Short Wave Enhances Mesenchymal Stem Cell Recruitment through Hypoxia-Inducible Factor-1 Signaling in Fracture Healing

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Abstract

Background As a type of high-frequency electrotherapy, a short wave can promote the fracture healing process; yet, its underlying therapeutic mechanisms still remain unclear. Purpose To observe the effect of short wave on mesenchymal stem cell (MSC) homing and its mechanisms associated with fracture healing. Materials and Methods For in vivo study, the effect of Short-Wave therapy in relation to fracture healing was examined in stabilized femur fractures model of 40 SD rats. Radiography was used to analyze the morphology and micro-architecture of the callus. Additionally, fluorescence assays were used to analyze the GFP-labeled MSC homing after treatment in 20 nude mice with a femoral fracture. For in vitro study, osteoblast from newborn rats simulated fracture site was first irradiated by the Short-Wave; siRNA targeting HIF-1 was used to investigate the role of HIF-1. Osteoblast culture medium was then collected as chemotaxis content of MSC, and the migration of MSC from rats was evaluated using wound healing assay and trans-well chamber test. The expression of HIF-1 and its related factors were quantified by q RT-PCR, ELISA, and Western blot. Results Our in vivo experiment indicated that Short-Wave therapy could promote MSC migration, increase local and serum HIF-1 and SDF-1 levels, induce changes in callus formation, and improve callus microarchitecture and mechanical properties, thus speeding up the healing process of the fracture site. Moreover, the in vitro results further indicated that Short-Waves therapy upregulated HIF-1 and SDF-1 expression in osteoblast and in the medium, as well as the expression of CXCR-4, β-catenin, F-actin and phosphorylation levels of FAK in MSC. On the other hand, the inhibition of HIF-1α was significantly restrained by the inhibition of HIF-1α in osteoblast, and it partially inhibited the migration of MSC. Conclusions These results suggested that short wave could increase HIF-1 in callus, which is one of the crucial mechanisms of chemotaxis MSC homing in fracture healing.

Introduction

Fracture healing is a biologically optimized process. Mesenchymal stem cell (MSC) are multipotent stromal cells that can differentiate into multiple cell types such as chondrocytes and osteocytes and thus have an important role in the bone healing process [1]. During the course of fracture healing, circulating MSC can receive signals from the injured tissue and migrate to the damaged sites. Over the last decade, several strategies for fracture healing have been investigated, including the stimulation of endogenous stem cell populations from the mature body [2, 3]. An alternative strategy is the use of exogenous stem cells, which can be obtained from connective tissues, and are controlled by the expression of molecules during MSC expansion, such as CXCR4 and complement 1q (C1q) [4, 5] or by certain chemicals [6], such as valproate or lithium that are involved in MSC homing and can trigger the expression of certain key factors. Since the mobilization is a kind of directional migration, both endogenous and exogenous MSC recruitment is related to the condition of the fracture site. Currently, researchers are focusing on improving the condition of MSC recruitment by using biological agents. Clinical-standard platelet products loaded membranes [7] and naringin [8] successfully support MSC colony formation and promote MSC migration in vitro. Furthermore, MSC migration can be improved under hypoxic conditions. Previous studies have
suggested that hypoxic conditions decrease the MMP secretion and increase CXCR4 expression \cite{9, 10}. However, the effect of physical agency therapy on MSC migration has not received adequate attention.

Short-Wave therapy is a type of high-frequency electrotherapy, which can promote the fracture healing process \cite{11, 12}. At these high frequencies, the electromagnetic energy is converted to thermal energy, which can induce heat (temperature over 40°C) to the treated area of the body, \cite{13} where the heating process affects blood flow \cite{14} and decreases pain \cite{15}. Accumulating evidence has indicated that both hypoxia and hypoxia-driven angiogenesis can be regulated by thermal therapy \cite{16}. The hypoxia-inducible factor (HIF) involved signaling pathway is activated under hypoxia. HIF protein, especially HIF-1α, has been associated with MSC migration and differentiation \cite{17}. As a key transcriptional regulator, HIF-1 can regulate the expression of multiple cytokines, such as stromal cell-derived factor 1 (SDF-1) \cite{10, 18}, and focal adhesion kinase (FAK) \cite{19}, which has a central role in the adaptation of MSC to hypoxia \cite{20, 21}.

