitors) significantly suppressed ESW-induced expression of phosphorylated FAK at tyrosine397 site, by ~2-fold (Fig. 5C and 5D). Inhibition of p38 activity by 15 μM SB203580 and JNK by 20 μM SP600125 did not affected ESW-promoted FAK activation. 25 μM H-89 (PKA inhibitor) and 50 μM AG490 (JAK inhibitor) had no
influence on FAK production (Fig. 5C and 5D). These findings suggest that Erk1/2, but not p38, JNK, PKA, or JAK, was essential for ESW-induced phosphorylation of FAK at tyr397 site.

**ESW enhances the activation of Wnt/β-catenin mediated by integrin α5 and β1**—We investigated another important bioactive molecule which might be responsible for ESW-induced cell adhesion and migratory capacity. Immunoblotting indicated that significantly increased β-catenin activation as demonstrated by phosphorylated β-catenin expression, peaking at 3 h after ESWT (Fig. 6A), by ~5-fold (Fig. 6C). Pretreatment with integrin siRNAs significantly reduced ESW-promoted phosphorylation of β-catenin expression (Fig. 6D). Nevertheless, U0126 did not affect ESW-promoted activation of β-catenin (Fig. 6D). We believe that Wnt/β-catenin was another signal pathway activated by ESW, possibly responsible for resisting the inhibitory effect of U0126, to maintain the integrin-mediated ERK dependent phosphorylation of FAK (Fig. 5D).

**ESW Promotion of ERK Phosphorylation was Mediated by Integrin α5 and β1**—ERK became activated in osteoblasts under ESW induction and inhibited when integrin siRNAs were present. Erk1/2 phosphorylation was increased in 2 h and the higher production of phosphorylated Erk1/2 persisted for 4 h (Fig. 7A and 7C). Integrin siRNAs could markedly attenuate ESW-induced ERK activation (Fig. 7B and 7D). Also U0126 abrogated the expression of phosphorylated Erk1/2 (Fig. 7B and 7D). These findings indicate that ERK activation was integrin-dependent under ESWT.

**DISCUSSION**

In the present study, we found that: I) an optimal extracorporeal shock wave (10KV for 500 impulses) promoted primary culture osteoblast adhesion and migration, II) α5β1 integrins played critical roles in ESW-induced osteoblasts adhesive and migratory capacity mostly by up-regulating the phosphorylation of FAK, III) Erk1/2 was essential for ESW-augmented osteoblast behaviors as one of the most important mediators for integrin α5β1-dependent FAK signaling pathway triggered by the exposure to ESW.

Interest in the application of ESWT to orthopaedic diseases was initially stimulated by the finding that after the modality was focused on the ureter, bone remodeling of the pelvis occurred (28). Ever since 1990s, shock waves have been used to treat musculoskeletal diseases especially delayed fracture healing and nonunion (5,10,29,30). Bone formation is the most important procedure during the bone regeneration period. Osteoblasts play a central role in bone formation by synthesizing multiple bone matrix proteins and by differentiation into osteocytes. Many investigators had committed to studying the effects of ESW on osteoblasts differentiation and proliferation. And the underlying mechanisms included the involvements of grow factors, gene expressions and several signal transduction pathways (15,17,23,31). Although the adhesion behavior as well as migration was also important for osteoblasts in ESW-induced fracture healing, there have been no investigations focused on these two aspects before. We focused on the effects of ESWT on the adhesion and migration of osteoblasts with aiming to reveal the underlying specific biochemical signals triggered by ESW.

Different shock waves could induce different outcomes (15,32). In our experiments, dose-dependent complications were observed and it was determined that optimal intensity of energy was 500 impulses at 10KV. Osteoblast adhesion refers to cell-cell adhesion and cell-ECMs adhesion. After ESWT, cell adhesion rate was much higher (80.24±2.3% vs 43.77±2.11% at 6h) than those without ESWT. The migration activity of osteoblasts was also significantly enhanced. Using the small interfering RNA technology, we knocked down the integrin α5 and β1 and observed the significant inhibitions of adhesion, spreading and migration in ESWT cells were observed.
These data indicated that ESW-induced adhesion, spreading, and migration partly depended on the expression integrins.

