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Original Article

CXCR4 enhances invasion and proliferation of bone marrow stem cells via PI3K/AKT/NF-κB signaling pathway

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Abstract: Osteosarcoma is the most common type of cancer that develops in bone, specifically; it is an aggressive malignant neoplasm. The purpose of this study is using superparamagnetic iron oxide nanoparticles (SPION) labeled bone mesenchymal stem cells (MSCs) to migrate into cancerous parts, then using alternating magnetic field to produce the high temperature to kill cancer cells in vitro. In order to enhance the invasion ability of MSCs, we successfully overexpressed CXCR4 in MSCs, we found the invasion behavior of CXCR4 overexpressed MSCs and CXCR4 overexpressed SPION labeled MSCs was enhanced when compared with MSCs. In addition, the proliferation of CXCR4 overexpressed MSCs and CXCR4 overexpressed SPION labeled MSCs. Then, we found that CXCR4 was able to enhance invasion related genes expression, including MMP9, MMP2, MMP13, MMP7, MMP10, MMP8, and MMP1. Among these genes, MMP9 and MMP2 were associated with PI3K/AKT/NF-κB signaling. The expression of MMP9 and MMP2 was decreased when PI3K/AKT signaling inhibitor LY294002 and NF-κB inhibitor PDTC were used respectively. Moreover the migrated of CXCR4 overexpressed MSCs and CXCR4 overexpressed SPION labeled MSCs were significantly reduced after LY294002 and PDTC used. These results suggest that CXCR4 overexpressed SPION labeled MSCs can be more easier migrate into cancerous parts; it may provide a promising method to treat the osteosarcoma.

Keywords: Bone marrow stem cells, SPION, CXCR4, invasion, PI3K/AKT/NF-κB

Introduction

Osteosarcoma, also called osteogenic sarcoma, is a cancerous tumor in a bone. Specifically, it is an aggressive malignant neoplasm that arises from primitive transformed cells of mesenchymal origin (and thus a sarcoma) and that exhibits osteoblastic differentiation and produces malignant osteoid [1, 2]. It usually starts in osteoblasts, which are a type of bone cell that becomes new bone tissue. Osteosarcoma is most common in adolescents [3]. It commonly forms in the ends of the long bones of the body, which include bones of the arms and legs. In children and adolescents, it often forms in the bones near the knee. Rarely, osteosarcoma may be found in soft tissue or organs in the chest or abdomen. Although individuals with localized osteosarcoma have an average 5-year survival of about 80%, however those with metastatic disease have much worse outcomes [4]. Its incidence is bimodally distributed by age with peaks in adolescence and in the elderly [5, 6]. Osteosarcoma incidence in childhood and adolescence seems to be relatively consistent throughout the world [7]. Thus some new, safe and effective therapeutic methods are need.

It has been demonstrated that cancer cells actively recruit mesenchymal cells into tumors and this recruitment is essential for the generation of a microenvironment that promotes tumor growth [8]. Since the mesenchymal cells can stimulate tumor growth and invasiveness, mesenchymal cells play an active role in cancer progression, induce chemotherapy resistance, and inhibit cancer cell apoptosis [9, 10]. Fu et

al. reported that bone mesenchymal stem cells (BMSCs) are locally adjacent to the tumor tissues and may interact with tumor cells directly, and BMSCs were able to promote osteosarcoma cell proliferation and invasion. So BMSCs can be worked as drug-delivery carriers.

Suspension of superparamagnetic iron oxide nanoparticles (SPION) is a FDA approved agents, which has been used to magnetically label cells, including stem cells and progenitor cells [11, 12]. Labeling stem cells with SPION allows for the non-invasive monitoring by MRI in both animal and human trials [13]. SPION-labeled cells can also be detected using Prussian blue stain to correlate histology to MRI. These studies suggest SPION-labeled stem cells are easier to detect.

In order to enhance the invasion ability of BMSCs, we try to overexpressed CXCR4 in BMSCs. CXCR4 expression has been found in many cancer types [14], and the CXCR4 receptor ligand system plays an important role in carcinoma progression by promoting tumor cell migration [15]. We hope the invasion ability of CXCR4 overexpressed BMSCs can be significantly increased. Taken together, in this study, we hypothesis that we used SPION-labeled CXCR4 overexpressed BMSCs to invade cancerous parts, then using alternating magnetic field to produce the high temperature to kill cancer cells in vitro. It may provide a promising method to treat the osteosarcoma.