The present study explored the effects of Short-Wave therapy on HIF-1 expression in fracture and MSC recruitment from peripheral blood during fracture healing. Accordingly, we applied Short-Wave treatment on the fracture in the animal model and assessed the healing of fracture using the radiographs. In addition, in vivo bioluminescent assays and callus histo-immunofluorescence were applied to assess the MSC migration. HIF-1 and related factors were also detected. siRNA targeted HIF-1α was transfected to clarify its effect in Short-Wave therapy (Figure 1). The aim of this study was to investigate the effect of Short-Waves therapy on MSCs recruitment and explore the underlying mechanisms of Short-Waves therapy on fracture healing.

**Materials And Methods**

**MSC Isolation and Identification**

MSC was isolated from femurs of 3 weeks old male SD rats, as previously described \cite{22}. Before performing any experiment, cells were passaged for 3 to 6 times. The surface marker expressions of MSC, including CD90, CD44, CD34, CD45, and CD11b/c, were analyzed by flow cytometry assay, as described in the previous study \cite{23, 24}.

**Animal Grouping**

A total of 40 male SD rats, 8-12 weeks old, weighing 350-500 g, were obtained from Laboratory Animal Center of Dalian Medical University, China. Rats were housed in an environment with a temperature of 22 ± 1 °C, relative humidity of 50 ± 1%, and a light/dark cycle of 12/12 hr.

After one week of adaptation, 40 SD rats were accurately weighed and randomly divided into two groups (n=20/group): Short-Wave treatment group (SW) and control group (Con). All rats underwent surgery in order to establish the femoral shaft fracture and intramedullary fixation model.
For *in vivo* bioluminescent assays to the MSC homing, 20 nude mice of femoral shaft fracture were injected MSC labeled by the GFP (MG), and then randomly divided into two groups, including a Short-Wave treatment group (MG+SW) and control group (MG) (n=10/group).

**Stabilized Fracture Model**

Stabilized right femur fractures were established by intramedullary fixation in 8 - 12-week-old male SD rats. A 0.25-mm titanium alloy pin was inserted inside the medullar canal of the femur. A three-point bending device was then used to produce closed fractures of the femoral shaft with a standardized force [25]. In 20 male nude mice, transverse osteotomy with a 1 mm bone fracture was created in the middle of the right femur in the same way. Bupremorphine was subcutaneously administered (0.5 mg/kg) for pain control.

**Cell Injection**

All the 20 nude mice received injections with $5 \times 10^6$/50μl GFP-labeled MSC (C57BL/6 mouse strain) (CYAGEN Company, China) three days after the femoral fracture. Cells were injected by tail vein using a microinjector.

**Short-Waves Treatment**

Three days after the operation, Short-Wave therapy was applied in a Short-Wave treatment group. Briefly, animals were fixed in an apparatus so that they could not turn around. The treatment regimen was applied to the right thigh. The shortwave generator (Curapuls 970, Netherlands) operated at a frequency of 27.12MHz. A micro-heat continuous-wave Short-Wave exposure for 10 min was applied once a day. A similar procedure was carried out in the control group; however, the device was turned off.

For the *in vitro* study, the isolated rat osteoblasts in treatment group underwent Short-Wave irradiation. The protocol of Short-Waves therapy irradiation on cells was based on a previous study [26]. The two non-contact applicators were of perpendicular contraposition and 10 centimeters away from each other. The cell culture flask was placed in the middle of the applicators. A micro-heat continuous-wave Short-Wave exposure for 90 min was applied twice on day 1 in the open air at 37°C. The control group received a sham Short-Wave treatment by turning off the Short-Wave generator. On day 2, the culture medium from each group was collected for HIF-1 and SDF-1 protein level analysis, and the medium was used in MSC cell wound healing assay and trans-well chamber test.

**Radiographic Assessment**

The fracture healing was assessed by plain anteroposterior radiographs at day 7, 14, 21 and 28. The same X-ray machine and settings were used for all radiographs every seven days after fracture. Bone defects were analyzed using Xsys software.
Micro-CT scanning was performed to assess callus. Quantification for the volumes of the bony calluses as determined as previously described \cite{27, 28}. The region of interest (ROI) was set within 800 μm (50 slices) around the defect edge. We applied a fixed threshold of less than 330 for new calcified cartilage or unmineralized cartilage. Three D microstructural image data were reconstructed by Inveon Research Workplace software. After 3D reconstruction, bone volume fraction (BV/TV) were automatically determined to confirm fracture healing.

**In Vivo Fluorescence Assays**

Ten nude mice per group received anesthesia and were examined by the IVIS imaging system at day 7 after the surgery. The identical parameter settings were used for all samples: f number: 1, field of view: 22, binning factor: 18, luminescent exposure (seconds): 10. The IVIS imaging examination and rates of photons were calculated and performed according to methods reported in a previous study \cite{29}.