Integrins, the cell surface receptors that comprise an expanding family of transmembrane heterodimers of an α- subunit and a β- subunit (33). They primarily mediate cell adhesion and migration, as well as being involved in cell proliferation, programmed cell death and differentiation (34). It was reported that impairment of integrin gene led to decreased expression of OPG, PEG2, and TGF-β1 (22). It has also been shown that ultrasound treatment at 125 mW/cm² for 10 min transiently increased the surface expression of α5 and β1 integrins in both MC3T3-E1 and primary osteoblasts (23). Integrin α5β1 is the classical fibronectin receptor and mediates critical interactions between osteoblasts and fibronectin (35). Flow cytometry and western blotting revealed that ESW increased the cell surface expression of α5 and β1 integrins. The α5β1 integrin-knock down in osteoblasts were insensitive to ESW stimulation and characterised by poor adhesion to plastic culture dishes and significantly impaired migratory capacity which caused severe defects in wound healing and transwell tests. Herein, in our study, the rapid increase in the expression of integrin α5 and β1 subunit mRNA after shock wave stimulation might be the initial step in the accelerated adhesion and migration of osteoblasts.

A number of important biological processes, including cell motility, are controlled by integrin-dependent signals and focal adhesion kinase (FAK) has been implicated in these processes as an important component of an integrin-dependent signaling pathway, which transmit signals from the extracellular matrix into the cytoplasm (24,36-38). Previous studies suggested that integrin β1 mediated expression of FAK regulated osteoblasts' behaviors (39), which played an important role in cell cycle progress and migration (34). Inhibition of endogenous FAK signaling resulted in reduced motility (40). Conversely, enhancing FAK signaling increases cell migration (38). The major autophosphorylation site of FAK, tyrosine 397, is absolutely required for enhancing cell migration (41,42). Also the Src recruitment into complex with FAK is required for cell migration by augmenting the other sites of phosphorylation on FAK (43). In our study, it was observed a marked elevation of FAK phosphorylation at Tyr397 peaking at 4 h following ESW-induced expression of integrin α5 and β1, and a slight increase of FAK phosphorylation at Tyr925 in 2 h after ESWT. ESW could not influence the expression of FAK phosphorylation at Tyr576/577. What's more, a significant decline in FAK phosphorylation after ESWT was observed after knocking down integrin genes. Thus, we believed that, FAK as a downstream reactor of integrin mediated signaling pathway played a crucial role in ESW-induced osteoblast adhesion and migration.

A short pulse of mechanical force induced gene expression and growth in MC3T3-E1 osteoblasts via an ERK 1/2 pathway (44). By transducing human osteoblastic with a pseudotyped retrovirus encoding a mutated Erk1 protein with a dominant negative action against both Erk1 and Erk2 (Erk1DN cells), cell adhesion, spreading and migration were inhibited, expression of the integrins was decreased (45). While there were also reports shown that Erk1/2 of MAPK signaling family was downstream of FAK (43,46). In our study, with optimal intensity of SW (500 impulses at 10KV), we found that Erk1/2 inhibitor U0126 didn’t influence the expression of integrin α5 and β1. In contrast, we obtained that both integrin α5 and β1 could trigger the phosphorylation of Erk1/2 which also played an important role in cell adhesive and migrative function as mediators. A possible explanation for the paradoxical findings in the present study is that shock wave as a mechanical wave with critical biomechanical properties can change the spreading capacity of osteoblasts, also alter integrin expressions (23). Furthermore, α5β1 integrin had been verified to be involved in
mechanotransduction pathways (47). Another possible explanation for the discrepancy is that ESW could activate Shc first (Fig. 11), and enhancing the Erk1/2 phosphorylation (48,49). Additional studies would be required to confirm these speculations.

The downstream signaling in the integrin pathway may involve the activation of β-catenin (47). It has been found that cyclical pressure-induced strain resulted in rapid phosphorylation of β-catenin in human articular chondrocytes (47). There are also studies showing that mechanical loading up-regulates Wnt/β-catenin genes (50,51). In our study, we found that β-catenin phosphorylation level significantly increased at 3 h after ESW-induced expression of integrin α5 and β1. The activation of β-catenin was abolished by knocking down integrin α5 and/or β1, rather than with U0126. It was consistent with the previous study that β-catenin was activated by integrin (47). As described above, U0126 inhibited the major phosphorylation site of FAK, but not the whole. We therefore speculate that Wnt/β-catenin might be another mediator in integrin-FAK signaling pathway in response to ESW (Fig. 8). The available evidence therefore suggests that Wnt/β-catenin signaling pathway provides an important contribution to bone cell adaptive responses to mechanical stimulation (52-54).

