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Original Paper

IL-8 Enhances Therapeutic Effects of BMSCs on Bone Regeneration via CXCR2-Mediated PI3k/Akt Signaling Pathway

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Key Words
Interleukin-8 (IL-8) • Tissue engineered bone (TEB) • Bone marrow mesenchymal stem cells (BMSCs) • CXCR2 • PI3k • Akt

Abstract
Background/Aims: Tissue engineering bone transplantation with bone marrow mesenchymal stem cells (BMSCs) is an effective technology to treat massive bone loss, while molecular regulation of the bone regeneration processes remains poorly understood. Here, we aimed to assess the role of interleukin-8 (IL-8) in the recruitment of host cells by seeded BMSCs and in the bone regeneration. ***Methods:*** A transwell assay was performed to examine the role of IL-8/CXCR1/CXCR2/PI3k/Akt on the migration potential of hBMSCs. The *in vitro* chondrogenic differentiation of hBMSCs was assessed by examination of 2 chondrogenic markers, Sox9 and type 2 collagen (COL2). mBMSCs were used in tissue engineered bone (TEB) with/without IL-8 implanted into bone defect area with CXCR2 or Akt inhibitors. Density and Masson staining of the regenerated bone were assessed. The chondrogenesis was assessed by expression levels of associated proteins, Sox9 and COL2, by RT-qPCR and by immunohistochemistry. ***Results:*** IL-8 may trigger *in vitro* migration of hBMSCs via CXCR2-mediated PI3k/Akt signaling pathway. IL-8 enhances osteogenesis in the TEB-implanted bone defect in mice. IL-8 induces chondrogenic differentiation of hBMSCs via CXCR2-mediated PI3k/Akt signaling pathway *in vitro* and *in vivo*. ***Conclusions:*** IL-8 enhances therapeutic effects of MSCs on bone regeneration via CXCR2-mediated PI3k/Akt signaling pathway.

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Introduction

Complex fractures can be caused by traffic accidents, resection of bone tumors, and debridement of a wide range of bone infections [1-5]. When the bone loss exceeds a volume threshold, the bone fails to repair the defect through autologous regeneration, and requires therapeutic approaches to maintain the limb length and restore limb function. Currently, the treatment of massive bone loss includes autologous bone transplantation, allograft bone transplantation, tissue engineering bone transplantation, et al. Although autologous bone graft is an ideal method of repair, the limited supply of bone source as well as bone surgery-associated infection, pain and other complications prevented its wide application [6]. Tissue engineered bone provides new strategies for the treatment of bone defects [7, 8]. However, the current tissue engineering bone suffers from several shortcomings like lack of osteoconductive and limited sources of seed cells [9]. The seed cells used for engineering bone are mainly osteoblasts, human umbilical cord mesenchymal stem cells, embryonic stem cells, adipose-derived stem cells, transgenic stem cells and bone marrow mesenchymal stem cells (BMSCs). BMSCs are easy to be obtained in a non-invasive way. The strong proliferative capacity *in vitro* and multiple differentiation potential of BMSCs make them ideal seed cells for massive bone repair. BMSCs that are used for decalcified bone matrix scaffold tissue engineering bone have been shown to have osteoinductivity and osteogenic, while its osteogenic potential remains poorly understood [10, 11].

The role of seed cells in the process of osteogenesis has been thought to stem from the proliferation and differentiation into osteoblasts by the seed cells on the tissue engineering bone to complete the bone repair [12-15]. However, recent studies showed that the seed cells contributed less to the later repaired tissues and most cells that participate into bone repair were derived from the host cells that had migrated to the site of injury to promote wound healing [13, 16-18]. It was suggested that seed cells release a variety of cytokines such as IL-2, IL-6, IL-8, SDF-1, and TNF to promote the migration and differentiation of host cells [13, 16-18]. Under physiological conditions, the concentration of IL-8 is extremely low, but could be rapidly induced by proinflammatory cytokines such as TNFa and IL-1b [19]. Thus, the role of IL-8 in the recruitment of host cells in the bone repair is interesting to be investigated.