**Histological Analysis and Immunofluorescence Imaging**

Twenty nude mice (MG: n=10, MG+SW: n=10) were sacrificed for histological analysis and immunofluorescence imaging at day 28 after operation. The femoral bone of nude mice and rats were sectioned, preserved, decalcification, and embedded in paraffin along the longitudinal axis. For morphological analysis, 5 μm slices were sectioned, deparaffinized, and stained using hematoxylin and eosin. The immunofluorescence staining was performed as previously described \cite{30}. Tissue slides of nude mice were stained with antibodies (A0516, Beyotime, China) against GFP to track exogenously delivered MSC labeled by GFP. A four-channel confocal laser scanning microscope was used to analyze all the samples. GFP-positive cells were automatically counted in five fields on × 100 magnification by ImageJ software. Sixteen rats were sacrificed for histological analysis at day 14 (n=4/group) and day 28 (n=4/group) after operation. It is on the same time end points for rats and mice.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

The plasma of SD rats received by heart puncture at day 3, 7 and 14 after operation (n=4/group/time point). The concentration SDF-1 in plasma of SD rats was analyzed using citrulline ELISA kit (CSB-E13414r, Cusabio Biotech, China). In culture media of osteoblasts (SD rat strain), the concentration of HIF-1 (SEA798Ra, USCN, China) and SDF-1 content was also detected using the ELISA kit.

**Quantitative Reverse Transcription-Polymerase Chain Reaction (q RT-PCR)**

Callus of sacrificed SD rats was collected and snap-frozen in liquid nitrogen at day 3, 7 and 14 after operation (n=4/group/time point). RNA isolation and subsequent cDNA synthesis (Bio-Rad, 170-8891) were performed as previously described \cite{31}. A total of 50 ng of cDNA was amplified with custom-designed q RT-PCR primers (Table 1) (Thermo Fisher Scientific). A melt curve was generated to analyze the purity of amplification products. The expression levels of mRNA were normalized to the average of β-actin. Relative expression of mRNA was evaluated by using the comparative CT method (ΔΔCt) \cite{32}.
Osteoblasts Culture and Identification

Osteoblasts were obtained from calvaria of one-day-old neonatal SD rats using the method of collagenase-pancreatic enzyme digestion as detailed in reference [33]. After two passages, alkaline phosphatase staining was utilized to identify the osteoblast cells (Figure 2c).

Small Interference RNA Transfection

We inhibited HIF-1α expression by siRNA in osteoblasts (SD rat strain). Synthetic siRNA oligonucleotide specific for HIF-1α (NM_024359) (59 to 39: UUUAUCAAGAUGGGAGCUCTT) and nontargeting siRNA were obtained from Sangon (Shanghai, China).

Osteoblasts Culture Medium

Osteoblasts (SD rat strain) were seeded in 6-well plates for 24 h to 80–90% confluence. Three kinds of interventions were provided: 1) Short-Wave continuous irradiation for 180 min; 2) 200 μmol/L CoCl_2-stimulated hypoxia condition in fracture; 3) siRNA inhibition of HIF-1α. Eight kinds of osteoblasts culture mediums were obtained from single or combined interventions for the follow-up experiment (Figure 1).

Wound Healing Assay

MSC (SD rat strain) was cultured on six-well plates to confluence and monolayers and wounded with a sterile 200 μL pipette tip. The cultures were washed with PBS to remove detached cells and stimulated with 8 kinds of osteoblasts culture medium fluid 1.5 ml in each well. Photographs were collected at 0, 24, and 48 hours.

Trans-well Chamber Test

The tests were performed in Boyden Chambers (Corning, 3422, Lowell, MA). Eight kinds of osteoblasts (SD rat strain) culture medium was seeded in the bottom chamber. The top chambers filled with MSC (SD rat strain) starved overnight were inserted. Twenty-four hours later, inserts were removed and washed. The cells that migrated to the bottom side were accumulated.

Western Blotting

Osteoblasts and MSC cells (both are SD rat strain) lysates were prepared, and western blots were performed as previously described [34]. The 30 μg of protein was loaded in each lane for reducing electrophoresis. Primary antibodies were used for β-actin (Sigma; A2228) diluted 1:10000, HIF-1α (Gene Tex; GTX127309) diluted 1:2000, SDF-1 (CST; 3740) diluted 1:1000, FAK (Sangon Botech; D160324) diluted 1:3000, phosphor-FAK (Sangon Botech; D160324) diluted 1:2000, F-actin (Abcam; Ab205) diluted 1:2000, β-catenin (wanleibio; WL0962a) diluted 1:500 and CXCR4 (Abcam; ab124824) diluted 1:1000.