Given the large amount of data suggest that integrin activation is one, if not the primary, early event. As a kind of mechanosensor, the existing of integrin α5β1 at the cell surface became activated via conformational changes which could be induced by mechanical stimuli (55,56,57). The conformational activation of integrin α5β1 might also play a crucial role in the process of ESW induced adhesion and migration of osteoblasts. Nidhi et al have demonstrated that PI3K signaling was responsible for the shear stress-induced conformational activation of integrin α5β1 leading to the opening of the hemichannels (55). Nevertheless, the mechanism on how integrins become activated which induced by ESW requires our further study (Fig. 8).

Many studies have shown that effective physical stimulation could induce a series of biological changes in osteoblasts, such as osteogenic effect (58,59), alteration of gene expressions (44,60), and related signal pathway changes (44,61). The beneficial effects for enhanced osteoblasts adhesion and migration may result from reorganization of actin cytoskeleton at a certain degree (23). The regulation of cell function by actin cytoskeleton is complex and may also involve integrin-mediated matrix binding and signal pathways (62-66). In addition, cells need to spread fully during migration (45), the enhanced ability of osteoblasts to spread would promote cell migration.

In summary, for the first time, to our knowledge, we found that extracorporeal shock wave promoted primary osteoblasts adhesion, spreading property and migratory capacity. ESW-induced functional activity of osteoblasts was via elevating both mRNA level and protein expression of integrin α5 and β1 subunits, particularly the β1 subunit involved in FAK signal pathway activation (Fig. 8). We have also further demonstrated that Erk1/2 signal pathway was essential for FAK signal cascades. Our findings provide additional insight into the mechanism by which ESW stimulated fracture healing. In addition, by specifically controlling cell and extracellular matrix organization using ESWT, this technology holds great potential for advancing the fabrication of complex engineered tissues in vitro. Although our understanding of ESW-induced adhesion and migration of osteoblasts is increasing, the picture is still far from being clear. There is still much to be learned before we fully understand the mechanisms induced by ESWT.

**REFERENCES**

1. Cacchio, A., Giordano, L., Colafarina, O., Rompe, J. D., Tavernese, E., Ioppolo, F., Flamini, S.,

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Shock Wave-induced Osteoblast Adhesion and Migration

Spacca, G., and Santilli, V. (2009) *J. Bone. Joint. Surg. Am.* 91, 2589-2597

2. Moretti, B., Notarnicola, A., Garofalo, R., Moretti, L., Patella, S., Marlinghaus, E., and Patella, V. (2009) *Ultrasound. Med. Biol.* 35, 1042-1049

3. Beutler, S., Regel, G., Pape, H. C., Machtens, S., Weinberg, A. M., Kremeike, I., Jonas, U., and Tscherne, H. (1999) *Unfallchirurg* 102, 839-847

4. Ikeda, K., Tomita, K., and Takayama, K. (1999) *J. Trauma.* 47, 946-950

5. Wang, C. J., Chen, H. S., Chen, C. E., and Yang, K. D. (2001) *Clin. Orthop. Relat. Res.* 387, 95-101

6. Wang, C. J., Huang, H. Y., Chen, H. H., Pai, C. H., and Yang, K. D. (2001) *Clin. Orthop. Relat. Res.* 387, 112-118

7. Birnbaum, K., Wirtz, D. C., Siebert, C. H., and Heller, K. D. (2002) *Arch. Orthop. Trauma. Surg.* 122, 324-330

8. Scott McClure DVM, P. D., and Christian, D. D. P. (2003) *Clinical. Techniques. in. Equine. Practice.* 2, 348-357

9. Rompe, J. D., Rosendahl, T., Schollner, C., and Theis, C. (2001) *Clin. Orthop. Relat. Res.* 387, 102-111

10. Rivilis, I., Milkiewicz, M., Boyd, P., Goldstein, J., Brown, M. D., Egginton, S., Hansen, F. M., Hudlicka, O., and Haas, T. L. (2002) *Am. J. Physiol. Heart. Circ. Physiol.* 283, H1430-H1438

11. Wang, F. S., Yang, K. D., Kuo, Y. R., Wang, C. J., Sheen-Chen, S. M., Huang, H. C., and Chen, Y. J. (2003) *Bone.* 32, 387-396

12. Chen, Y. J., Wurtz, T., Wang, C. J., Kuo, Y. R., Yang, K. D., Huang, H. C., and Wang, F. S. (2004) *J. Orthop. Res.* 22, 526-534