IL-8 is a member from CXC cytokine family [20]. IL-8 can be produced by a variety of cells, such as macrophages, epithelial cells, airway smooth muscle cells and endothelial cells [20]. MSCs also secrete IL-8, which regulates MSC function in both autocrine and paracrine manners [20]. IL-8 effects through binding to its receptors CXCR1 or CXCR2 [20]. Both CXCR1 and CXCR2 are members of the G-protein coupled receptor (GPCR) family and have a similar transmembrane membrane structure [20]. Activation of CXCR1 or CXCR2 by IL-8 activates the intracellular PI3K and MAPK pathway, and then exert effects in a cell-type-dependent manner [21]. PI3K pathway activation leads to Akt phosphorylation, which is critical for cell survival, angiogenesis and cell migration [22-24]. However, the role of IL-8 in osteogenesis repair through its receptor CXCR1 or CXCR2 remains to be elucidated [24-29]. A previous study has shown that IL-8 can recruit BMSCs to repair cartilage defects [30]. Another study showed that IL-8 promotes BMSCs to differentiate into cartilage via CXCR2 [31]. Therefore, in the current study, we aimed to study the effects of IL-8 on therapeutic effects of MSCs on bone regeneration and its underlying mechanisms.

Materials and Methods

Human BMSCs and in vitro migration experiments

Bone marrow was obtained from healthy volunteers who signed the informed consent form. Fresh bone marrow was diluted with 0.1 M phosphate buffer (Gibco, Carlsbad, CA, USA) and the nucleated cells in bone marrow were obtained by density gradient centrifugation. The obtained cells were cultured in DMEM/F12 medium (GE Healthcare Life Sciences HyClone Laboratories, Utah, UT, USA) containing 12% fetal bovine

serum in an incubator at a concentration of 5% carbon dioxide at 37 ° C to obtain adherent spindle-shaped swirl-like growth cells, which could be induced to differentiate into Bone, fat and cartilage. Adherent culture was used to obtain bone marrow mesenchymal stem cells (hBMSCs). Transwell chambers (8 μm pore size, Corning Co., NY, USA) were added to 200 μl of hBMSCs at a concentration of 1 $X10^5$ / ml and then 500 μl of drug-containing DMEM/F12 medium was added to a 24-well plate. Control, IL-8, anti-CXCR1 antibody (aCXCR1, R&D System, Los Angeles, CA, USA), SB225002 (Selleck Chemicals, Houston, TX, USA), LY294002 (Selleck Chemicals) and MK22-6 (Selleck Chemicals), weere added in 3 replicate wells. After 36 hours, cells in the upper part of the small cell membrane were wiped off and fixed with paraformaldehyde. The cells were then stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) and examined.

Cartilage-induced culture of hBMSCs

The third generation hBMSCs were used to differentiate into cartilage. The chondrogenic-inducing medium was a medium containing 1 × insulin iron selenide (Gibco), 1% double antibody, 50 mg / mL ascorbic acid and 10 ng / mL transforming growth factor-beta3. In order to form stereotactic culture of hyaline cartilage, 0.5 ml cell suspension with a concentration of $5×10^5$ cells / ml was transferred to a 15 ml polypropylene centrifuge tube and centrifuged at 150 g for 5 min at room temperature. MK-2206 was used as 400uM, SB225002 was used as 10 uM, which were added to IL-8 (100 ng / ml). The incubation was done in a 5% CO_2 incubator at 37 °C. Twenty-four hours after the cell mass aggregation, the bottom of the centrifuge tube was flicked so that the cartilage ball from the bottom of the tube is suspended in the liquid. Chondrocytes were collected for analysis of cartilage-associated transcription factors and marker proteins after 7 days' culture. Pathological analysis of cartilage ball collected at 28 days.