Statistical analysis
Statistical analysis was performed by SPSS 22.0 for Windows (SPSS, Chicago, USA). The results are shown as the mean value ± standard deviation. The differences between groups were analyzed by t-test or analysis of variance (ANOVA). Two-tailed $P$ values were computed, and $P<0.05$ was considered to be statistically significant.

**Results**

**MSC identification**

After 3 to 6 passages, MSC extracted from SD rats were in fusiform shape, arranged in bundles or whorls (Figure 2A). The results of flow cytometry demonstrated that over 90.2% and 83.5% of the mononucleated cell colonies isolated from the bone marrow of SD rats were positive for fibroblastic marker CD90 and MSC marker CD44, respectively; and negative for hematopoietic lineage markers (CD45 and CD11b/c) and the endotheliocyte lineage marker CD34 (0.36%, 0.29%, and 0.93%, respectively) (Figure 2B).

**Short-Wave treatment enhancing fracture healing**

To examine the role of Short-Wave treatment and MSC during fracture repair, we generated a closed femoral shaft fracture model. Radiographic examinations showed a normal bone healing process in all rats. The radiographic analysis demonstrated that the SW group showed better fracture healing than the Control group (Figure 3A). In addition, the quantitative measurement density of the fracture gap showed that SW groups were significantly larger than the Control group at day 14 ($P = 0.033$) and 21 ($P = 0.026$). Nevertheless, no significant difference was found between day 7 ($P = 0.152$) and 28 ($P = 0.163$).

MicroCT analysis showed greater amount of bony callus was found in the SW groups than in the Control group. Three-D reconstructed microCT images at day 14, and 28 post-fracture are shown in Figure 3C. The bone volume fraction (BV/TV) in the SW group was significantly higher at day 14 ($P = 0.045$) and day 28 ($P = 0.049$). (Figure 3D).

**Short-Waves therapy treatment promoting MSC homing in vivo**

In order to observe the MSC homing to the fracture, nude mice were injected with MSC expressing the GFP report gene. IVIS imaging system recorded radiant efficiency in the leg at day 7 post-operation. Higher cell migration was more common in animals receiving Short-Wave irradiation with total radiant efficiency $[p/s] / [\mu W/cm^2]$ compared to those not exposed to wave irradiation ($1123.1 \pm 116.0$ vs $878.2 \pm 79.2$, $P = 0.023$; Figure 4A & 4B). Besides, GFP distribution analysis showed that MSC was not uniformly distributed throughout the body and had a tendency to migrate to organs such as lung, liver, and tail, which was observed in both groups.

Next, we quantified the homing of exogenously delivered MSC to the fracture site by using immunofluorescent staining and histological analysis. All the nude mice were sacrificed, and the callus
tissue was prepared for immunofluorescence staining at 28 after the operation. Significantly more GFP-MSC cells were counted in the femora of animals treated with Short-Wave irradiation (GFP-positive cells per 100× field 56.3 ± 26.8 VS 184.3 ± 21.2, \( P = 0.003 \); Figure 4C & 4D).

**Short-Wave therapy changes the gene expression in callus**

The results above indicated that Short-Wave therapy enhanced MSC migration to the fracture. Low oxygen occurs in the fracture following bony injury\[^{[35]}\]. Therefore, the involvement of HIF-1 and its related factors (SDF-1, F-actin, and FAK) were investigated after Short-Wave therapy using q RT-PCR. No significant differences in HIF-1 were detected between the two groups at day 3 (\( P = 0.706 \)) and 14 (\( P = 0.602 \)) post-therapy, yet its expression significantly increased in the SW group at day 7 compared to the control group (\( P = 0.002 \); Figure 5A). Moreover, compared to the control group, the expression of SDF-1 increased in the SW group at day 7 (\( P = 0.044 \)) and 14 (\( P = 0.016 \)). In addition, a significant increase of F-actin was found at day 7 in SW group (\( P = 0.038 \)), while there was no difference at day 3 (\( P = 0.728 \)) and day 14 (\( P = 0.835 \)). For FAK, it seemed that Short-Wave therapy led to increases at days 3 and 7; nevertheless, there was no statistical difference between the two groups at day 3 (\( P = 0.051 \)), day 7 (\( P = 0.142 \)) and day 14 (\( P = 0.287 \)).