13. Wang, F. S., Wang, C. J., Chen, Y. J., Chang, P. R., Huang, Y. T., Sun, Y. C., Huang, H. C., Yang, Y. J., and Yang, K. D. (2004) *J. Biol. Chem.* 279, 10331-10337

14. Jun, H., Da, X., Aibin, Z., and Jiangnan, Z. (2006) *Prog. Biochem. Biophys.* 33, 452-457

15. Steinbach, P., Hofstädtler, F., Nicolai, H., Rössler, W., and Wieland, W. (1992) *Ultrasound. Med. Biol.* 18, 691-699

16. Tuckwell, D. S., and Humphries, M. J. (1993) *Crit. Rev. Oncol. Hematol.* 15, 149-171

17. Schneider, G. B., Whitson, S. W., and Cooper, L. F. (1999) *Bone.* 24, 321-327

18. Cloveer, J., Dodds, R. A., and Gowen, M. (1992) *J. Cell. Sci.* 103 (Pt 1), 267-271

19. Gronowicz, G. A., and McCarthy, M. B. (1995) *Endocrinology* 136, 598-608

20. Hynes, R. (2002) *Cell.* 673-687

21. Siebers, M. C., Walboomers, X. F., van den Dolder, J., Leeuwenburgh, S. C., Wolke, J. G., and Jansen, J. A. (2008) *J. Mater. Sci. Mater. Med.* 19, 861-868

22. Wang, L., Zhao, G., Oliives-Navarrete, R., Bell, B. F., Wieland, M., Cochran, D. L., Schwartz, Z., and Boyan, B. D. (2006) *Biomaterials* 27, 3716-3725

23. Yang, R. S., Lin, W. L., Chen, Y. Z., Tang, C. H., Huang, T. H., Lu, B. Y., and Fu, W. M. (2005) *Bone* 36, 276-283

24. Guan, J. L. (1997) *Matrix. Biol.* 16, 195-200

25. Tang, C. H., Yang, R. S., Liou, H. C., and Fu, W. M. (2003) *J. Bone. Miner. Res.* 18, 502-511

26. Cheng, S. L., Lai, C. F., Fausto, A., Chelliaiah, M., Feng, X., McHugh, K. P., Teitelbaum, S. L., Civitelli, R., Hruska, K. A., Ross, F. P., and Avioli, L. V. (2000) *J. Cell. Biochem.* 77, 265-276

27. Wang, F. S., Wang, C. J., Huang, H. J., Chung, H., Chen, R. F., and Yang, K. D. (2001) *Biochem. Biophys. Res. Commun.* 287, 648-655

28. Uslu, M. M., Bozdogan, O., Guney, S., Bilgili, H., Kaya, U., Olcay, B., and Korkusuz, F. (1999) *Bull. Hosp. Jt. Dis.* 58, 114-118

29. Schaden, W., Fischer, A., and Sailler, A. (2001) *Clin. Orthop. Relat. Res.* 387, 90-94
30. Wang, C. J., Weng, L. H., Chou, W. Y., Hsu, S. L., Ko, J. Y., Ko, S. F., and Huang, C. C. (2011) *Am. J. Sports. Med.*, doi: 10.1177/0363546511404201

31. Hofmann, A., Ritz, U., Hessmann, M. H., Alini, M., Rommens, P. M., and Rompe, J. D. (2008) *J. Trauma.** 65, 1402-1410

32. Seidl, M., Steinbach, P., Worle, K., and Hofstadter, F. (1994) *Ultrasonics.** 32, 397-400

33. Brakebusch, C., Bouvard, D., Stanchi, F., Sakai, T., and Fassler, R. (2002) *J. Clin. Invest.** 109, 999-1006

34. Damsky, C. H., and Ilic, D. (2002) *Curr. Opin. Cell. Biol.* 14, 594-602

35. Moursi, A. M., Globus, R. K., and Damsky, C. H. (1997) *J. Cell. Sci.** 110 (Pt 18), 2187-2196

36. Schlaepfer, D. D., Hauck, C. R., and Sieg, D. J. (1999) *Prog. Biophys. Mol. Biol.** 71, 435-478

37. Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M., and Weed, S. A. (2000) *Oncogene.** 19, 5606-5613

