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# Remote control of the recruitment and capture of endogenous stem cells by ultrasound for in situ repair of bone defects

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#### ABSTRACT

Stem cell-based tissue engineering has provided a promising platform for repairing of bone defects. However, the use of exogenous bone marrow mesenchymal stem cells (BMSCs) still faces many challenges such as limited sources and potential risks. It is important to develop new approach to effectively recruit endogenous BMSCs and capture them for in situ bone regeneration. Here, we designed an acoustically responsive scaffold (ARS) and embedded it into SDF-1/BMP-2 loaded hydrogel to obtain biomimetic hydrogel scaffold complexes (BSC). The SDF-1/BMP-2 cytokines can be released on demand from the BSC implanted into the defected bone via pulsed ultrasound (p-US) irradiation at optimized acoustic parameters, recruiting the endogenous BMSCs to the bone defected or BSC site. Accompanied by the daily p-US irradiation for 14 days, the alginate hydrogel was degraded, resulting in the exposure of ARS to these recruited host stem cells. Then another set of sinusoidal continuous wave ultrasound (s-US) irradiation was applied to excite the ARS intrinsic resonance, forming highly localized acoustic field around its surface and generating enhanced acoustic trapping force, by which these recruited endogenous stem cells would be captured on the scaffold, greatly promoting them to adhesively grow for in situ bone tissue regeneration. Our study provides a novel and effective strategy for in situ bone defect repairing through acoustically manipulating endogenous BMSCs.

# 1. Introduction

Bone defects remain a major clinical challenge [1,2]. The healing often fails when the large bone defect exceeds host self-repair capacity, especially for those with trauma, congenital anomalies, and tissue resection due to cancer [3–5]. Conventional surgical reconstructive procedures using autograft or allograft bone tissues may repair the defects to some degree in anatomical and functional outcomes [6,7]. But these treatment options occasionally bring with some considerable risks

to the patients [8–11]. For instance, the use of autograft is hampered by the limited availability, unpredictable incorporation, and donor site morbidity [12–14]. Allograft materials have several limitations such as disease transfer, limited availability and immunological rejection [8,9, 13,15]. Tissue engineering approaches provide a promising strategy for bone tissue regeneration [3,16,17], which not only provides mechanical support and biological function, but also effectively solves the problems such as the source and rejection of bone defect grafts. Ideally, the strategy combines three essential elements, including scaffolds, stem

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cells, and growth factors, to produce a tissue engineered construct for bone healing [18,19]. Among these, the use of exogenously supplied stem cells may generally be necessary because only cells can create bone and help to overcome recalcitrant healing [20–24]. In recent years, scholars have used various carriers to graft exogenous bone marrow-derived mesenchymal stem cells (BMSCs) into bone defect sites for bone repair [23,24]. However, the exogenously transplanted stem cells for tissue engineering have posed impediments due to their invasive donor biopsies, labor-intensive, time-consuming cell culture steps and potential malignant transformation [25,26].

For these reasons, some simpler in situ tissue regeneration approaches are attracting more and more attentions by using of the body's own stem cells or endogenous stem cells [27–29]. The success of in situ tissue regeneration relies on effective recruitment of endogenous stem or progenitor cells into the implanted scaffolds and subsequent induction of their differentiation into functional bone. To achieve it, the sustained delivery of biological cues, such as bioactive molecules, from the implanted scaffold is important for providing an appropriate microenvironment that can direct host stem and progenitor cells to home to the implant [22]. Moreover, an ideal scaffolding system for in situ tissue regeneration still needs to support their expansion and differentiation into a desired tissue type [30]. For this purpose, a well-designed combination of biological cues with biomaterial scaffolds would provide

appropriate microenvironments within the implanted scaffold. Among various bioactive molecules, stromal cell derived factor-1 (SDF-1) and bone morphologic protein-2 (BMP-2) possess the most widely used active agents for bone tissue regeneration [31-40]. The former has been shown to be a strong bioactive chemoattractant which can attract BMSCs and hematopoietic stem cells (HSCs) to injured tissues, and the latter plays a central role in bone-tissue engineering because of its potent bone-induction ability [41–43]. To date, numerous documents have demonstrated that the sustained release of SDF-1 $\alpha$ , BMP-2 or their combination within an implanted scaffold could generate a high concentration gradient of these factors and drive efficient stem cell migration into the implant or be conducive to their bone differentiation [37-40]. Unfortunately, these current systems are largely less satisfactory for their limited efficacy since the local drug delivery is mainly dependent on passive release from the implanted scaffold, resulting in accumulation of migrated cells mostly at the periphery of the scaffold, much less helping them to adhere onto the scaffold for growth.

Here, we engineered an acoustically responsive scaffold system that not only can control the release of bioactive molecules for recruitment of endogenous BMSCs, but also promote the capture of the recruited BMSCs to the scaffolds via resonant gradient field induced trapping force. As shown in Fig. 1, SDF-1 and BMP-2 were loaded into alginate hydrogels formed via ionic cross-linking with divalent  $Ca^{2+}$  ions.



**Fig. 1.** Schematic design of BSC-mediated endogenous BMSCs repairing bone defect by US. ARS was fabricated with PLA and embedded into calcium alginate hydrogels which contain SDF-1 and BMP-2 chemokines, resulting in the formation of BSC. When BSC was implanted into the bone defect and received with p-US irradiation in the fibrovascular stage of bone repair, SDF-1 and BMP-2 could be released on demand from BSC due to the acoustically-induced disruption of ionically cross-linked hydrogels, recruiting the endogenous BMSCs towards the bone defected site. Accompanied with daily acoustic irradiation by p-US for 14 days, the alginate hydrogels were gradually degraded and ARS was exposed to these recruited endogenous BMSCs. After that, another set of s-US irradiation was used to induce the resonance of ARS to produce the circumferential acoustic trapping force to capture these recruited endogenous stem or progenitor cells, facilitating their adhesive growth for in situ bone tissue regeneration.

Ultrasound (US) could release these loaded bioactive factors on demand by disrupting ionically cross-linked hydrogels and promote the degradation of hydrogels. Meanwhile, the acoustically responsive scaffolds (ARS) made of polylactic acids (PLA) were embedded in the hydrogels. When the biomimetic scaffold complex (BSC, SDF-1/BMP-2-loaded hydrogels embedded with ARS) was implanted into the bone defect, it was daily received pulsed ultrasound (p-US) irradiation to destruct the alginate hydrogels and to release the loaded SDF-1/BMP-2 for recruitment of distant endogenous BMSCs to the site of bone defect. Accompanied by the release of bioactive molecules and the degradation of hydrogels, ARS were exposed to the recruited host stem cells or progenitor cells. Then, another set of sinusoidal continuous wave ultrasound (s-US) stimulation was applied to induce the resonance of ARS, which produces the acoustic trapping force to capture these endogenous stem or progenitor cells on the scaffold, facilitating their adhesive growth and osteogenesis for in situ bone tissue regeneration.

# 2. Materials and methods

# 2.1. Materials and reagents

Alginate was purchased from Pronova (USA). Calcium sulfate (CaSO<sub>4</sub>), alizarin red S (ARS), indocyanine green (ICG) and crystal violet were obtained from Sigma-Aldrich (USA). Calcein AM, propidium iodide (PI) and recombinant rat SDF-1 $\alpha$ /CXCL12 $\alpha$  were purchased from Beyotime Institute of Biotechnology (China). Albumin bovine serumfluorescein isothiocyanate (BSA-FITC) and alkaline phosphatase (ALP) were purchased from Solarbio (China), silicone isolators from Grace Bio-Labs (USA), ALP assay kit from Sciencell (USA), MEM without phenol red from Invitrogen (USA), pacific Blue anti-rat CD45 antibody and APC anti-mouse/rat CD29 antibody from Boilegend (America). Recombinant human/mouse/rat BMP-2 (C012) was bought from Novoprotien (China), cell counting kit-8 (CCK8) from Dojindo Molecular Technologies (Japan), complete culture medium of bone marrow mesenchymal stem cells from Cyagen Biosciences (USA), rat BMP-2 ELISA kit and rat SDF-1α/CXCL12α ELISA kit from Jonln (China). The SD rats were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd (China).