Preparation of mBMSCs

Four-week-old male fvb mice (Third Military Medical University Animal Experimental Center) were killed, after which tibial bone marrow cells were obtained under aseptic conditions. Mouse bone marrow mesenchymal stem cells (mBMSCs) were obtained by whole-bone culture, as described [32]. The phenotype of mBMSCs were evaluated by flow cytometry analysis of the surface markers Sca-1, CD90, CD105, CD34, CD45 and HLA-DR, and inducible differentiation of mBMSCs into osteocytes, adipocytes and chondrocytes with Osteocyte Differentiation Tool (American Type Culture Collection (ATCC), Rockville, MD, USA; Catalog number: PCS-500-052), Adipocyte Differentiation Toolkit (ATCC; Catalog number: PCS-500-050) and Chondrocyte Differentiation Tool (ATCC; Catalog number: PCS-500-051), respectively. Alcian blue staining, Von kossa staining and Oil red O staining were performed for detecting differentiated chondrocytes, osteocytes and adipocytes, respectively.

Construction of mouse tissue engineered bone (TEB) with/without mouse IL-8

The mBMSCs were transduced with adeno-associated virus (AAV) carrying recombinant mouse IL-8 to obtain mBMSCs/IL-8 under the control of a CMV promoter. Briefly, the control scrambled construct or mouse IL-8 coding sequence was cloned into a pAAV-CMV-RFP plasmid (Clontech, Mountain View, CA, USA). To generate AAVs, HEK293T cells (ATCC) were co-transfected with 10 μg of the prepared plasmids and 5 μg each of packaging plasmids using Lipofectamine 3000 (Invitrogen, St. Louis, MO, USA). The viruses were purified using CsCl density centrifugation and the titrated with a quantitative densitometric dot-blot assay. For generating TEB, a 2X2X2 mm-size demineralized bone matrix (DBM) was prepared. The DBM was placed in ultra-low adsorption 24-well plates (Cornin Co.) and 10 μl of mBMSCs or mBMSCs/IL-8 at a concentration of $1X10^6$ / ml was applied to the DBM and placed in a 5% CO_2 incubator at 37 °C for 1 hour. After turning the DBM upside down, 10 ul of mBMSCs or mBMSCs/IL-8 at a concentration of $1X10^6$ / ml was again dropped onto the DBM and placed in a 5% CO_2 incubator at 37 °C for 1 hour. Media is changed every 1-2 days. TEB was removed after one week of culture.

Tissue engineering bone repair of long bone defects in mice

The male eGFP mice of 12 weeks of age (Third Military Medical University Animal Experimental Center) were anesthetized by intraperitoneal injection of 0.5% sodium pentobarbital at a dose of 15 mg / kg. The animals were fixed in a prone position and skin preparation was performed in the conventional leg surgery area with iodophor disinfection. The right limbs knees were put at 90°, after which a longitudinal incision of about 1 cm in length was made on the lateral side of the knee joint. The skin and subcutaneous

tissue were incised and the white muscle line was revealed. Blunt dissection of the rectus femoris muscle and semitendinosus muscle space along the white line was performed to fully expose the femur (Including the greater trochanter), and the screws were firmly fixed in the femoral shaft resection of 2mm length of the backbone and periosteum, to allow the TEB to be implanted into the bone defects. The right lower limb was protected against immobilization to allow free movement, eating and drinking. SB225002 (1 mg / kg / 2 days) and MK-2206 (100 mg / kg / week) were given in the corresponding groups. Morphological histological analysis of the samples taken 14 days after operation.

Micro-CT analysis

Specimens were fixed in paraformaldehyde for 48 hours, after which MicroCT examination was applied.

Histology and immunohistochemistry

After the sample was fixed in paraformaldehyde for 48 hours, it was decalcified in 10% EDTA decalcification solution for 2 weeks. After decalcification was complete, internal fixation was removed and paraffin specimens were prepared into 4um-thickness consecutive sections, for H&E, Masson and Safranin O staining, which were evaluated by three pathologists independently. For immunohistochemistry, sections were washed three times with PBS and then incubated with goat anti-COL2 (Santa Cruz Biotechnology, Dallas, TX, USA), goat anti-Sox9 (Santa Cruz Biotechnology) overnight at 4 °C. After washing with PBS, anti-goat secondary antibody was shown as a red fluorescent donkey. Primary antibody and secondary antibody dilution ratio was 1:100. DNA were stained by DAPI (Sigma-Aldrich). The images were obtained by Lycra laser confocal microscope and analyzed.