38. Schaller, M. D. (2001) *Biochim. Biophys. Acta.* 1540, 1-21

39. Nakayamada, S., Okada, Y., Saito, K., Tamura, M., and Tanaka, Y. (2003) *J. Biol. Chem.* 278, 45368-45374

40. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) *Nature** 377, 539-544

41. Owen, J. D., Ruest, P. J., Fry, D. W., and Hanks, S. K. (1999) *Mol. Cell. Biol.** 19, 4806-4818

42. Sieg, D. J., Hauck, C. R., and Schlaepfer, D. D. (1999) *J. Cell. Sci.** 112 (Pt 16), 2677-2691

43. Chaturvedi, L. S., Marsh, H. M., and Basson, M. D. (2007) *Am. J. Physiol. Cell. Physiol.** 292, C1701-C1713

44. Hatton, J. P., Pooran, M., Li, C. F., Luzzio, C., and Hughes-Fulford, M. (2003) *J. Bone. Miner. Res.** 18, 58-66

45. Lai, C. F., Chaudhary, L., Fausto, A., Halstead, L. R., Ory, D. S., Avioli, L. V., and Cheng, S. L. (2003) *J. Bone. Miner. Res.** 15, 1501-1509

46. Yee, K. L., Weaver, V. M., and Hammer, D. A. (2008) *IET. Syst. Biol.** 2, 8-15

47. Lee, D. Y., Yeh, C. R., Chang, S. F., Lee, P. L., Chien, S., Cheng, C. K., and Chiu, J. J. (2008) *J. Bone. Miner. Res.** 23, 1140-1149

48. Robinson, J. A., Chatterjee-Kishore, M., Cullen, D. M., Zhao, W., Li, C., Kharode, Y., Sauter, L., Babij, P., Brown, E. L., Hill, A. A., Akhter, M. P., Johnson, M. L., Recker, R. R., Komm, B. S., and Bex, F. J. (2006) *J. Biol. Chem.** 281, 31720-31728

49. Tzima, E., Pozo, S., Hartzell, S. J., Chien, S., Schwartz, M. A. (2001) *EMBO. J.* 20,4639–4647

50. Warden, S. J., Favaloro, J. M., Bennell, K. L., McMeeken, J. M., Ng, K. W., Zajac, J. D., and
Wark, J. D. (2001) *Biochem. Biophys. Res. Commun.* **286**, 443-450

59. Rubin, C. T., Gross, T. S., McLeod, K. J., and Bain, S. D. (1995) *J. Bone. Miner. Res.* **10**, 488-495

60. Chen, Y. J., Wang, C. J., Yang, K. D., Chang, P. R., Huang, H. C., Huang, Y. T., Sun, Y. C., and Wang, F. S. (2003) *Febs. Lett.* **554**, 154-158

61. Plotkin, L. I., Mathov, I., Aguirre, J. I., Parfitt, A. M., Manolagas, S. C., and Bellido, T. (2005) *Am. J. Physiol. Cell. Physiol.* **289**, C633-C643

62. Humphries, M. J., Travis, M. A., Clark, K., and Mould, A. P. (2004) *Biochem. Soc. Trans.* **32**, 822-825

63. Tarone, G., Hirsch, E., Brancaccio, M., De Acetis, M., Barberis, L., Balzac, F., Retta, S. F., Botta, C., Altruda, F., and Silengo, L. (2000) *Int. J. Dev. Biol.* **44**, 725-731

64. Klingbeil, C. K., Hauck, C. R., Hsia, D. A., Jones, K. C., Reider, S. R., and Schlaepfer, D. D. (2001) *J. Cell. Biol.* **152**, 97-110

65. Hsia, D. A., Lim, S. T., Bernard-Trifilo, J. A., Mitra, S. K., Tanaka, S., den Hertog, J., Streblow, D. N., Ilic, D., Ginsberg, M. H., and Schlaepfer, D. D. (2005) *Mol. Cell. Biol.* **25**, 9700-9712

66. Schlaepfer, D. D., Jones, K. C., and Hunter, T. (1998) *Mol. Cell. Biol.* **18**, 2571-2585

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**FOOTNOTES**

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2The abbreviations used are: ESW, extracorporeal shock wave; SW, shock wave; SD, Sprague-Dawley; RT-PCR, reverse transcription polymerase chain reaction; FAK, focal adhesion kinase; ERK, extracellular regulated protein kinases; siRNA, small interfering RNA; ECM, extracellular matrix proteins; BMPs, bone morphogenetic proteins; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CD29/Itgb1, Integrin β1; CD49e, Itga5, Integrin α5; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl -terrazolium bromide; DAPI, 6-diamidino-2-phenylinde