Materials and methods

MSCs isolation and culture

Briefly, one-hundred-gram female rat was sacrificed with chloroform anesthesia. Both sites, femora and tibia were aseptically removed. Then cut at both ends of femora and tibia to gain access to the marrow cavity. Thereafter, the bone marrows were flushed out using DMEM medium, supplemented with 10% (v/v) FBS, 1% glutamine (v/v), 1% (v/v) penicillin-streptomycin. The released cells were collected and cultured in 10 cm cell culture plate. Cells were allowed to attach for 2 days, when after the non-adherent cell population was removed and the culture medium was replaced with fresh medium. The culture medium was changed every two days. The 4th passage MSCs were used to do experiments.

Construction of CXCR4 overexpressed MSCs

The 4th passage MSCs were seed into 6-well culture plate with a concentration of 2×10^5/ml, then CXCR4-lentiviral-vector was added plus with 8 μg/ml polybrene, and cultured overnight then changed the medium with fresh medium. Finally the CXCR4 overexpressed MSCs were obtained.

Preparing for SPION labeled MSCs and identification

SPION (50 μg/ml) and polylysine (1.5 μg/ml) were added into DMEM medium and shocked for 60 minutes at room temperature. Then equal volume of DMEM plus with FBS, EGF, the final concentration of SPION, polylysine, FBS, EGF is 25 μg/ml, 0.75 μg/ml, 10%, 10 ng/ml. Then this kind of medium was used to culture MSCs and CXCR4 overexpressed MSCs for 48 hours. Following washed with PBS for 3 times. SPION labeled MSCs and CXCR4 overexpressed MSCs were fixed with 4% paraformaldehyde for 1 hour, then stained with Prussian blue for 30 minutes, and washed with distilled water for 3 times, stained with nuclear fast red for 10 minutes.

RT-PCR

Total RNA was extracted from cultured MSCs, CXCR4 overexpressed MSCs, SPION labeled MSCs, CXCR4 overexpressed SPION labeled MSCs using TRIzol and determined its concentration was determined. Total RNA (0.5 μg) was used as a template to prepare cDNA. The mRNA expression of target genes was quantified using SYBR Premix EX Taq on the ABI 7500 sequence detection system. PCR was performed with the following thermocycling conditions: An initial 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Finally, the $2^{-\Delta\Delta Ct}$ method was performed to calculate the relative expression. The primer sequences as following.

CCK-8 assay

About 5×10^3 MSCs, CXCR4 overexpressed MSCs, SPION labeled MSCs, CXCR4 overexpressed SPION labeled MSCs were seeded into 96-well plates respectively. Cell proliferation was evaluated using Cell Counting Kit-8 (KeyGEN biotech, Nanjing, China) in accordance

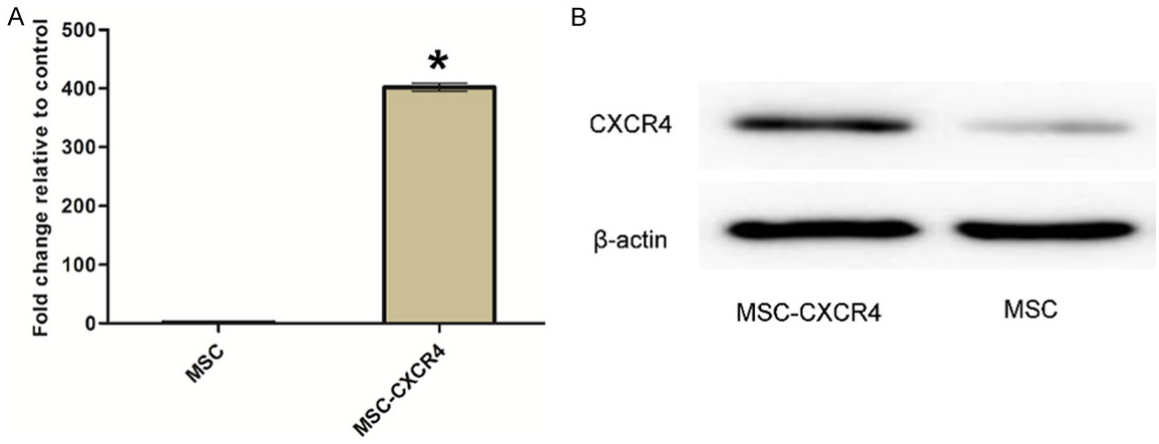

Figure 1. The construction of CXCR4 overexpressed MSCs. A. The detection of CXCR4 expression in MSCs and CXCR4 overexpressed MSCs by RT-PCR. B. The detection of CXCR4 expression in MSCs and CXCR4 overexpressed MSCs by western blotting.