# 2.2. Preparation and characterization of BSC

ARS was made of PLA through 3D printing technology by Dongguan Qidian Technology Co. Ltd. The ARS was a disk structure, with an outer diameter of 4.2 mm. The diameter of each single cylindrical scaffold was 300 µm and the distance between two parallel cylindrical scaffolds was kept 1 mm apart. Considering that there are not similar molecules available with the same molecular weight for the in vivo imaging detection. We used BSA-FITC and ICG as model molecules to investigate the release of SDF-1/BMP-2 in our study. The alginate hydrogels were fabricated by the following procedure. Firstly, 70 mg/ml alginate polymers in ddH<sub>2</sub>O were added with 5  $\mu$ l/ml BSA-FITC, 0.5 mg/ml ICG, 3 µg/ml SDF-1, 3 µg/ml BMP-2 or 6 µg/ml SDF-1/BMP-2 (each concentration is 3  $\mu$ g/ml). Then, the mixture solution was cross-linked by adding into 4 mg/ml CaSO4. The formed hydrogels (30  $\mu l)$  were cast as disks (4.5 mm diameter, 2 mm thickness) with ARS using the silicone isolators to form BSC. BSC were equilibrated overnight in serum-free DMEM without phenol red before use. The concentrations of alginate polymers and CaSO<sub>4</sub> were adjusted to optimize their ratios in the experiments of the US-triggered drug release. The scanning electron microscope (SEM, VEGA3 TESCAN SEM, Brno, Czech Republic) was used to observe the surface structure of the scaffold and the BSC.

# 2.3. Simulations of acoustic field and acoustic radiation force

The involved physical simulation processes were performed according to our previous reports [44]. Briefly, a single PLA scaffold immersed in the PBS was excited at its resonance frequency to generate the localized acoustic field to further apply the acoustic radiation force to suspended cells. The physical model for the above physical process can be divided into an acoustic step and particle trapping step, and the simulation procedure was implemented in the finite element package COMSOL Multiphysics 5.2a. The waves after transmission through scaffolds in aqueous media were characterized with the previously reported methods [44,45].

### 2.4. Cell culture and cell viability assay

The BMSCs were harvested from the femora of SD rats. Femurs were aseptically removed, and bone marrow was flushed with PBS using a sterile syringe. Bone marrow samples were centrifuged for 5 min at 1000 g and then placed in complete BMSCs culture medium (Cyagen Biosciences Inc, Sunnyvale, CA, USA) with 10% fetal bovine serum and 1% penicillin and streptomycin, then incubated at 37 °C in an incubator with 5% CO<sub>2</sub> for 48 h. Subsequently, the nonadherent cells were discarded, and the adherent cells were allowed to grow to 80% confluence. These cells were defined as passage one cells (P1). P3 cells were used for all experiments.

CCK-8 was used to determine the cell viability of BMSCs received with US irradiation. In brief,  $3 \times 10^3$  cells were seeded in 96-well plates and allowed to adhere overnight. The cells were then irradiated by p-US at different acoustic intensities at 0.1 MPa, 0.2 MPa, 0.3 MPa or 0.4 MPa, s-US at different acoustic intensities at 0.1 MPa, 0.2 MPa or 0.3 MPa for 2 min or the cells on ARS irradiated by s-US. After incubation for 6 h, the medium was removed and 10 µl of CCK-8 was added into every well and further incubated for 1 h. After that, the optical density of the cells was measured using a microplate reader (BioTek Synergy4, USA) at an absorbance of 450 nm. The relative cell viability was calculated as (As - $A_b$ )/( $A_c - A_b$ ) imes 100%, where  $A_s$  is the absorbance of the well containing irradiated cells, culture medium and CCK-8 solution; Ab is the absorbance of blank wells; Ac is the absorbance of the well containing nonirradiated cells, culture medium, and CCK-8 solution. And the cell viability was also test for 7 days' daily US irradiation. The cells subjected to s-US or p-US irradiation were stain with calcein AM/PI staining after 6 h US irradiation.

The cell viability of SDF-1, BMP-2 and SDF-1 + BMP-2 groups, and under p-US or s-US irradiation was determined with CCK-8.

# 2.5. p-US-triggered drug release

Ultrasound stimulation was generated by a custom-made transducer at a center transmit frequency of 1.5 MHz. The transducer (20 mm diameter) was driven by a function generator (DG4162, Rigol, Suzhou, China) connected to a power amplifier (LZY-22+, MINI, USA). The BSCs replenished with 2 ml PBS were placed in a 24-well plate. Degassed water was used to cover the area between the transducer and the well plate. To optimize the acoustic parameters for drug release from BSC, FITC-labeled BSA was taken as a model drug. BSC with different alginate/Ca<sup>2+</sup> concentration ratios were acoustically stimulated by p-US with 0.3 MPa acoustic intensity. The BSC with 70 mg/ml alginate and 4 mg/ml calcium were acoustically stimulated at different acoustic intensities from 0 MPa to 0.4 MPa for different duration from 0 min to 20 min. The supernatant was collected and the absorbance at 493 nm was measured on a Multimode Plate Reader for quantitative analysis of the released FITC-BSA. To determine the acoustic thermal effect of p-US, BSC were stimulated at a defined acoustic intensity of 0.1 MPa, 0.2 MPa, 0.3 MPa or 0.4 MPa for 20 min. To determine the acoustic thermal effect of s-US, BSC were stimulated at a defined acoustic intensity of 0.1 MPa, 0.2 MPa or 0.3 MPa for 20 min. The changes of temperature were detected by Fluke thermal imager (Fluke, USA) every 5 min. After that, the release of SDF-1 and BMP-2 from BSC were determined. Briefly, SDF-1/BMP-2-loaded BSC was daily irradiated by p-US for 20 min at 0.3 MPa acoustic intensity for 9 days. 1 ml supernatant was collected every day

and 1 ml PBS was supplemented. Both SDF-1 and BMP-2 in the supernatant was quantitatively analyzed by enzyme linked immunosorbent assay.

#### 2.6. Osteogenic differentiation of BMSCs

The osteogenic differentiation capability of BMSCs were evaluated by using osteogenic differentiation medium (Cyagen Biosciences Inc.) supplemented with conditioned medium from BSCs loading with SDF-1, BMP-2, SDF-1/BMP-2 with or without p-US treatment. The conditioned medium was prepared as follows. The osteogenic differentiation medium with BSC was irradiated by p-US for 20 min at 0.3 MPa. Then the irradiated medium was transferred to a new Eppendorf tube as the conditioned medium. The culture medium without conditioned medium from BSCs with SDF-1/BMP-2 were used as the control. After osteoblast differentiation induction for 4 days, the culture media in the groups with p-US treatment were collected into a 96-well plate. The alkaline phosphatase (ALP) activity was determined with the alkaline phosphatase assay kit (Sciencell, USA). After co-incubation of substrates and pnitrophenol for 30 min at 37 °C, the ALP activity was determined at the wavelength of 405 nm. At the same time, the cells in the groups with p-US treatment were fixed in 4% paraformaldehyde, followed by staining with ALP solution (Solarbio, China) for 20 min. The ALP-positive cells were visualized by microscope. As for the assay of mineralization nodules, BMSCs in groups with or without p-US treatment were fixed with 4% paraformaldehyde solution and washed with PBS for three times after 14-day osteoblast induced differentiation. 2% (wt/v) Alizarin Red S (Cyagen Biosciences, China) solution was added into the fixed cells. After being incubated for 30 min at 37 °C, the samples were air-dried and images were acquired by microscope. To quantify the orange-red coloration of Alizarin Red S, 10% acetic acid was added to the cells. After 30 min incubation, the cells were transferred to an oven at 85 °C for 10 min. Then, the sample were neutralized with 10% ammonium hydroxide and centrifuged for 15 min at 16,000g. 200 µl of sample solution was added to 96-well plates and measured at a wavelength of 405 nm with a microplate reader.

The osteogenic differentiation capability of BMSCs were evaluated by using osteogenic differentiation medium (Cyagen Biosciences Inc.) with s-US irradiation, p-US irradiation or ARS with or without s-US irradiation. After osteoblast differentiation induction for 14 days, the mineralization nodules were assayed with Alizarin Red S staining and visualized by microscope.