In addition, SDF-1 in plasma was detected by ELISA (Figure 5B). After 7 days of treatment with Short-Wave therapy, SDF-1 was increased by 1.5 fold compared to the control group (\( P = 0.044 \)).

**Short-Wave therapy promotes HIF-1 expression in osteoblasts in vitro**

We evaluated the HIF-1 in the culture medium fluid of osteoblasts under a Short-Wave in vitro by ELISA. The results showed that the expression of HIF-1 significantly increased following Short-Wave treatment (\( P = 0.047 \)), especially under CoCl\(_2\) stimulated hypoxic conditions (\( P = 0.018 \)). Next, we collected the osteoblasts and measured the expression of HIF-1 by western blot. We found higher expression of HIF-1 after Short-Wave irradiation, both under normoxia (\( P = 0.021 \)) and hypoxic condition (\( P = 0.046 \)) (Figure 6A & C). Also, the expression of SDF-1 in the medium improved following Short-Wave therapy under normoxia (Figure 6B & D). The HIF-1 expression was inhibited by HIF-1\(\alpha\)-SiRNA. The expression of SDF-1 in osteoblasts and its concentration in culture media of osteoblasts was decreased after treating cells with HIF-1\(\alpha\)-SiRNA.

**The migratory effect of HIF-1 on MSC under Short-Wave irradiation**

As shown in Figure 7, MSC cultured in normoxia with the medium fluid of SW irradiated osteoblasts infected vector for 24h, and at 48h it showed higher migration compared to control cells cultured in the medium fluid without SW irradiation (\( P < 0.05 \)). The same tendency was also seen in simulated hypoxic conditions at 24h (\( P < 0.05 \)), but not at 48h. The results of analysis see in Table 2.

Additionally, to determine the effects of the HIF-1 in Short-Wave treatment on MSC migration, we used siRNA to inhibit HIF-1 in cultured osteoblasts. Briefly, siRNA reversed the above effect (Figure 7, Table 2).
Migration-correlation factor expression in MSC

Next, we examined whether the medium fluid of osteoblasts irradiated by Short-Wave could affect the gene expression of con-cultured MSC. The gene expression of CXCR4, β-catenin, FAK, and F-actin in MSC were analyzed using qRT-PCR (Figure 8A) and western blot (Figure 8B & C). As a receptor, CXCR4 was increased under normoxia ($P = 0.011$), especially in hypoxic conditions ($P = 0.015$). In addition, it decreased under HIF-1 inhibited medium both under normoxia ($P = 0.900$) and hypoxia ($P = 0.046$). The Short-Wave-irradiation medium also provided a positive effect on the expression of β-catenin ($P = 0.013$) and F-actin ($P = 0.031$) in MSC under hypoxia; yet, no statistical difference was observed under normoxia (each $P > 0.05$). The rise of F-actin and β-catenin were restrained since osteoblasts were transplanted in HIF-1 siRNA under normoxia and hypoxia (each $P > 0.05$). Additionally, no statistical difference was observed in FAK between the two groups (each $P > 0.05$). Nevertheless, phosphorylation levels of FAK were much higher in the Short-Wave group under normoxia ($P = 0.040$), which also decreased since MSC cultured in the medium of HIF-1 restrained osteoblasts (each $P > 0.05$).

Discussion

The clinic application of high-frequency treatment can accelerate the resolution of haematoma and fracture healing. Nevertheless, the underlying mechanisms are not fully understood. Previously, it has been reported that fracture healing is associated with an increase in calcium phosphate mineral salt deposition, which occurs 2 to 4 weeks after injury [36, 37]. In this study, we found that high-frequency Short-Wave irradiation could promote the healing process, including the promotion of bony callus formation and the MSC migration. These healing characteristics were observed 1 to 3 weeks after the injury.

MSC has a vital role in the process of fracture healing [38]. The early stage of healing is mainly mediated by the biological actions of MSC [38]. Preclinical studies have suggested that transplanted primary or endogenous MSC can migrate to the fracture site after injury and restore the fracture callus volume and biomechanical properties of the bone in mice [39]. Injection of MSC by intravenous or intra-arterial injection is commonly used to treat the bone injury in mice. Yet, systemic administration has low therapeutic efficacy because only a small percentage of MSC can reach the target tissue [40, 41]. Hence, one of the solutions to improve therapeutic efficacy is to promote MSC migration and homing.