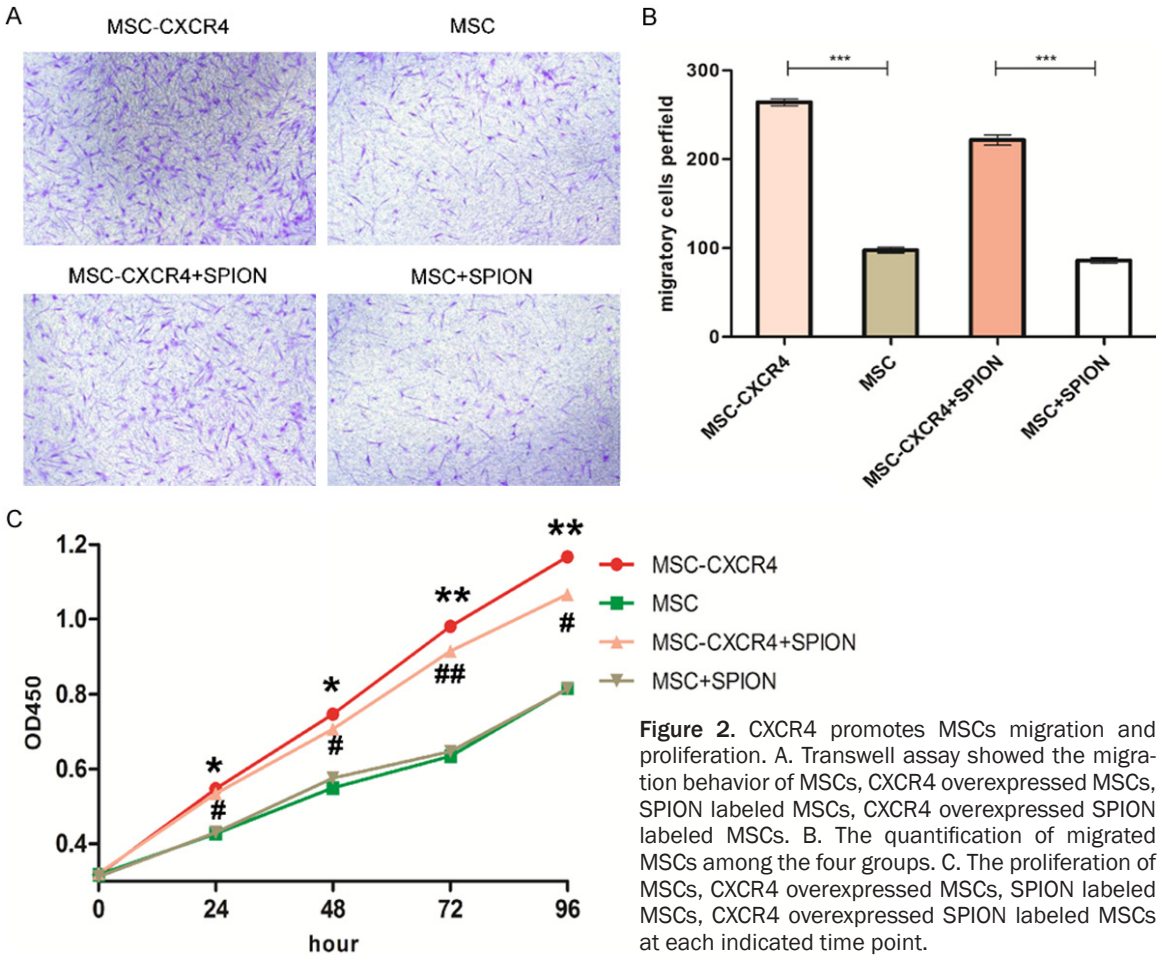

Figure 2. CXCR4 promotes MSCs migration and proliferation. A. Transwell assay showed the migration behavior of MSCs, CXCR4 overexpressed MSCs, SPION labeled MSCs, CXCR4 overexpressed SPION labeled MSCs. B. The quantification of migrated MSCs among the four groups. C. The proliferation of MSCs, CXCR4 overexpressed MSCs, SPION labeled MSCs, CXCR4 overexpressed SPION labeled MSCs at each indicated time point.

with the manufacturer's instructions. The cell proliferation curves were plotted by the absorbance values after 24, 48, 72, 96 hours culture.