Quantitative RT-PCR

The quantitative RT-PCR assay is summarized as follows. Total RNA was extracted by total RNA extraction kit (Qiagen, Valencia, CA, USA) according to instructions. Total RNA is transcribed by reverse transcription kit (Qiagen). The primers used for RT-qPCR were: GAPDH (sense: AGGGCTGCTTTTAACTCTGGT, anti-sense: GGCATGGACTGTGGTCATGAG); Sox9 (sense: CACAAGAAAGACCACCCGGA, antisense: GGAAATGTGCGTCTGTTCGG); COL2 (sense: GGGCAGAGGCAGGAAACTAA, antisense: TGTAGGACACACGCAGTTCC). The average of three cycles was used to calculate gene expression, with GAPDH as an internal control.

Statistical analysis

All values represent the mean ± standard deviation (SD). Statistical analysis of group differences was carried out using a one-way analysis of variance (ANOVA) test (SPSS 19.0, Chicago, IL, USA) followed by the Fisher's Exact Test to compare two groups. A value of $p<0.05$ was considered statistically significant after Bonferroni correction.

Results

IL-8 triggers in vitro migration of hBMSCs via CXCR2

We purchased hBMSCs and put the cells in culture (Fig. 1A). In order to examine whether IL-8 (IL-8) plays a key role in the migration of hBMSCs, as well as through which receptor (CXCR1 or CXCR2) hBMSCs may respond to IL-8, we performed a transwell migration study. The hBMSCs were cultured in the upper chamber of the transwell, while the lower chamber contained either control media (CTL), or media with IL-8 (IL-8), or media with IL-8 and antibody against CXCR1 (aCXCR1; IL-8+aCXCR1), or media with IL-8 and SB225002 (a specific inhibitor for CXCR2; IL-8+ SB225002), or media with IL-8 and LY294002 (a specific inhibitor for PI3k; IL-8+ LY294002), or media with IL-8 and MK-2206 (a specific inhibitor for Akt; IL-8+ MK-2206), respectively (Fig. 1B). The migrated cells after 24 hours' culture were determined, shown by representative images (Fig. 1C), and by quantification (Fig. 1D). Our data showed that IL-8 significantly increased the migrated hBMSCs, while suppression of CXCR2 by SB25002, but not suppression fo CXCR1 by aCXCR1, abolished the effects of IL-8 (Fig. 1C-D). Moreover, suppression of PI3k/Akt signaling also abolished the effects of IL-8

(Fig. 1C-D). Together, these data suggest that IL-8 may trigger *in vitro* migration of hBMSCs via CXCR2-mediated PI3k/Akt signaling pathway.

IL-8 induces chondrogenic differentiation of hBMSCs via CXCR2-mediated PI3k/Akt signaling pathway in vitro

In order to assess *in vitro* chondrogenic differentiation of hBMSCs by IL-8, hBMSCs were treated with either control media (CTL), or media with IL-8 (IL-8), or media with IL-8 and SB225002 (IL-8+ SB225002), or media with IL-8 and MK-2206 (IL-8+ MK-2206), respectively (Fig. 2A). Then the hBMSCs were subjected to chondrogenic differentiation media in micromass culture for 7 days, followed by alcian blue staining (Fig. 2B). The chondrogenic differentiation was assessed by examination of two chondrogenic markers, Sox9 and COL2, by RT-qPCR in the differentiated hBMSCs (Fig. 2C). Our data showed that IL-8 significantly increased the Sox9 and COL2 levels in differentiated hBMSCs, while suppression of CXCR2 by SB25002, or suppression of Akt signaling by MK-2206, similarly abolished the effects of IL-8 (Fig. 2C). These data suggest that IL-8 induces chondrogenic differentiation of hBMSCs via CXCR2-mediated PI3k/Akt signaling pathway *in vitro*.