#### 2.7. Quantitative real-time polymerase chain reaction analysis

The quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the differential expression of osteogenesis-relative genes, including Runx-2, OCN or OPN. After the osteoblast in a 6-well plate were induced to differentiate for 7 days, the total mRNAs of these cells were extracted. The concentrations of mRNAs were measured and the primers of target genes were added (Table S1). Then each sample was reverse-transcribed using the cDNA synthesis kit (Thermofisher, America). In addition, the Phanta Max Super-Fidelity DNA polymerase (Vazyme, China) and an ABI 7500 Sequencing Detection System (LightCycler® 96 System, Roche, Swiss) were applied to amplify the cDNA via qRT-PCR assay according to the manufacturer's protocol.

#### 2.8. Adipogenic differentiation of BMSCs

The adipogenic differentiation capability of BMSCs were evaluated by using adipogenic differentiation medium (Cyagen Biosciences Inc.) supplemented with conditioned medium from BSCs with SDF-1, BMP-2, SDF-1/BMP-2 after p-US treatment. The culture medium without conditioned medium from BSCs with SDF-1/BMP-2 were used as the control. As for the assay of lipid droplets, BMSCs were fixed with 4% paraformaldehyde solution and washed with PBS for three times after 14-day adipogenic differentiation. Oil red (Cyagen Biosciences, China) solution was added into the fixed cells. After being incubated for 30 min at 37  $^{\circ}$ C, the samples were air-dried and images were acquired by microscope.

#### 2.9. In vitro recruitment of BMSCs by p-US

Transwell experiments were performed to quantitatively investigate the migration of BMSCs co-incubated with BSC received with or without p-US irradiation. In brief, BMSCs were seeded into the upper chambers of transwell inserts (24-well insert, pore size 8  $\mu$ m, Corning Incorporated) at the density of 5  $\times$  10<sup>4</sup> in 200  $\mu$ l serum-free DMEM. The BSCs with SDF-1, BMP-2, SDF-1/BMP-2 or without chemokines were placed on the lower chambers with complete culture medium. Subsequently, BSCs were stimulated with or without p-US irradiation (1.5 MHz, 0.3 MPa) for 2 min. After 12 h incubation at 37 °C in a 5% CO<sub>2</sub>-humidified incubator, the cells that migrated to the lower membrane surface were fixed, stained with 0.1% crystal violet, photographed by light microscope (Leica DMI4000B, German). The migrated BMSCs to the lower membrane surface were digested, stained with APC- labeled CD29 antibody (1:100) at 4 °C for 30 min, and then counted by a flow cytometer (Beckman Coulter CytoFLEX S).

The short-distance recruitment of BMSCs was also evaluated by improved wound healing experiments. Briefly,  $1 \times 10^5$  BMSCs were seeded onto the 6-well plates. A pipette tip and cotton swabs were used to make circular scratch wound with 4.5 mm in diameter in a confluent monolayer of BMSCs. The floated cells were washed out twice with PBS and resupplied with fresh medium. Then, BSCs with SDF-1/BMP-2 were placed on the circular cell-free regions, followed with or without p-US irradiation for 2 min at 0.3 MPa twice at 6 h interval. After another 6 h incubation, the BSCs were stained with calcein AM and PI, and photographed by fluorescence microscope.

# 2.10. In vitro capture of BMSCs by s-US

To verify the capture ability of BMSCs by ARS irradiated by US, a microfluidic chamber (30 mm  $\times$  3 mm  $\times$  3 mm) containing a single 30mm long ARS was fabricated and mounted on the stage of an inverted optical microscope (TE2000-U, Nikon). ARS was stained with PI and BMSCs were stained with calcein AM. The fluorescently labeled BMSCs were infused into the chamber through a 5-ml syringe. Acoustic stimulation by s-US was applied to the ARS at different transmit frequency 1.4 MHz, 1.5 MHz or 1.6 MHz with 0.2 MPa acoustic pressure intensity for 5 min. Images and videos of the movement of BMSCs around the ARS under the simulated acoustic field were taken by Nikon imaging software (NIS-Advanced, Nikon) through a charge-coupled device digital camera (CoolSNAP HQ2, Photometrics). To examine whether the capture of BMSCs by ARS can favor their adhesive growth on the ARS, BMSCs were mixed into 1% of chitosan hydrogels and then the ARS was embedded into the hydrogels. The hydrogels containing ARS and BMSCs was placed on the culture plate with culture medium, followed by s-US stimulation for 2 min each day at the acoustic intensity of 0.2 MPa. ARS in chitosan hydrogels with BMSCs which did not receive with acoustic stimulation was used as the control. After 7 days, the ARS were taken out and observed under the light microscope. The number of BMSCs which adhesively grew on the ARS were counted by Image J software from three random fields. The morpohlogy of BMSCs was observed under fluorescence microscope after 6 h and 24 h with s-US irradiation or without s-US irradiation with FITC-phalloidin (Solarbio, China) and DAPI staining (Biosharp, China).

# 2.11. Hydrogel degradation by p-US

To verify the degradation of hydrogels under acoustic irradiation *in vitro*, the hydrogels were placed in the 24-well plates and daily stimulated 20 min at an acoustic intensity of 0.3 MPa for 12 consecutive days.

Then, hydrogels were filtered through 100-mesh sieve under gravity for 30 min and weighed. To verify the degradation of hydrogels *in vivo*, ICG-loaded alginate hydrogel (0.5 mg/ml of ICG) was subcutaneously implanted into rats' legs. The implantation site was exposed for 20 min ultrasonic stimulation every day at 0.3 MPa for 12 consecutive days. Images were acquired every day using an *in vivo* imaging system (IVIS Spectrum, PerkinElmer, Ex = 710 nm and Em = 800 nm). ICG-loaded alginate hydrogel which did not receive with ultrasonic stimulation was used the control. All the experiments and analysis were performed in triplicate, n = 3. Then, the BSCs were obtained and the swelling and degradation of BSCs were observed and photographed.

# 2.12. Animal modeling

Male rats were used to build the femoral bone defect model. Briefly, rats were anesthetized through a nose cone, in which 2–2.5% isoflurane was constantly provided with oxygen through a vaporizer. After anesthesia and routine preparation, a midline sagittal incision was made on the posterolateral region left hind limb to expose the femur. The 2 mm thickness, 4.5 mm bone defects were created using electric hand drill (Deguqmnt, China). The surgical field was irrigated with saline and BSCs or ARS were implanted. The incision line was sutured. All animal experiments were performed under the guidelines approved by the Animal Study Committee of Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences.

# 2.13. In vivo recruitment and capture of BMSCs by US

Twelve mature male rats (18 weeks old, n = 3 for each group) with the femoral bone defects were randomly divided into the following four groups: (i) BSC: the defect was implanted with BSC with SDF-1/BMP-2 but did not receive with ultrasonic stimulation, (ii) BSC + p-US: the defect was implanted with BSC with SDF-1/BMP-2 and received with p-US stimulation, (iii) ARS: the defect was implanted with ARS without chemokines and received with s-US stimulation, (iv) ARS + s-US: the defect was implanted with ARS without chemokines and received with s-US stimulation. Ultrasonic stimulation was applied for 20 min each time, once each day, from the 2nd day post-implantation to the 8th day, with the optimized parameters at 1.5 MHz driving frequency, 0.3 MPa acoustic intensity (p-US) with 50% duty cycle or 0.2 MPa acoustic intensity (s-US). One week after implantation, the BSCs or scaffolds were harvested for analysis. For direct evaluation of cell recruitment ability, the cells were stained with calcein AM and photographed by fluorescent microscope. For further analysis of cell phenotype, cells were isolated from the BSCs or scaffolds using a syringe plunger, digested in Trypsin-EDTA (0.25%) (Gibco, America) for 10 min on ice with vortex twice. Cells were passed through a 40 µm cell strainer, centrifuged and resuspended in FACS buffer (PBS containing 0.5% FBS). The cells were stained with Pacific Blue-labeled CD45 antibody and APC- labeled CD29 antibody (1:100) at 4 °C for 30 min, and then analyzed by a flow cytometer (Beckman Coulter CytoFLEX S).