So far, only a few studies have focused on investigating the MSC migration in the electric field, especially in the direct-current electric field. Griffin and Zimolag have observed that in an external direct current electric field, MSC directly migrated toward the cathode [42, 43]. Moreover, Banks et al have discovered that cells display a highly elongated phenotype conversion and consistent perpendicular alignment to the electric field vector accounting for the effects of electric field strength [44]. Furthermore, Liu et al and Zhao and his team found that the applied electromagnetic field might be useful to control or enhance the migration of MSC during bone healing [45, 46]. Nevertheless, as a kind of high-frequency electrotherapy, areas of exposure could not be polarized by Short-Wave therapy, i.e., the migration of MSC might not be associated with field stress. Short-Wave therapy is generally used for thermal effects that provoke or
enhance cellular activity resulting from energy-absorbing in oscillating electrical fields [47]. Previous studies have shown that thermal stress can modulate some molecules affected by hypoxia [48, 49]. For example, HIF-1α, a transcription factor that is positively correlated with the acute temperature changes in organs, such as the brain, liver, kidney, and gonad tissues, can regulate the cellular response to hypoxia stress [50] that is significantly increased in osteoblasts [51]. In the current study, we found an increased expression of HIF-1 in callus and osteoblasts exposed to Short-Wave therapy. Consequently, we assume that the regulation of the hypoxia pathway directs MSC behavior to promote tissue regeneration. High expression of HIF-1a and BMP-2 promote the migration of MSC to the bone defect area [52]. Therefore, in a number of tissue engineering strategies, HIF-stabilized biological materials are used to improve MSC migration and survival [53, 54]. In this study, it was found that exogenous MSC homing increased in fractures exposed to Short-Wave irradiation in vivo. Besides, we discovered that the MSC migration was improved by the cultural medium of osteoblast exposed to Short-Waves therapy irradiation in vitro, which could be further improved in stimulated hypoxia in fracture and restrained by inhibiting HIF-1α expression of culture osteoblasts. Therefore, we believe that HIF-1 is a key factor in the healing process activated by short-wave treatment. However, the effect of Short-Wave treatment on fracture healing of HIF-1a inhibitor was not observed in vivo, which is a limitation of the current study. Although the results of tests in vitro and in vivo were of mutual corroboration, the conclusion seemingly was not solid enough. A research of HIF-1a conditional knockout rats of fracture with Short-wave therapy will be conceived in the future.

The homing of CXCR4-positive progenitor cells in circulation is up-regulated since the increase of HIF-1 induces SDF-1 expression. As a cell growth-stimulating factor, SDF-1 belongs to the CXC subfamily of chemokines [55, 56]. SDF-1 can activate CXCR4, a G protein-coupled receptor [57]. Progenitor cell recruitment to injured tissues can be prevented if SDF-1 in ischemic tissue or CXCR4 on circulating cells were blockaded [55, 58]. In the bone marrow, discrete regions of the anoxic chamber have increased SDF-1 expression and progenitor cell tropism [59]. Over the last ten years, numerous studies have confirmed that SDF-1/CXCR4 has a pivotal role in the biologic and physiologic functions of MSC [60]. In this study, we found increased expression of SDF-1 in callus and blood exposed to irradiation, which serves as a chemoattractant to recruit CXCR4-expressed MSC both in circulation and fracture site. FAK is the downstream protein kinase in the CXCR4 signaling cascade, which can integrate extracellular signaling and cellular migration [61]. Additionally, the cytoskeleton network partly detects the biomechanical characterization of living cells. It has been suggested that F-actin transmutation affects cell morphology and migration [62]. In the current study, the expression of F-actin and phosphorylated FAK increased in the medium of cultured osteoblasts under Short-Wave irradiation (Figure 9). The migration of MSC was improved on the molecular level when it was cultured in the medium of osteoblast irradiated by Short-Wave. Therefore, Short-Waves treatment improved the local chemotaxis for MSC, which might be the underlying mechanism.

**Conclusion**
Short-Wave therapy could increase HIF-1 in callus, which is one of the crucial mechanisms of chemotaxis MSC homing in fracture healing.

Declarations

Ethical Review Committee Statement

All animal studies (including the rats and mice euthanasia procedure) were done in compliance with the regulations and guidelines of Affiliated Zhongshan Hospital of Dalian University institutional animal care.

Consent for publication

Not applicable.

Availability of data and material

Authors do not wish to share the data for the moment since the further study is in progress.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

Conception and design (DMY, CC); analysis and interpretation of the data (CC, QZ); drafting of the article (DMY QWW); critical revision of the article for important intellectual content (CC, SL, HWL); final approval of the article (all authors); statistical expertise (QZ, HWL); obtaining funding (DMY); administrative, technical, or logistical support (CC, QWW).