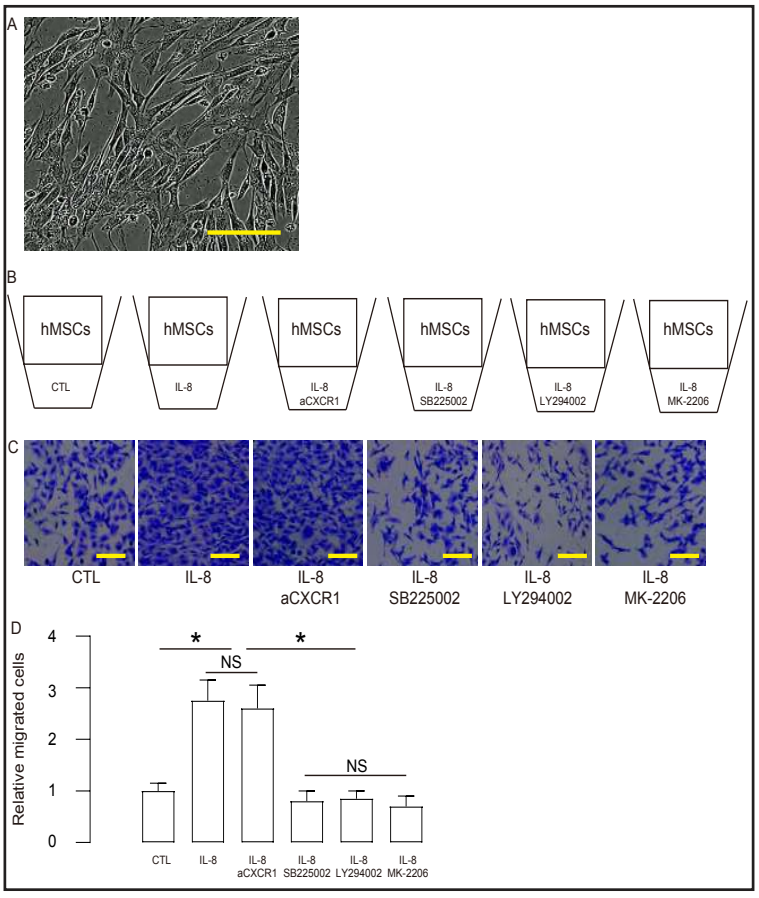

Fig. 1. IL-8 triggers in vitro migration of hBMSCs via CXCR2. (A) hBMSCs in culture. (B) A transwell migration study in which hBMSCs were cultured in the upper chamber of the transwell, while the lower chamber contained either control media (CTL), or media with IL-8 (IL-8), or media with IL-8 and antibody against CXCR1 (aCXCR1; IL-8+aCXCR1), or media with IL-8 and SB225002 (a specific inhibitor for CXCR2; IL-8+ SB225002), or media with IL-8 and LY294002 (a specific inhibitor for PI3k; IL-8+ LY294002), or media with IL-8 and MK-2206 (a specific inhibitor for Akt; IL-8+ MK-2206), respectively. (C-D) The migrated cells after 24 hours' culture were determined, shown by representative images (C), and by quantification (D). *p<0.05. NS: non-significant. N=5. Scale bars are 20uM.

Isolation and characterization of mBMSCs

Next, we isolated mouse BMSCs (mBMSCs) for *in vivo* study (Fig. 3A). The surface marker analysis for Sca-1, CD90, CD105, CD34, CD45 and HLA-DR confirmed an BMSC phenotype (Fig. 3B). Also, mBMSCs were able to be induced to differentiate into chondrocytes, adipocytes or osteocytes (Fig. 3C). Hence, the phenotype of the isolated mBMSCs was confirmed.