#### 2.14. Bone repairing of femoral defects in rats

Thirty mature male rats (18 weeks old, n = 5 for each group) were used to building the femoral bone defect model and were randomly divided into six groups: (i) Sham: no scaffold implantation and any ultrasonic stimulation, (ii) ARS + p-US + s-US: the defect was implanted with ARS without chemokines and received with ultrasonic stimulation, (iii) BSC: the defect was implanted with BSC with SDF-1/BMP-2 but did not receive with ultrasonic stimulation, (iv) BSC + p-US: the defect was implanted with BSC with SDF-1/BMP-2 and only received with p-US stimulation, (v) BSC + s-US: the defect was implanted with BSC with SDF-1/BMP-2 and only received with s-US stimulation, (vi) BSC + p-US + s-US: the defect was implanted with BSC with SDF-1/BMP-2 and received with p-US and s-US stimulation. Acoustic irradiation by p-US was applied from the 2nd day after BSC implantation for 14 days, once each day with the optimized p-US parameters at 1.5 MHz driving frequency, 0.3 MPa acoustic intensity, 50% duty cycle and 20 min exposure duration. From the 15th day on, s-US was utilized for another 14 days, once each day with the following parameters: 1.5 MHz driving frequency, 0.2 MPa acoustic intensity, 20 min exposure duration. All the rats were anesthetized through a nose cone when treating with US, in which 2–2.5% isoflurane was constantly provided with oxygen through a vaporizer. During the treatment, ultrasound gel was placed on the rat's skin, which acted as both a lubricant and an energy conductor. And it could reduce the pressure around lesion and the pain when treated with US with a thick layer gel on the skin. After 3 months, all of the rats were sacrificed and the femurs were harvested for further examination.

# 2.15. $\mu CT$ examination

The femur samples were scanned by  $\mu$ CT scanner (Bruker, SkyScan 1176, Germany) at the following parameters: beam energy 60 kV, electrical current 100  $\mu$ A, resolution 18  $\mu$ m. Scanning was performed in a cone-beam acquisition mode. The image slices were reconstructed by NRecon program package v.1.6.8.0 (SkyScan, Kontich, Belgium). The 3D surface rendering image were made using the CT-Analyzer program (version 1.12.10.0, Skyscan, Aartselaar, Belgium). During reconstruction, dynamic image range, post-alignment value, beam hardening, and ring-artifact reduction were optimized for each experimental set. Then the quantitative evaluation of newly formed bones were analyzed according to the parameters such as trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), bone volume-to-tissue volume (BV/TV), number of trabecular bone (Tb.N), bone mineral density (BMD), bone surface area density (BS/BV) using the analysis software.

# 2.16. Biomechanical testing

The stiffness of hydrogels and BSCs were measured with universal testing machine (INSTRON 5982). The hydrogels and BSCs were not hydrated for the duration of the test. The elastic modulus was calculated from the slope of the stress-strain curve, in the initial 0-50% linear elastic deformation range. A total of n = 3 hydrogels or BSCs per group were measured. Indentation test was taken to evaluate the mechanical properties of the new bone. Samples were prepared with cool mosaic method using liquid hardener (Gaopin, G900800, China). For the mechanical testing, the samples were placed under a plane dynamometer system (Kistler, 9129AA) with new bone regions facing up using six-axis manipulator (Epson) for operation. The indentation was performed in the axial direction of the vessel using a spherical flat indenter with a diameter of 1 mm and the velocity of 0.1 mm/s. The initial point was 1 mm away from the new bone of sample plane, and the actual pressing depth was 2 mm, the pressing time was kept for 20 s. Data were analyzed using Dynoware 2.6.5.16 software (Kistler). Also, atomic force microscopy was taken to evaluate the microscopic mechanical properties of the newly formed bones by using of bimodal atomic force microscopy (AM-FM), which was carried on an Asylum Research MFP-3D-Bio atomic force microscopy (AFM). Briefly, the bones were first embedded in resin, and then cut into 10 mm cuboid using chainsaw. Afterward, the specimens were polished using carbide papers to obtain smooth surface. The AC-160TS (Olympus, Taiwan) probes with manufactured spring constant of 26 N/m and free resonant frequency of 300 kHz were used in AM-FM experiments for all specimens. The cantilever spring constants of these probes were calibrated using thermal fluctuation method [46] before measurement. All the data was collected on the edge of the specimen.

# 2.17. Histological analysis

After micro-CT scanning, samples were decalcified in 15% ethylene diamine tetraacetic acid (EDTA) (Servicebio, China) for 10 weeks before

they were embedded in paraffin, after dehydration in a series of ethanol and xylene. Sections with a thickness of 4  $\mu$ m were cut and subjected to H&E (Servicebio, China) and masson's tricolor (Servicebio, China) for the evaluation of newly formed bone under light microscope. Major organs were collected and sectioned for H&E staining for biosafety analysis.

#### 2.18. Statistical analysis

The data in current study was analyzed with Prism 8.0 (GraphPad) via student's t-test and one-way analysis of variance (ANOVA). P < 0.05 was the significance level.

## 3. Results

#### 3.1. Fabrication and characterization of the ARS and BSC

A 4.2-mm-diameter disk-shape acoustically responsive scaffold (ARS) with mesh-like structure was designed and fabricated with polylactic acids (PLA), in which the diameter of each single cylindrical scaffold was 300  $\mu$ m and the distance between two parallel cylindrical scaffolds was kept 1 mm apart (Fig. S1). As shown in Figs. 2a and S1, the rough surface could be observed from the scanning electron microscopy (SEM), which would be beneficial for cell adhesion and nutrition exchange [47,48]. The ARS was further embedded in the drug-loaded



Fig. 2. Acoustically-controlled chemokine release from BSC. (a) SEM image of a diskshaped ARS with a mesh-like internal structure and rough surface. Scale bar = 1 mm. (b) Light-microscopic image of BSC with ARS embedded into alginate hydrogels. Scale bar = 1 mm. (c) Schematic illustration of acoustically-induced disruption of ionically cross-linked hydrogels and the release of SDF-1 and BMP-2 chemokines via p-US irradiation. (d) Graphical illustration of some of the variables involved in constructing p-US waveforms. (e) SEM images of alginate hydrogels after being irradiated by p-US at different acoustic intensity or duration, revealing the formation of pores in the hydrogel. Scale bars = 100  $\mu$ m. (f-g) Thermal effect of BSC irradiated by p-US (f) or s-US (g) at the different acoustic intensity and duration, showing an elevated but controllable temperature changes. (h-i) The cumulative release curves of FITC-BSA from BSC treated with different acoustic intensity (h) for 10 min or for different duration at 0.3 MPa (i) (n = 3). (j) The cumulative release curves of SDF-1 and BMP-2 from BSC irradiated by p-US at 0.3 MPa acoustic intensity for 20 min (n = 3). (k) Weight loss rate curves of BSC treated with or without daily p-US irradiation at 0.3 MPa acoustic intensity for 20 min for 12 consecutive days (n = 3). Data in (f-k) were expressed as each replicate of three independent experiments. P values in (f-i) were determined by oneway ANOVA with Turkey's multiple comparisons test for multiple comparisons. P values in (j) and (k) were determined by two-tailed student's t-test. \*, P < 0.05; \*\*\*, P < 0.001.

hydrogels which made of alginate, a polysaccharide that forms a hydrogel via ionic cross-linking of calcium binds to guluronic acid chains (Fig. 2b) [49]. Upon receiving with US stimulation, the calcium-dependent cross-links in the alginate hydrogels would be disrupted, greatly accelerating the release of drugs encapsulated in the hydrogels (Fig. 2c). To examine it, we first designed and optimized a set of pulsed US stimulation parameters for drug release (named as p-US): pulse repetition frequency (PRF) = 1.5 MHz, pulse width = 333 ms, pulse interval = 333 ms (Fig. 2d). An elevated temperature could be observed with the increase of duty cycle, duration, and acoustic intensity amplitude, but the heating effects caused by p-US could be controlled below 37 °C at 0.3 MPa acoustic intensity and 50% duty cycle (even if duration for 20 min), and no apparent damages to cell viability were observed for these BMSCs irradiated by p-US with acoustic pressure intensities below 0.3 MPa (duty cycle = 50%, duration = 2 min),



even for six days culture with continuous p-US irradiation, confirming its safety in the *in vitro* condition (Figs. 2f, 2g, S2, S3b, S3d, S4a). Interestingly, the obvious structure disruption of hydrogels was also found to be duration- and intensity-dependent. The stronger US energy or the longer duration was used, the more and larger pores would be produced in the hydrogels (Fig. 2e). To test whether the drugs encapsulated in the hydrogel could be effectively released in a p-US-triggered manner, we used FITC-labeled bovine serum albumin (FITC-BSA) as a model drug and encapsulated them into the hydrogel. Irradiation of FITC-BSA-loaded hydrogel by p-US greatly accelerated these protein release out of hydrogel (Fig. 2h and i). Similar duration- and intensity-dependent released curves of FITC-BSA could be also observed in Fig. 2h and i. The ratios of alginate and calcium were optimized and found the 70 mg/ml alginate and 4 mg/ml calcium concentrations was appropriate for US-controlled drug release (Figs. S5 and S6). Thus, the