**FIGURE LEGENDS**

**FIGURE 1.** Optimal Intensity of ESW (10 KV for 500 impulses) accelerated osteoblasts adhesion. Data are presented as the mean ± SD in triplicate independent experiments (n = 3). The data has shown that at the time 2 h, 4 h, 6 h, 8 h, and 10 h after ESW treatment the number of adhesive osteoblasts were significant higher than those without ESW treatment. p<0.01 as compared to the control group at the same period. While the siItgb1 was added prior to ESW treatment, the promotion of adhesion of osteoblasts by ESW was inhibited. p<0.01 as compared to ESW group at the same period. p>0.05 as compared to the control group at the same period. It was also observed that siItga5 also inhibited the ESW-induced adhesion, although not as significantly as siItgb1 did. The promotion
of adhesion induced by ESW was abrogated while integrin α5 and β1 subunits were silenced. $p<0.01$ as compared to ESW group at the same period.

**FIGURE 2.** ESW promoted migration of osteoblasts as shown in transwell tests and wound healing assays. The promotion could be inhibited by both siItgb1 and siItga5. Primary cultured osteoblasts were divided into six groups randomly, those were cells with (SW) (Fig. 2 B1, B2, B3) or without 10KV for 500 impulses ESWT (Control) (Fig.2 A1, A2, A3), and with negative siRNA control (SW + siRNA control) (Fig.2 C1, C2, C3), siItga5 (SW+siItga5) (Fig. 2 E1, E2, E3), siItgb1 (SW+siItgb1) (Fig.2 D1, D2, D3) or both siItga5 and siItgb1 (Fig. 2 F1, F2, F3) for 6h prior to ESWT. Results from the wound healing assays (Fig.2 G) and transwell tests (Fig.2 H) were consistent. Data are presented as the mean ± SD (N = 6). a, $p<0.01$; b, $p<0.05$; c, $p>0.05$ as compared to the control group. #, $p>0.05$ as compared to ESW group. Bar=100 μm.

**FIGURE 3.** ESW-induced elevations of mRNA level of α5 and β1 integrins of osteoblasts, peaking at 1 h (Fig. 3B). The specific inhibitors for signal transduction pathways had no influence on the integrins expression (Fig. 3C). Representative electrophoretic image were depicted respectively (Fig. 3 A, C). The osteoblasts were harvested to extract total RNA 0.5, 1, 2, 4, 8, and 12 h after 500 impulses at 10 KV shock wave treatment. The cells without ESWT were run as control groups. After standardization of housekeeping gene expression, equal amount of cDNA from each sample were subjected to 36 cycles to amplify Itga5 and Itgb1 mRNA expressions. The values of the control group were normalized to 100%. a, $p>0.05$; b, $p<0.01$; c, $p<0.05$ as compared to the control group at certain time period. In addition, several signal transduction pathway inhibitors were added to the samples for 1 h prior to ESWT. 2 h after ESWT the samples were collected to extract RNA and analysis whether the Itga5 and Itgb1 mRNA were influenced by signal pathway inhibitors listed above. Our data showed that no influence on the expressions of Itga5 or Itgb1 mRNA were observed under the conditions with or without inhibitors ($p>0.05$).

**FIGURE 4.** ESW enhanced integrin α5 and β1 subunit proteins production in 2 h according to the data from flow cytometry analysis and western blotting. For flow cytometry, osteoblasts from experimental groups and the control group were stained with PE-conjugated anti-rat Itga5 or Itgb1 antibody under the guidance of the manufacturer. It’s shown that both of Itga5 and Itgb1 proteins increased significantly in 2h in the experiment group (Fig.4 A). *, $p=0.021$, #, $p<0.01$ as compared to the blank control group. Samples with or without ESWT and those associated with siItga5 and/or siItgb1 prior to ESW were subjected to RIPA lysis. Western blotting was applied to analysis itga5 and itgb1 expression levels changed in the protein extractions. The same results were shown as those indicated from flow cytometry (Fig.4 B, C). In addition, the data from the ESW plus siRNA groups indicated that the siRNA reagents for both Itga5 and Itgb1 were effective (Fig. 4 B, C). a, $p<0.05$, b, $p<0.01$ as compared to the blank control group. #, $p<0.01$ as compared to ESW group. Results are presented with mean values ± SE calculated from four paired triplicate experiments.