IL-8 enhances osteogenesis in the TEB-implanted bone defect in mice

Mouse tissue engineered bone (TEB) with/without overexpression of mouse IL-8 (mBMSCs; mBMSCs/IL-8) was prepared (TEB; TEB/IL-8). A 2mm-length resection of

bone was generated at the right femur of the eGFP-transgenic mice, after which TEB, or TEB/IL-8, or TEB with SB225002, or TEB with MK-2206 was implanted into the bone defect. Morphological histological analysis of the samples were taken 14 days after operation by micro-CT (Fig. 4A). The density of the regenerative bone was determined, showing that IL-8 significantly increased the density of the regenerative bone, while either SB225002 or MK-2206 significantly decreased it (Fig. 4B). Masson staining was performed, showing that IL-8 significantly increased the collagen (Masson+ area) in the regenerated bone, while either SB225002 or MK-2206 significantly decreased it (Fig. 4C). Thus, IL-8 enhances osteogenesis in the injured mouse bone via CXCR2-mediated PI3k/Akt signaling pathway.

IL-8 enhances chondrogenesis-associated proteins in the injured mouse bone

Finally, the chondrogenesis in the regenerated bone was assessed by examination of two chondrogenic markers, Sox9 and COL2, by RT-qPCR (Fig. 5A), and by immunohistochemistry (Fig. 5B). Our data showed that IL-8 significantly increased the Sox9 and COL2 levels in the regenerated bone, while suppression of CXCR2 by SB25002, or suppression of Akt signaling by MK-2206, similarly abolished

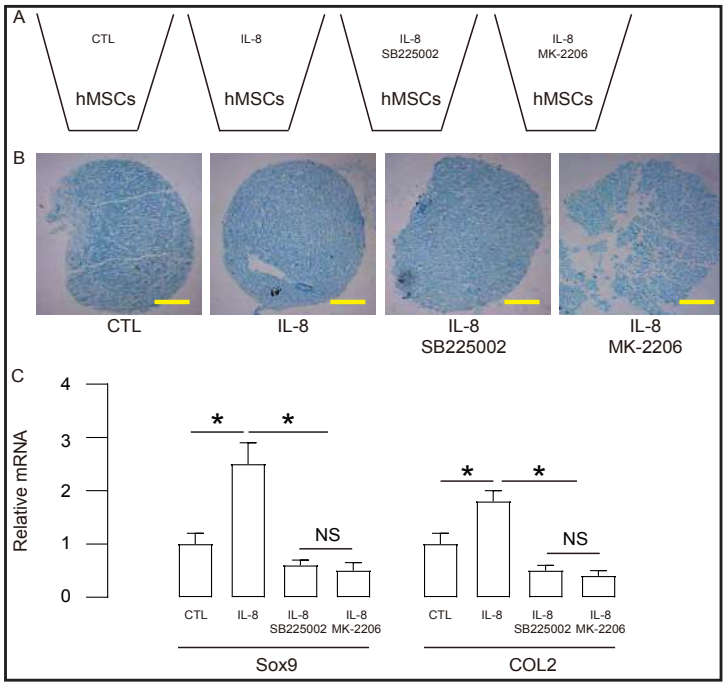

Fig. 2. IL-8 induces chondrogenic differentiation of hBMSCs via CXCR2-mediated PI3k/Akt signaling pathway in vitro. (A) hBMSCs were treated with either control media (CTL), or media with IL-8 (IL-8), or media with IL-8 and SB225002 (IL-8+ SB225002), or media with IL-8 and MK-2206 (IL-8+ MK-2206), respectively, and then in vitro chondrogenic differentiation of hBMSCs was assessed in 7 days. (B) Alcian blue staining. (C) RT-qPCR for Sox9 and COL2 in the differentiated hBMSCs. *p<0.05. NS: non-significant. N=5. Scale bars are 50uM.