> Fig. 3. SDF-1 and BMP-2 promoted BMSCs migration and osteogenic differentiation. (a-b) Representative images (a) and quantification (b) of BMSCs stained with alkaline phosphatase. These cells were cultured for 4 days in osteogenic differentiation medium from BSC loading with SDF-1, BMP-2 or combination of SDF-1 and BMP-2 after p-US irradiation, respectively. Scale bar = 800 µm. (c-d) Representative images (c) and quantification (d) of BMSCs stained with alizarin red S after 14-day culture in osteogenic differentiation medium from BSC loading with different chemokines after p-US irradiation. Scale bar = 800  $\mu$ m. (e)The schematic depiction of BMSCs migration in the transwell system. The BSC was put in the bottom of the lower chamber and received with or without p-US irradiation at 0.3 MPa acoustic intensity for 2 min for promoting the release of SDF-1 and BMP-2. (f-g) Representative images (f) and quantification (g) of the BMSCs migrated to the lower chamber of transwell after 12 h ultrasonic stimulation. These cells in (f) were stained with 0.2% crystal violet. Scale bar = 400µm. Data in (b), (d) were expressed as means  $\pm$  s.d. of three independent experiments. Data in (g) were expressed as means  $\pm$  s.d. of five independent experiments. P values in (b) were determined by one-way ANOVA with Turkey's multiple comparisons test for multiple comparisons. P values in (d) were determined by Brown-Forsythe and Welch ANOVA tests. P values in (g) were determined by two-way ANOVA with Sidak's multiple comparisons test for multiple comparisons. \*, P < 0.05; \*\*, P < 0.01; \*\*\*\*, P < 0.0001.

optimal hydrogel components and p-US parameters for drug release were determined as alginate = 70 mg/ml, calcium = 4 mg/ml for hydrogel formation, acoustic pressure intensity = 0.3 MPa, duty cycle = 50%, duration = 20 min for p-US irradiation. After that, we displaced FITC-BSA with SDF-1 and BMP-2 bioactive molecules in the hydrogels and fabricated SDF-1/BMP-2-loaded hydrogels. The cumulative releases of SDF-1 and BMP-2 from BSC with daily ultrasonic stimulation for 20 min were higher than that without ultrasonic stimulation (Fig. 2j). Notably, the weight loss rate of hydrogel was also accelerated after p-US stimulation, reaching almost 100% degradation after 12 days (Fig. 2k).

#### 3.2. Osteogenic differentiation and cell migration

To evaluate osteogenic differentiation ability of BMSCs exposed to medium from BSC loading with SDF-1 or/and BMP-2 which was irradiated using p-US. Fig. 3a and b confirmed their higher ALP activity for the BMSCs treated with medium from BSC loading with SDF-1 or BMP-2 than the untreated control group, but the combination treatment with medium from BSC loading with SDF-1/BMP-2 achieved the highest ALP activity in the BMSCs. Alizarin red S staining revealed that without p-US irradiation, there were no differences of calcium nodules formation among the BMSCs exposed to medium from BSC loading with SDF-1 or/ and BMP-2 (Fig. S7). However, BMSCs treated with medium from BSC loading with BMP-2 irradiated using p-US had more calcium nodules, compared to those treated with medium from BSC loading with SDF-1 or the control group irradiated using p-US (Figs. 3c, 3d, S8). Moreover, the BMSCs treated with medium from BSC loading with SDF-1/BMP-2 irradiated using p-US had the most calcium nodules in these groups (Figs. 3c, 3d, S8), which was significantly more than that in the group without p-US irradiation (OD.  $_{p-US(+)} = 1.408 \pm 0.076$ , OD.  $_{p-US(-)} =$  $1.130 \pm 0.120$ , P = 0.0077). Furthermore, the expression levels of Runx-2, OPN and OCN genes were also detected by qRT-PCR in these treated BMSCs (Table S1, Supporting Information). As shown in Fig. S9, BMSCs exposed to the conditional medium from BSC loading with SDF-1/BMP-2 showed the highest expression levels of Runx-2, OPN and OCN genes. Significantly less lipid droplets were also observed in p-US-irradiated BMSCs treated with medium from BSC loading with SDF-1/BMP-2 than these SDF-1- or BMP-2-exposed cells or untreated control cells (Fig. S10). In addition, only p-US or s-US treatment of BMSCs did not promote osteogenic differentiation through alizarin red S staining or qRT-PCR assay (Fig. S11).

Next, we further evaluated the cell migratory response of stem cells to SDF-1 and BMP-2 released by p-US stimulation, we plated exogenous BMSCs into the transwell insert and put the BSC loading with SDF-1 or/ and BMP-2 in the bottom of the lower chamber. Irradiation by p-US was applied to the BSC at the above optimal parameters for 2 min to promote the release of chemokines from BSC (Fig. 3e). Obviously, significantly more BMSCs migrated toward the bottom compartment after 12 h in comparison to the ones which did not receive with ultrasonic stimulation. It is notable that BSC loading with both SDF-1 and BMP-2 attracted the most BMSCs to the bottom chamber after p-US irradiation, achieving 6.44-, 1.71- or 2.00-fold higher than control or those with only SDF-1 or BMP-2 at the same ultrasonic stimulation, respectively (Fig. 3f and g). Additionally, compared to groups without p-US irradiation, the groups with p-US irradiation had increased number of BMSCs migrating into the lower chamber of transwell (Fig. 3f and g). No apparent damages to cell viability were observed for these BMSCs exposed to SDF-1 or/and BMP-2, or medium from BSC loading with SDF-1 or/and BMP-2 irradiated by p-US (acoustic pressure intensity = 0.3 MPa, duty cycle = 50%, duration = 2 min) (Fig. S4d).

# 3.3. In vitro BMSCs capture and osteogenic differentiation of ARS with s-US

Our previous study has demonstrated the acoustically responded hollow glass cylindrical shell structure can capture sub-wavelength

polystyrene particles or cells (5  $\mu$ m) on the inner surface, attributing to the significantly enhanced acoustic trapping force originating from the resonant excitation of low order circumferential modes intrinsically existing in the cylindrical shell [14]. Here, we examined whether the ARS could capture BMSCs to its outer surface through exciting the ARS at the resonance frequency since the structure of ARS was soft and made of biocompatible PLA but not glass (Fig. 4a). Before testing it, we first determined the resonance frequency of ARS to excite enhanced gradient field and generate trapping force. As shown Fig. 4b, the experimental transmission spectra at incidence for the single PLA cylinder, showing a remarkable dip at the frequency of 1.5 MHz. The numerical pressure field at the frequency of 1.5 MHz is shown in Fig. 4c. It is clearly observed that the field in radial direction is gradient and its energy decays away from the surface of the cylinder, while the field in the circumferential direction is standing wave and forms four orders. That means that the resonant dip originates from the excitation of the fourth circumferential resonance mode in the PLA cylinder (Fig. 4d). The acoustic radiation force distribution for cells with radius of 5 µm placed around the cylinder at the resonant frequency is shown in Fig. 4e, which is calculated based on the Gor'kov formula [50]. It clearly revealed that the distributions of acoustic radiation forces are centrosymmetric around ARS. Cells can experience a stable trap acoustic radiation force at the eight pressure minima. To avoid overheating effect on the cells during their trapping, we optimized the sinusoidal continuous wave acoustic parameters (s-US) as follows, frequency: 1.5 MHz, duration: 2 min, acoustic pressure intensity: 0.2 MPa (Fig. S12). To confirm the cell capture capability of ARS under s-US stimulation, we firstly examined the performance of single ARS in a microfluidic chamber at 1.4 MHz, 1.5 MHz or 1.6 MHz excitation frequencies (Fig. S13). Obviously, ARS could capture BMSCs with s-US at the frequency of 1.5 MHz, and the number of captured cells increased with the extension of the ultrasonic duration (Fig. 4f and h). By contrast, hardly BMSCs could be captured on the ARS when the ultrasonic frequency was tuned to non-resonant 1.4 MHz or 1.6 MHz (Fig. 4f and h and Movie S1). The two-dimensional mesh-like ARS was also confirmed its capture capability of BMSCs at the frequency of 1.5 MHz with s-US, but not without s-US irradiation at non-resonant frequency (Fig. 4g and Movie S2). Interestingly, significantly more BMSCs could adhesively grow on the surface of ARS after once-a-day s-US irradiation for successive 7 days, with 5.94-fold higher than ARS that did not receive with s-US, and BMSCs on ARS with s-US stimulation showed better spreading ability and lateral expansion than these on ARS without s-US stimulation (Figs. 4i, 4j, S14). An elevated temperature could be observed with the increase of duration or acoustic intensity amplitude, but the heating effects caused by s-US could be controlled below 37 °C at 0.2 MPa acoustic intensity (even if duration for 20 min) (Figs. 2g and S2b). No apparent damages to cell viability were observed for these BMSCs irradiated by s-US with acoustic pressure intensities below 0.2 MPa, even for 5 days culture with daily s-US irradiation, confirming its safety in the in vitro condition. (Figs. S3a, S3c, S4a). Thus, s-US parameters for BMSCs capture of ARS were determined as frequency = 1.5 MHz and duration = 20 min.