**FIGURE 5.** The evaluations of phosphorylation levels of focal adhesion kinase surrounding Tyr397, Tyr576/577 and Tyr925. After experiment groups direct exposure to 10KV for 500 impulses ESWT, we collected the extracts at 0.5, 1, 2, 4, or 8 h. Samples without ESWT were set as the control group. Then the extracts were quantified in triplicate using western blotting and normalized by β-actin expression (Fig. 5A). A marked elevation of FAK phosphorylation at Tyr397 peaking at 4 h was observed, and we also observed a slight increase of FAK phosphorylation at Tyr925 in 2 h after ESWT. ESW had no influence on the expression of FAK phosphorylation at Tyr576/577 (Fig. 5A). A
representative electrophoretic image of study on FAK phosphorylation at Tyr397 influenced by siRNAs of integrins is also depicted (Fig. 5B). 4 h after the optimal dose of ESWT, a decline in the expression of phosphorylated FAK at Tyr397 was observed in the group with siRNA pretreatment. However, total protein expression levels of FAK were not affected by silencing of Itga5 and/or Itgb1 (Fig.5B). Moreover, several specific cell signal pathway inhibitors, namely PD98059, U0126, LY294002, SB203580, SP600125, H-89, AG490, were also added to the osteoblasts for 1h respectively before ESWT. Both bands of total FAK and β–actin showed equal amounts of proteins were subjected to protein electrophoresis (Fig. 5C). The data indicated that both PD98059 and U0126, different from other inhibitors listed, inhibited ESW-induced FAK phosphorylation at Tyr397 (Fig. 5D). Data represent the mean±SE in triplicate independent experiments (n = 3). The values of the control group were normalized to 100%. a, p>0.05; b, p<0.05; c, p<0.01 as compared to the control group at the same time. d, p<0.01 ; e, p>0.05 versus ESW group.

FIGURE 6. Enhancement of β-catenin activity by ESW (500 impulses at 10KV) stimulation after elevation of integrin α5 and β1 expression. ESW raised β-catenin phosphorylation in 3 h (Fig. 6A). Erk1/2 inhibitor U0126 did not alter the activation of β-catenin (Fig. 6B). Cytosolic extracts of osteoblasts treated with ESW in the presence of U0126 (with final concentration of 20 μM) for 60 min prior to ESW were subjected to western blotting. Phosphorylated β-catenin and β-catenin were probed with anti-p-β-catenin and β-catenin primary monoclonal antibodies, respectively. (Fig. 6C) Note that in comparison with the control, ESW exposure markedly elevated the activation of β-catenin. Further studies on the relationship between expressions of integrins and β-catenin by transfection shown that knocking out integrins led to baseline level expression of activation of β-catenin (Fig. 6B, 6D). (Fig. 6D) Summary of the results (mean ±SE, n=4, triplicate in each experiment). a, p>0.05 as compared to the control at the same period. b, p<0.01 as compared to the control at the same period. c, p>0.05 as compared to the ESW group. d, p<0.01 as compared to the ESW group.

FIGURE 7. ESW activated ERK phosphorylation after treatment. Erk1/2 phosphorylation was increased in 2h (Fig. 7A, 7C). The higher production of phosphorylated Erk1/2 persisted for 4h (Fig. 7A, 7C). Osteoblasts were serum deprived for 12 h before treatment with 10KV for 500 impulses in the absence or presence of U0126, siRNAs for the indicated time. Western blotting analysis was performed with antibodies against ERK and its phosphorylated forms (p-ERK) (Fig. 7B). (Fig. 7C, 7D) Summary of the results (mean ±SE, n=4, triplicate in each experiment). a, p>0.05 as compared to the control at the same period. b, p<0.01 as compared to the control at the same period. c, p>0.05 as compared to the control group. d, p<0.01 as compared to the ESW group.