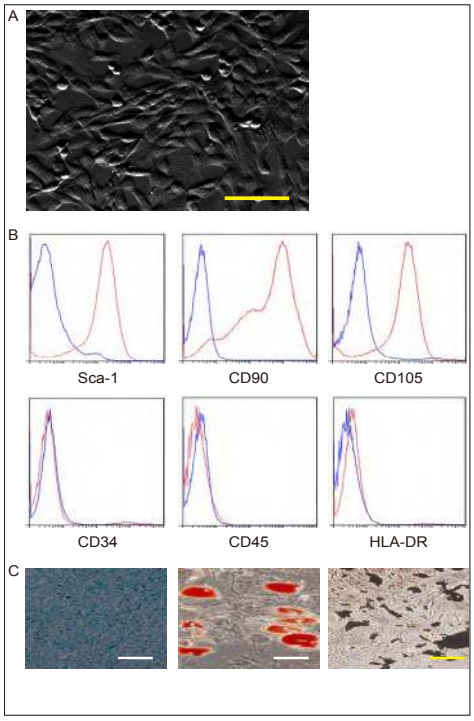

Fig. 3. Isolation and characterization of mBMSCs. (A) Isolated mouse BMSCs (mBMSCs) in culture. (B) Flow cytometry for Sca-1, CD90, CD105, CD34, CD45 and HLA-DR in mBMSCs. Red: antibody. Blue: Isotype. (C) chondrocyte differentiation by Alcian blue staining (left), adipocyte differentiation by Oil red O staining (middle) and osteocyte differentiation by Von kossa staining (right). N=5. Scale bar is 20uM in A and 50uM in C.

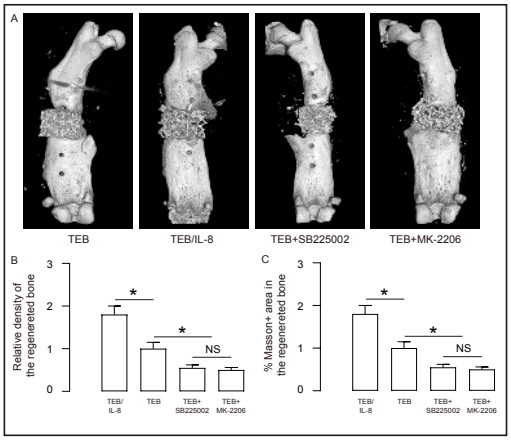

Fig. 4. IL-8 enhances osteogenesis in the TEB-implanted bone defect in mice. (A) Mouse tissue engineered bone (TEB) with/without overexpression of mouse IL-8 (mBMSCs; mBMSCs/IL-8) was prepared (TEB; TEB/IL-8). A bone resection was generated at the right femur of the eGFP-transgenic mice, after which TEB, or TEB/IL-8, or TEB with SB225002, or TEB with MK-2206 was implanted into the bone defect. Morphological histological analysis of the samples was taken 14 days after operation by micro-CT. (B) The density of the regenerative bone. (C) Masson staining. *p<0.05. NS: non-significant. N=10.