To evaluate osteogenic differentiation ability of BMSCs on ARS with s-US. Alizarin red S staining revealed that BMSCs on ARS with s-US had more calcium nodules, compared to those on ARS without s-US (Fig. S15a). Furthermore, qRT-PCR revealed that the Runx-2 mRNA level of BMSCs on ARS with s-US significantly increased (Table 1, Fig. S15b).

#### 3.4. In vivo US-triggered drug release from BSC for stem cell recruitment

Next, we further examined the capability of drug release from BSC in the *in vivo* environment. For the convenience of observation, ICG was used as a model drug loaded in the alginate hydrogel of BSC which was subcutaneously transplanted into the rats. The ICG-loaded BSCs were received with or without daily 20-min p-US irradiation, followed by evaluation the drug diffusion from BSC via an In Vivo Imaging System



Fig. 4. Capture of BMSCs by ARS combining with s-US. (a) Schematic illustration of the capture of BMSCs by ARS with s-US irradiation. (b) The experimental transmission spectra at incidence for the single PLA cylinder, both showing a remarkable dip at the frequency of 1.5 MHz. (c) The simulated normalized pressure field at the resonant frequency was shown. The field in radial direction is gradient and its energy decays away from the surface of the cylinder, while the field in the circumferential direction is standing wave and forms four orders. (d) The simulated acoustic radiation force map exerted on cells around the cylinder at the resonant frequency. The color map and arrows indicate the magnitude and direction of the acoustic radiation force. (e) The radial (blue line) and circumferential (red line) components of the acoustic radiation force at the surface of cylinder. These blue hollow circles denote the stable trapping positions. (f) Snapshot images of single ARS trapping BMSCs from the beginning to 120 s. ARS was irradiated by s-US at resonate 1.5 MHz or non-resonate 1.4 MHz and 1.6 MHz frequencies. BMSCs were stained with calcein AM. Scale bar = 300  $\mu$ m. (g) Snapshot images of the ARS trapping BMSCs with or without s-US at the resonat frequency. Scale  $bar = 150 \ \mu m$ . (h) Quantitative analysis of the number of BMSCs attached onto the surface of ARS in (f),(n = 3). (i) Lightmicroscopic images of local ARS received with or without s-US irradiation at 1.5 MHz for daily 2 min for continuous 7 days. A large number of attached BMSCs could be observed on the surface of ARS received with s-US irradiation but not on the ARS without s-US irradiation. Scale bar = 100 µm. (j) Quantitative analysis of the number of BMSCs attached onto the surface of ARS in (i),(n = 3). Data in g is expressed as means  $\pm$  s.d. of three independent experiments. P values in (h) were determined by one-way ANOVA with Turkey's multiple comparisons test for multiple comparisons. P values in (j) were determined by two-tailed student's t-test. \*\*, *P* < 0.01.

(IVIS Spectrum). As shown in Fig. 5a and b, significantly larger drug diffusion areas were able to be observed in the BSC-transplanted rats received with p-US irradiation from day 1 to day 5 than these rats without p-US irradiation. Quantitative analysis revealed there were comparable fluorescence signal intensities at the BSC-transplanted site, suggesting more drugs were released from p-US-irradiated BSCs since

they diffused larger areas than that of non-irradiated BSCs (Fig. 5c). Also, we examined the degradation rate of alginate hydrogels when they were daily exposed to p-US for more days. From Fig. 5a and d, we can see that the degradation rate of alginate hydrogel in rats was significantly accelerated under the condition of p-US irradiation, without any fluorescence signals existing at the BSC-transplanted site after 12 days. By



Fig. 5. In vivo drug release for recruitment and capture of host stem cells. (a) In vivo fluorescence images of rats treated with or without daily 20-min p-US irradiation for 12 days, revealing gradually increased fluorescent areas in the rats received with p-US irradiation from day 0 to day 5 and indicating the accelerated degradation of alginate hydrogel in rats received with p-US irradiation. The ICG-loaded BSCs were used and subcutaneously transplanted into the rats. These circles marked with white-dotted lines denote the initial site of BSCs. The representative images of rats at day 0, day 1, day 2, day 3, day 4, day 5, day 7, day 10 and day 12 were presented. (b-c) Quantitative analysis of the fluorescent areas (b) and total radiant efficiency (c) for the rats received with or without p-US irradiation. (d) Quantitative analysis of the mean radiant efficiency of the rats received with or without p-US irradiation. (e) Fluorescence images of BSCs isolated from rats received with or without daily 20-min p-US irradiation after 7 days. The host cells recruited into the hydrogels were stained with calcein AM. Scale bar = 50  $\mu$ m. (f) Flow cytometry analysis of the percentage of CD45 negative and CD29 positive cells in these recruited host cells. (g) Fluorescence images of ARS isolated from rats received with or without daily 20-min s-US irradiation after 7 days. The host cells adhesively grew on the surface of scaffolds were stained with calcein AM. Scale bar = 50  $\mu$ m. (h) Flow cytometry analysis of the percentage of CD45 negative and CD29 positive cells in these adhesively grown host cells. Data in (b), (c), (d), (f) and (h) were expressed as each replicate of three independent experiments. P values were determined by two-tailed student's t-test. \*, P < 0.05

contrast, still fluorescence signals retained at the BSC-transplanted site for those non-irradiated rats after 12 days. Considering the *in vitro* results that the daily ultrasonic stimulation with p-US greatly accelerated loss rate of hydrogel weight, it is reasonable to infer that p-US stimulation can promote the degradation of alginate hydrogel in the rats, resulting in the faster decay of fluorescence signals, which was also confirmed in Fig. S16.

To further investigate whether the p-US-triggered release of SDF-1 and BMP-2 from BSC can recruit host stem or progenitor cells *in vivo*, we implanted the SDF-1/BMP-2-loaded BSC into a 4.5 mm femoral defect in the rats and received with or without daily p-US irradiation. One week later, these implanted BSC were removed and stained with calcein AM for labelling of these recruited host cells. It clearly showed there were significantly more stained cells in the BSC received with daily p-US irradiation than that without p-US irradiation (Fig. 5e), consistent with the *in vitro* BMSCs recruitment results (Fig. S17). Flow cytometry assay revealed 17.9% of recruited host cells were negative for CD45 but positive for CD29 in the p-US irradiated BSC, confirming their stem or progenitor cell phenotype. By contrast, only 1.95% of CD45 negative and CD29 positive cells could be found in the BSC that did not receive with p-US irradiation (Fig. 5f). Similar experiments were also used to test the in vivo cell capture ability of ARS by s-US stimulation, using ARS instead of BSC implanted into the femoral defect of the rats and received with or without daily s-US irradiation for 7 days. Numerous cells could be observed on the ARS scaffolds received with s-US irradiation but not on the one without s-US irradiation (Figs. 5g and S18). Flow cytometry assay after isolating these ARS-attaching cells with trypsin digestion revealed 4.59% of CD45 negative and CD29 positive stem or progenitor cells on the s-US irradiated ARS, approximately 12.4-fold higher than the stem cell number on the ARS without s-US irradiation (Fig. 5h). Thus, our results indicated the SDF-1 and BMP-2 can be released on demand from BSC by p-US irradiation and effectively recruit host stem or progenitor cells towards BSC *in vivo*, and ultrasonic stimulation by s-US irradiation can actively capture these endogenous stem or progenitor cells to adhesively grow on the ARS.