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**Abbreviations**

MSC: mesenchymal stem cell

HIF: hypoxia-inducible factor

SDF-1: stromal cell-derived factor

CXCR: C-X-C chemokine receptor

FAK: focal adhesion kinase

SW: Short-Waves therapy treatment group

Con: control group

MG: MSC labeled by the GFP group

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Tables

Table 1. Oligonucleotide product size and accession numbers for q RT-PCR

| Gene     | Product size | Accession number |
|----------|--------------|------------------|
| HIF-1    | 105          | NM_021704        |
| SFD-1    | 126          | NM_001033        |
| FAK      | 130          | NM_013081        |
| F-actin  | 127          | NM_001109        |
| CXCR4    | 116          | NM_009911        |
| β-catenin| 155          | NM_053357        |
| β-actin  | 103          | NM_007393        |

Table 2. Migration test *in vitro*

|                          | Wound region (%WR)\textsuperscript{a} | Number of migrated cell 24h |
|--------------------------|----------------------------------------|-----------------------------|
|                          | 24h                                    | 48h                         |                             |
| Vector                   | 31.4 ± 1.1                             | 19.8 ± 0.8                  | 319 ± 13                    |
| Vector+SW                | 27.7 ± 1.5\textsuperscript{b}          | 8.84 ± 1.3\textsuperscript{b} | 420 ± 15\textsuperscript{b} |
| Hif-1-SiRNA              | 44.9 ± 1.7                             | 38.7 ± 3.7                  | 221 ± 11                    |
| Hif-1-SiRNA+SW           | 46.1 ± 1.4                             | 40.0 ± 1.5                  | 229 ± 12                    |
| CoCl\textsubscript{2}+Vector | 45.6 ± 0.9                        | 32.2 ± 3.0                  | 244 ± 10                    |
| CoCl\textsubscript{2}+Vector+SW | 35.4 ± 2.2\textsuperscript{c}       | 33.8 ± 3.4                  | 374 ± 17\textsuperscript{c} |
| CoCl\textsubscript{2}+Hif-1-SiRNA | 45.9 ± 1.2                     | 42.6 ± 1.7                  | 221 ± 11                    |
| CoCl\textsubscript{2}+Hif-1-siRNA+SW | 43.7 ± 1.6                      | 38.8 ± 1.8                  | 207 ± 13                    |

\textsuperscript{a}: %WR = (the size of wound region / total size of image) \times 100. \textsuperscript{b}: Vector VS Vector+SW at the same time point \( P < 0.05 \). \textsuperscript{c}: CoCl\textsubscript{2}+Vector VS CoCl\textsubscript{2}+Vector+SW at the same time point \( P < 0.05 \).

Figures
Figure 1

Schematic diagram of Short-Waves promoting fracture healing by chasing HIF-1 and promoting MSC migration. SW: Short-Wave treatment, HIF-1: hypoxia-inducible factor 1; SDF-1: stromal cell-derived factor 1; CXCR4: C-X-C motif receptor 4; PI3K: phosphatidylinositol 3-kinase; Erk: extracellular regulated protein kinases; FAK: focal adhesion kinase; MSCs: mesenchymal stem cells.
Figure 2

Factors expression in MSC. (A) MSC (SD rat strain) was fed with different kinds of osteoblasts culture medium. The gene expression of CXCR4, β-catenin, FAK, and F-actin in MSC detect by q RT-PCR. (B) The presence of the factors as protein of MSC was detected by western blot. (C) Quantification of protein bands from western blot films. The quantification will reflect the relative amounts as a ratio of each protein band relative to the lane's loading control beta-actin. The data of western blot represent mean ± SD. Differences between the control group and SW group were assessed by performing a t-test. *P <0.05. Con: control, SW: Short-Wave treatment.
Migration in vitro. In wound Healing Assay, MSC (SD rat strain) was fed with 8 different kinds of osteoblasts culture medium. Photographs were collected at 0, 24, and 48 hours. Additionally, the chemotactic effect of culture medium of short wave irradiated osteoblasts on MSC in vitro analyzed by trans-well assay. Eight kinds of osteoblasts (SD rat strain) culture medium was seeded in the bottom chamber. The top chambers filled with MSC (SD rat strain) starved overnight were inserted. Twenty-four hours later, the cells that migrated to the bottom side were accumulated.
Figure 4