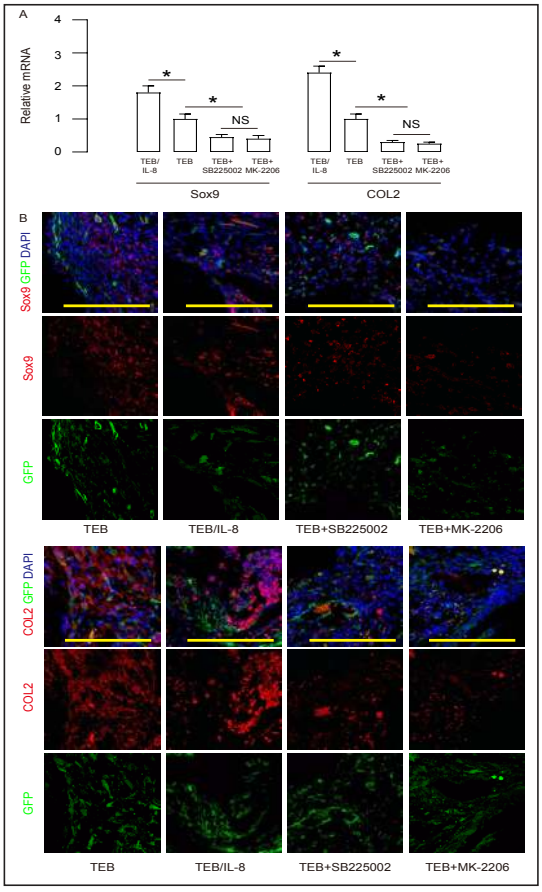

Fig. 5. IL-8 enhances chondrogenesis-associated proteins in the injured mouse bone. (A-B) Chondrogenesis in the regenerated bone was assessed by examination of two chondrogenic markers, Sox9 and COL2, by RT-qPCR (A), and by immunohistochemistry (B). *p<0.05. NS: non-significant. N=10. Scale bars are 50uM.

the effects of IL-8 (Fig. 5A-B). These data suggest that IL-8 enhances chondrogenesis via CXCR2-mediated PI3k/Akt signaling pathway *in vivo*.

Discussion

Bone regenerative capacity is limited, and is unable to repair large bone defects through its own regeneration. Tissue engineered bone graft is one of the ideal ways to repair large bone defects. At present, the technology of tissue engineering bone is yet perfect, and the mechanism of repairing the bone defect using tissue engineering bone remains poorly understood. A previous study found that seed cells contributed less to the final repair of bone tissue, compared to host-derived cells [12]. Here, we showed that BMSCs on tissue engineering bone release a large amount of IL-8, while some studies have shown that IL-8 is a chemotaxis to MSCs *in vitro* [33-36]. In this study, transwell experiments were done to confirm that IL-8 has a chemotactic effect on BMSCs. Chemotaxis of IL-8 disappeared after SB225002 treatment on BMSCs but remained after aCXCR1 treatment, suggesting that IL-8 binds to CXCR2 but not CXCR1 to induce chemotaxis. LY294002 pretreatment of BMSCs inhibited PI3K activity, resulting in disappearance of IL-8 chemotaxis, suggesting that chemotaxis may require activation of PI3K signaling pathway. Moreover, Akt-specific antagonist MK-2206 abolished the effects of IL-8, suggesting that IL-8 enhances bone regeneration via CXCR2-mediated PI3k/Akt signaling pathway. Interestingly, the *in vivo* experimental results verified our findings from *in vitro* experiments.

Endochondral ossification is the mechanism by which most fractures heal. Tissue engineered bone may also repair bone defects through endochondral ossification. Tissue engineered scaffold around the visible cartilage causes collagen accumulation, chondrocytes hypertrophy different from normal chondrocytes. Repairing of bone defects by tissue engineered bone may be initiated by seed cells that secret a variety of cytokines and inflammatory cytokines in the local inflammatory environment. The secreted cytokines may recruit the host cells to the bone defect area, in which the interaction between the seed cells and host cells differentiate into chondrocytes to secrete a large amount of collagen and cartilage matrix, to form cartilage mineralized osteoblast, and induce chondrocyte to osteoblast transformation [37].

Here, we found that tissue engineering bone could repair large segmental defects in animal models and the experimental results showed that IL-8 promote osteogenesis, in a CXCR2/PI3k/Akt-dependent manner.

Bone regeneration primarily occurs through endochondral bone formation, in which mesenchymal condensations determine the bone domain by forming growth cartilage to allow chondrocytes generate and proliferate. The transcription factor Sox9 is specifically expressed in osteochondroprogenitors in the mesenchymal condensations. Sox9 transcriptionally activates major cartilaginous matrix proteins such as aggrecan and COL2. Thus, Sox9 and COL2 are two markers for the chondrogenic differentiation [38]. Both *in vitro* and *in vivo* experiments that analyzed Sox9 and COL2 confirmed the results that IL-8 enhances bone regeneration via CXCR2-mediated PI3k/Akt signaling pathway.

To summarize, here using both *in vitro* and *in vivo* tools, we were able to demonstrate a critical role of IL-8 in the bene regeneration through tissue engineering bone technology. Moreover, we found that the involved molecular signaling tranduction was CXCR2-mediated PI3k/Akt signaling pathway.

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Disclosure Statement

No conflict of interests exists.

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