# 3.5. Bone defect repair in rats

To further explore the bone defect repair efficacy of BSC combined



with p-US and s-US irradiation, a femoral defect rat model was developed and received with six kinds of different treatments, including (i) Sham: no scaffold implantation and any ultrasonic irradiation, (ii) ARS + p-US + s-US: the defect was implanted with ARS without chemokines and received with p-US and s-US irradiation, (iii) BSC: the defect was implanted with BSC with SDF-1 and BMP-2 chemokines but did not receive with ultrasonic irradiation, (iv) BSC + p-US: the defect was

Fig. 6. In vivo bone defect repair performance of BSC in rats. (a) Schematic illustration of in vivo therapeutic schedule of with BSC combined with p-US and s-US irradiation. After implantation of ARS or BSC, the p-US was first applied for 2 weeks, and then s-US was used for another 2 weeks. After 3 months, the rats were sacrificed and the femur samples were isolated. (b) Representative µCT 3D reconstructed images (top row) and sagittal images (bottom row) of femoral defects in rats received with different treatment groups. (c-e) Quantitative analysis of the mineralization presented in BV/TV (c), BV (d) and BMD (e) values. n = 5 animals for all groups. P values in (c–e) were determined by one-way ANOVA with Turkey's multiple comparisons test for multiple comparisons. \*\*, P < 0.01. (f) Histological staining images of the femoral defects after 3 months, showing better osteogenesis in the rats received with BSC combined with p-US and s-US irradiation (right panel). Scale bar = 1500 µm. Highmagnification images of the regions highlighted by the red box are shown in the left panel. Scale bars = 250  $\mu$ m. (g) Masson's trichrome staining images of the regenerated bone in femur. Large amounts of fibroblastic connective tissue (CT) and mature bone island (NB) can be observed in the rats treated with BSC combined with p-US and s-US irradiation, but not in the other groups (right panel). Scale bar = 1500 µm. Highmagnification images of the regions highlighted by the black box are shown in the left panel. Scale bar = 250  $\mu$ m. BV/TV: bone volume-to-tissue volume. BV: bone volume. BMD: bone mineral density. CT: connective tissue. S: BSC or ARS. NB: mature bone island.

implanted with BSC with SDF-1 and BMP-2 chemokines and only received with p-US irradiation, (v) BSC + s-US: the defect was implanted with BSC with SDF-1 and BMP-2 chemokines and only received with s-US irradiation, (vi) BSC + p-US + s-US: the defect was implanted with BSC with SDF-1 and BMP-2 chemokines and received with p-US and s-US irradiation. As can be seen from the *in vivo* therapeutic schedule, ultrasonic irradiation by p-US was firstly applied for 14 days and then followed by s-US for another 14 days (Fig. 6a). Then, the femurs were

harvested to assess the newly-formed bone within the defect by using of a commercially available microcomputed tomography ( $\mu$ CT) system. As shown in Figs. 6b and S19, the sham group showed minimal new bone formation within the defect, revealing the self-repairing efficacy, while defects treated with BSC, ARS + p-US + s-US, BSC + p-US or BSC + s-US groups only showed limited benefits for new bone formation. By contrast, BSC + p-US + s-US group exhibited the strongest bone repair efficacy, achieving nearly complete repair of bone defects after 3



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Fig. 7. Biomechanical measurement of newly formed bone in femoral defects. (a) Pressure time curves of newly formed bones in rats' femoral defects among different groups. The dashed area denote the energy required to cause a new bone to fracture. (b) The fracture strain of different groups, n = 3. P values were determined by one-way ANOVA with Turkey's multiple comparisons test for multiple comparisons. \*, P <0.05; \*\*, P < 0.01; \*\*\*, P < 0.001. (c) The topography and elasticity of rats' femur determined by AFM from sham and BSC + p-US + s-US groups. Bone normal reagions were used as the control when determining the topography and elasticity of each rats' femur. Scale bar = 1  $\mu$ m. (d) The Young's moduli of rats' femur determined by AFM from sham and BSC + p-US + s-US groups. Orange stands for the Young's moduli from bone defect reagions and gray for the Young's moduli from bone normal regions.

months. Significantly increased percent of bone volume (bone volume to-tissue volume, BV/TV), bone volume (BV), bone mineral density (BMD), trabecular number (Tb. N), and trabecular thickness (Tb. Th.) and trabecular separation (Tb. Sp.) were also found in the newly formed bone of rats received with BSC + p-US + s-US treatment in comparison with other groups (Fig. 6c–e, S20). All of these data from our *in vivo*  $\mu$ CT data indicated that bone defects treated with BSC + p-US + s-US produced significantly higher bone mass in trabecular bone and better mineralization architecture in rats, compared to rats treated with BSC, ARS + p-US + s-US, BSC + p-US or BSC + s-US groups after 3 months.

In addition, the bone histomorphometry analysis by H&E and Masson's trichrome staining showed that rats treated with BSC or/and single ultrasonic stimulation appeared numerous visible areas of osteoid and dense connective tissue at the defect site (Fig. 6f and g). By contrast, extensive formation of new bone that was organized similar to healthy bone was observed in the group treated with BSC + p-US + s-US (Figs. 6f, 6g, S21). No inflammation or necrosis was observed from all the groups, revealing both BSC and ultrasonic stimulations had a good biosafety (Fig. 6f). Besides, there were not significant histological morphological and pathological changes observed in the main organs of all of these groups from H&E histological staining of heart, liver, spleen, lung and kidney (Fig. S22).

# 3.6. Biomechanical examination

To test the biomechanical properties, the newly formed bone samples were placed under a plane dynamometer system. The dynamic indentation testing was performed in the axial direction of the bone with a diameter of 1 mm and the velocity of 0.1 mm/s. The pressure time curve of new bones in rats' femoral defects among different groups were showed in Fig. 7a. From these pictures, we can see that loading of minimal force could cause new bone fracture in the sham group, revealing the poor bone self-repairing quality after 3 months. Newly formed bones in the defects treated with BSC, ARS + p-US + s-US, BSC + p-US or BSC + s-US groups just showed limited benefits. By contrast, BSC + p-US + s-US group exhibited the best bone repair quality, achieving nearly normal bone biomechanical performance after 3 months. Significantly increased fracture strain was also found in the newly-formed bone of rats received with BSC + p-US + s-US, achieving 1395.76  $\pm$  74.02 MPa versus 419.11  $\pm$  125.54 MPa, 586.03  $\pm$  77.42 MPa, 835.46  $\pm$  73.84 MPa for BSC, ARS + p-US + s-US, BSC + p-US groups, respectively (Fig. 7a and b). The Young's moduli of the newly formed bone samples from sham and BSC + p-US + s-US groups were also evaluated by bimodal atomic force microscopy. Note that, the value of Young's modulus was not calibrated by reference sample. It clearly revealed there was significantly lower Young's moduli in the newly formed bone in comparison to its surrounding normal bone for the sham group although the new bone appeared a similar topography with surrounding normal bone (P < 0.05) (Fig. 7c). The median Young's moduli values decreased to 72.18% of surrounding tissue after 3 months (Fig. 7d). Interestingly, the newly formed bone from BSC + p-US + s-USgroup showed a comparable Young's moduli with its surrounding normal bone, achieving 99.23% of surrounding tissue after 3 months (Fig. 7e and f). These fibrils were in short length, which might account for the mineral plates covering the underlying collagen (Fig. S23). The stiffness of new bone was far harder than hydrogel of BSC (Fig. S24). All of these data indicated that the bone defects treated with BSC + p-US + s-US resulted in the new bone formation with significantly better biomechanical performance in comparison with sham, BSC, ARS + p-US + s-US or BSC + p-US groups after 3 months.