Factors expression in osteoblasts in vitro. Osteoblasts of neonatal SD rats were fed in four kinds of medium. Short-Wave irradiation was provided to the cells in SW group. (A & B) The concentration of HIF-1 and SDF-1 in culture medium of osteoblasts were analyzed by ELISA. (C & D) The expression of HIF-1 and SDF-1 in the osteoblasts was analyzed by western blot. Data represent mean ± SD. Differences between the control group and Short-Wave treatment group were assessed by performing a t-test. *P <0.05. Con: control; SW: Short-Wave treatment
Figure A: Bar graphs showing the fold change of HIF-1 and FAK over time (3d, 7d, 14d) for control (Con) and stimulated with water (SW) conditions. The graphs display a significant increase in the fold change for both HIF-1 and FAK in the SW condition compared to the control at 7d and 14d.

Figure B: Bar graph showing the level of SDF-1 in plasma for control (Con) and stimulated with water (SW) conditions. The graph displays a significant increase in SDF-1 levels in the SW condition compared to the control at 14d.
Figure 5

Factors expression in vivo. (A) Callus of sacrificed SD rats was collected at day 3, 7 and 14. The expression of HIF-1, SDF-1, FAK, and F-actin in callus tissue detected by q RT-PCR (n=4/group/time point). (B) The concentration of SDF-1 in plasma of sacrificed SD rats detect by ELISA at day 3, 7 and 14 (n=4/group/time point). Data represent mean ± SD. Differences were assessed among the four groups on each day by performing one way ANOVA. *P <0.05. Con: control; SW: Short-Wave treatment.
Figure 6

Tracing MSC homing in nude mice. (A) Fluorescence assays in vivo. Nude mice of femoral shaft fracture were injected MSC labeled by the GFP (MG), and then randomly divided into two groups, including a Short-Wave treatment group (MG+SW) and control group (MG) (n=10/group). SC-GFP were examined by an IVIS imaging system seven days after operation. (B) Analysis of total radiant efficiency in the right femur. (C) Homing of MSC-GFP to the fracture site, analyzed by histological analysis and immunofluorescence imaging at 28 days after the operation. The GFP marked MSC show red accounted for Cy3 labeled antibody. (D) Six high magnification fields (100×) were randomly observed, and the number of GFP-positive cells was counted and analyzed. Significantly more GFP-MSC cells were counted in femora of animals treated with Short-Wave irradiation. Data represent mean ± SD. Differences were assessed by performing a t-test. *P < 0.05. MG: injected MSC labeling with the GFP; SW: Short-Wave treatment.
Figure 7

Radiographs and microCT of the femur. (A) Radiographs of SD rat's femur. Callus formation was seen in the control group as well as SW group (n=4/group). (B) The normalized radiographic density of femur in control and different treatment. The higher radiographic density turned out in SW group at a day14 and days21 post-operation. After 28 days, no obvious fracture gap was found in each group. (C) Micro CT images of the fracture (SD rats). More callus and narrow gap was seen in SW group at day14 (n=4/group) (D) Analysis of ROI bone volume fraction (BV/TV) in control and SW group. Data represent mean ± SD. Differences were assessed on each day by performing one way ANOVA. *P <0.05. Con: control; SW: Short-Wave treatment.
Figure 8

MSC cells used in this study. (A) MSC from SD rat had a fusiform shape and was arranged in bundles or whorls. (B) MSC established from cells in the primary culture were stained for high-affinity receptors CD90, CD44, and the low-affinity receptor CD34, CD45, CD11b/c with specific antibodies, and were then analyzed using flow cytometry. (C) The alkaline phosphatase staining method was used to identify osteoblasts.
Flowchart of the study design. Stabilized femur fractures were established in 40 SD rats. The effect of Short-Wave on a fracture healing was examined by radiographs, microCT analysis and histological method. The expression HIF-1 and other factors in callus was tested by q RT-PCR. SDF-1 in plasma evaluated by ELISA. To analyze the MSC migration in healing, in vivo fluorescence assays and immunofluorescence were used after treatment in 20 nude mice with a femoral fracture. For in vitro study, osteoblast simulated fracture site was first irradiated by the Short-Wave; CoCl2 in medium stimulated hypoxia condition; siRNA targeting HIF-1 was used to investigate the role of HIF-1. Osteoblast culture medium was then collected as chemotaxis content of MSC, and the migration of MSC was evaluated using wound healing assay and trans-well chamber test. The expression of HIF-1 and its related factors were quantified by q RT-PCR, ELISA, and Western blot. SW: Short-Wave treatment, MG: GFP-labeled MSC. Image element in the flowchart mainly comes from https://app.biorender.com/.