FIGURE 8. Hypothetical model elucidating the regulation of phosphorylated FAK expression through integrin alpha5 and beta1 mediated MEK-ERK1/2 dependent pathway after ESWT. ESW directly stimulate integrin alpha5 and beta1 mRNA expressions inside the cell nucleus, then the integrins protein expression is increased. Inteagins induce MEK1/2 to phosphorylates ERK1/2, then the activated ERK1/2 phosphorylates FAK, and enhance its binding to the corresponding sites located in the adhesion sites, finally resulting in the enhancement of adhesion and migration. Wnt/beta-catenin signal pathway may also be involved in ESW-induced integrin-FAK signaling. The conformational activation of existing integrin alpha5/beta1 complexes at the osteoblasts surfaces may also be an additional potential mechanism which requires our further study.
Figure 1

![Graph showing cell number over time for different conditions: Control, 10KV (500), SW+sihgb1, SW+silga5, SW+silga5&bb1. The graph plots cell number per filed against time in hours, with data points and error bars indicating variability.](image-url)
Figure 2

Shock Wave-induced Osteoblast Adhesion and Migration

G

Migration distance

H

Migration Number (per field)

Control ESWSiRNA Control slitgb1 slitgα5 & b1 slitgα5 & b1

Control ESWSiRNA Control slitgb1 slitgα5 & b1 slitgα5 & b1

ESW

ESW

18
Figure 3

A

| Time (hr) | 0.5 | 1   | 2   | 4   | 8   | 12  |
|----------|-----|-----|-----|-----|-----|-----|
| Integrin α5 |     |     |     |     |     |     |
| Integrin β1 |     |     |     |     |     |     |
| GAPDH     |     |     |     |     |     |     |
| Shock Wave| -   | +   | -   | +   | +   | -   |

B

Relative Expression

| Time (hrs) | 0.5 | 1   | 2   | 4   | 8   | 12  |
|------------|-----|-----|-----|-----|-----|-----|
| Igα5       | b   | a   | b   | b   | b   | b   |
| Igβ1       | b   | b   | b   | b   | b   | b   |

C

| Integrin α5 |       |       |
|-------------|-------|-------|
| Control     |       |       |
| ESW         |       |       |
| Integrin β1 |       |       |
| GAPDH       |       |       |
| Shock Wave  |       |       |
| PD98059     |       |       |
| U0126       |       |       |
| LY294002    |       |       |
| SB203580    |       |       |
| SP600125    |       |       |
| H-89        |       |       |
| AG490       |       |       |

D

1h after shock wave or not

Legend:
- b
- a

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Figure 4

A

Fluorescence Intensity

Integrin β1
Integrin α5

0 0.5 1 2 4 8 12

Time (hrs)

B

Fold stimulation/control

Control ESW SW+sttGas

C

Fold stimulation/control

Control ESW SW+sttGbb1

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Figure 5

A

| Protein | ESWT or not for 4hrs |
|---------|---------------------|
| p-FAK   |                     |
| FAK     |                     |
| β-actin |                     |

Vehicle Control, ESW, ESW+Sl1ga5, ESW+Sl1ga5-m1

B

C

4hrs after treatment

p-FAK\text{\textsuperscript{Y397}}
FAK
β-actin

Vehicle Control, PD98059, U0126, LY294002, SB203580, SP600125, H-89, AG490

ESW

D

| Protein | Fold stimulation/control |
|---------|--------------------------|
| p-FAK   | c, d                     |
| FAK     | c, d                     |
| β-actin | c, d                     |

Control, ESW, PD, U0126, LY, SB, SP, H-89, AG490

ESW

Shock Wave
Figure 6

A  Hours  1  2  3  4  
ESW  -  +  -  +  +  
p-β-catenin  
β-catenin  
β-actin  

B  

C  

D  

Vehicle  Control  ESWT  
U0126  
siltga5  
siltgb1  
siltga5+b1  

Fold stimulation/Control

Time (hrs)

1h  2h  3h  4h
Figure 7

A

| Hours | ESW | Control |
|-------|-----|---------|
| 1     |     |         |
| 2     |     |         |
| 3     |     |         |
| 4     |     |         |

B

|          | p-Erk1/2 | Erk1/2 | β-actin |
|----------|----------|--------|---------|
| ESW      |          |        |         |
| Control  |          |        |         |

C

![Bar chart showing fold stimulation/Control over time (hrs)]

D

![Bar chart showing fold stimulation/Control with different treatments and SW indicated]
Optimal Intensity Shock Wave Promotes the Adhesion and Migration of Rat Osteoblasts via Integrin \( \beta 1 \)-mediated Expression of Phosphorylated Focal Adhesion Kinase

Jian-kun Xu, Hong-jiang Chen, Xue-dong Li, Zhong-lian Huang, Huan Xu, Hai-long Yang and Jun Hu

*J. Biol. Chem. published online May 31, 2012*

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