# 4. Discussion

BMSCs-based tissue engineering has provided promising therapeutical options for patients with tissue defects through a variety of ways [51-55]. For instance, freshly isolated stem cells can be

transplanted directly into tissue and induced their differentiation into a desired cell type [21,56]. In many cases, stem cells are able to be genetically engineered to express specific genes or pre-differentiated into a particular cell type in order to enhance lineage-specific differentiation [57]. Literatures have reported that the direct injection of BMSCs from different tissue sources were able to engraft and regenerate to repair bone defects after implantation in different types of animals [58, 59]. In addition to direct stem cell injection, researchers have used tissue engineering approaches by delivering stem cells on biodegradable scaffolds [15,60]. In this case, several groups show that pre-differentiating or pre-manipulating stem cells in vitro prior to implantation could be beneficial to generate a particular cell phenotype [61,62]. In spite of these successful cases, exogenous stem cells-based tissue engineering encountered many unconquerable problems, such as insufficient source, sophisticated technological process and risk of tumorigenesis. By contrast, endogenous stem or progenitor cells combined with functional scaffolds provide a promising alternative to cell-based strategies, especially for bone tissue engineering. Researchers designed different scaffolds for repairing bone defects, such as magnetic lanthanum-doped hydroxyapatite/chitosan scaffolds, aptamer-bilayer scaffolds and biomimetic nanosilica-collagen scaffolds [36,63]. Although these studies can promote the recruitment of endogenous stem cells to some degree, but these works cannot actively capture endogenous BMSCs onto the scaffolds, a key step for bone repairing in the tissue engineering field. In this study, we designed an acoustically responsive scaffold to capture endogenous BMSCs onto the scaffolds via a focused field-based acoustic tweezer technology, contributing to the significantly enhanced acoustic radiation force around scaffold surfaces which originates from the resonant excitation of low order circumferential modes [14]. The highly localized field around scaffolds and enhanced acoustic tapping force make stem cells move towards the scaffold, facilitating their adhesive growth and osteoblastic differentiation on the scaffold.

It is notable that the effective cell-trapping distance for ARS is limited, making it difficult to capture distant cells beyond its acoustic field arrange. Therefore, it is another important issue to effectively recruit host stem or progenitor cells to be close to ARS in the in vivo condition. To address it, we embedded the ARS scaffold into the hydrogels which loaded SDF-1 and BMP-2 factors in this study. In fact, BMP-2 has been approved by the U.S. Food and Drug Administration and used for promoting bone formation in clinical practice, such as anterior lumbar interbody fusion, tibial fractures, and sinus augmentation. Research has also shown that BMP-2 can accelerate BMSCs migration through CDC42/PAK1/LIMK1 pathway [64]. Document also demonstrated that SDF-1 is an important factor involved in tissue repair by mediating migration of BMSCs to the site of tissue injury [65]. The combined choice of SDF-1 and BMP-2 attributes to their cooperative functions not only in favoring the migration of BMSCs, but also in promoting their osteogenic differentiation of BMSCs (Fig. 3a-d). Notably, the calcium ions in the alginate hydrogels could also be released upon receiving with p-US stimulation. It was reported that the exposure of primary osteoblasts or osteoblast-like cells to high levels of Ca<sup>2+</sup> in the extracellular fluid may stimulate osteoblast proliferation, differentiation and matrix mineralization capacity via activation of mitogen-activated protein kinases and/or c-Jun N-terminal kinase [66]. In this study, we found that the osteogenic related genes were significantly up-regulated in the BMSCs on BSC loading with SDF-1/BMP-2 with p-US treatment. In fact, Runx-2 is not only an early marker of osteogenic differentiation, but also can act as a transcription factor to stimulate osteogenic differentiation-related proteins (such as OCN, OPN) [67].

However, too high doses of BMP-2 can possibly result in some unintended side effects, such as ectopic bone formation, nerve damage, and significant inflammation. Thus, the release of the factors can be triggered on demand by acoustic irradiation to avoid the unintended side effects, attributing to the utilization of alginate hydrogels which were able to be disrupted by acoustic energy. Indeed, our study demonstrated duration- and intensity-dependent protein release from BSCs by p-US irradiation (Fig. 2h and i), resulting in more BMSCs migrating toward the bottom chamber from the transwell insert *in vitro* or more host stem or progenitor cells recruiting to the BSC *in vivo* (Fig. 5e–h). Interestingly, alginate hydrogels would be degraded completely after 14-day p-US irradiation, which was important for exposing the ARS to these recruited host stem cells for further cell capture by s-US irradiation.

In our study, we used 3D printing to fabricate the ARS, making it possible accurately control the structure. The rough surface of ARS greatly facilitates cell adhesion, nutrient exchange and blood vessel regeneration [68-71]. The transmission spectrum of the scaffold showed that its low-order resonance frequency was about 1.5 MHz. Further simulation of the acoustic field and acoustic radiation force by Comsol showed that the resonance excitation of the inherent low-order circumferential mode of the ARS was highly localized on the surface of ARS, indicating that the resultant force potential had a large gradient and significantly enhanced the directed movement of cells. As expected, our *in vitro* and *in vivo* experiments confirmed that stem cells could be captured onto the ARS under s-US irradiation. Due to the strong gradient of acoustic intensity, the power used to manipulate the cells were relatively small, which will reduce the damage to normal cells or healthy tissues. More importantly, the acoustic energy used in our study is relative low (<0.5 MPa), which did not produce tissue damage [72]. Thus, ARS designed in this study had the advantages of low frequency, low input power and non-invasiveness in the remote capture of BMSCs under acoustic irradiation.

Given that the repairing of bone defect is a multi-stage physiological process [22], p-US irradiation was used in the fibrovascular stage of bone repairing to mediate the release of SDF-1 and BMP-2 from BSC for efficient recruitment of distant endogenous BMSCs. In the bone formation stage of bone repairing, s-US irradiation was used to capture these recruited BMSCs. In order to avoid the hydrogel obstructing the movement of BMSCs onto the ARS when s-US irradiation, successive 14-day p-US stimulation (20 min for each day) was used in this study to accelerate the degradation of calcium alginate hydrogel. Thus, the ARS embedded inside BSC could be exposed to these recruited host stem or progenitor cells before bone formation stage. Our in vitro and in vivo experiments confirmed that the hydrogel completely degraded after 12 days of p-US treatment (Figs. 2k, 5a, 5d, S16). Besides, the synergistic effect of SDF-1 and BMP-2 could effectively promote the osteogenic differentiation in the early stage of bone repair before the hydrogel was completely degraded (Fig. 3a-d, S9). As showed in Figs. 6 and 7, implanted BSC combined with p-US for drug release and s-US stem cells for capture could significantly repair the bone defect.

#### 5. Conclusion

In summary, we designed an ARS and embedded it into SDF-1/BMP-2 loaded alginate hydrogel to form BSC to recruit endogenous BMSCs and to capture them onto the scaffold for in situ bone tissue regeneration. The p-US irradiation was used to disrupt the alginate hydrogel for accelerating its degradation and releasing the encapsulated SDF-1/BMP-2 cytokines, recruiting distant host endogenous BMSCs towards the bone defected site. After these cells were recruited and the hydrogel was degraded in the fibrovascular stage of bone repairing, the ARS was exposed to these recruited stem cells. The s-US irradiation was then used to capture these cells and help them adhesively grow onto the scaffold, greatly favoring the repairing of bone defects. Taken together, our study presents a novel and effective strategy for manipulating endogenous stem cells for bone defect repairing.

# CRediT authorship contribution statement

Yanni He: Project administration, Methodology, Data curation, Formal analysis, Writing – original draft, Visualization, Funding acquisition. Fei Li: Project administration, Validation, Methodology, Data curation, Writing – original draft, Software. **Peng Jiang:** Project administration, Validation, Methodology, Data curation, Writing – original draft, Software. **Feiyan Cai:** Writing – review & editing, Software. **Qin Lin:** Methodology, Data curation, Software. **Meijun Zhou:** Validation, Methodology, Funding acquisition. **Hongmei Liu:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Fei Yan:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

# Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.08.012.

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