Transwell assay

MSCs, CXCR4 overexpressed MSCs, SPION labeled MSCs, CXCR4 overexpressed SPION

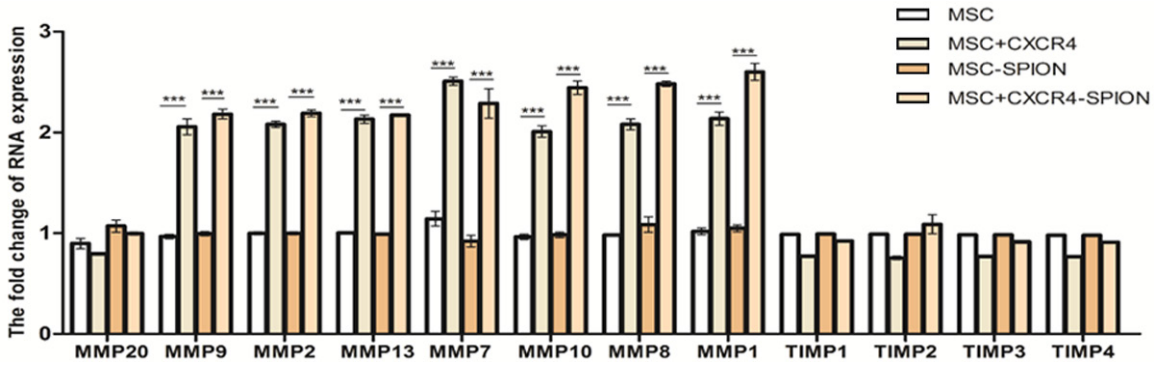

Figure 3. The expression of MMP20, MMP9, MMP2, MMP13, MMP7, MMP10, MMP8, MMP1, TIMP1, TIMP2, TIMP3 and TIMP4 in MSCs, CXCR4 overexpressed MSCs, SPION labeled MSCs, CXCR4 overexpressed SPION labeled MSCs.

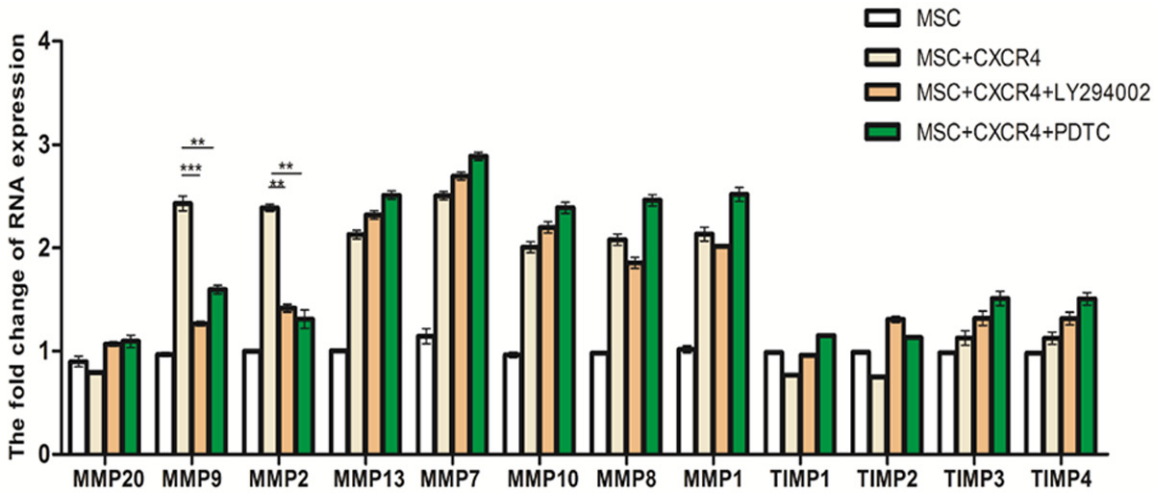

Figure 4. The expression of MMP20, MMP9, MMP2, MMP13, MMP7, MMP10, MMP8, MMP1, TIMP1, TIMP2, TIMP3 and TIMP4 in MSCs, CXCR4 overexpressed MSCs, CXCR4 overexpressed MSCs treated with LY294002, CXCR4 overexpressed MSCs treated with PDTC.

labeled MSCs (2×10^5) in 100 μl of serum-free DMEM medium were cultured in transwell plates. Then, the lower chamber of culture inserts was filled with 10% FBS-DMEM medium. Cells were cultured for 24 h, fixed with methanol for 30 min and stained with hematoxylin for 20 min. Finally, migrated cells were counted in five random fields under an inverted microscope.

Western blotting

Total protein was collected from MSCs, CXCR4 overexpressed MSCs, CXCR4 overexpressed MSCs treated with LY294002, CXCR4 overexpressed MSCs treated with PDTC by using a lysis buffer and quantified by bicinchoninic acid (BCA) method. Cell lysates were separated using 10% SDS-PAGE and transferred to the polyvinylidene difluoride membrane. The membranes were immersed into PBST solution supplemented with 5% non-fat milk for 2 h. Then, the membranes were incubated with antibody-MMP2, antibody-MMP9 respectively, at 4 centigrade overnight. All membranes were incubated with appropriate second antibody at room temperature for 1 h and visualized by enhanced chemiluminescence.

Statistical analyses

The statistical calculations were performed by using GraphPad Prism 4 software (GraphPad Software, San Diego, CA). And statistical significance was assessed by One-way ANCOVA, with significance accepted at the $P <$

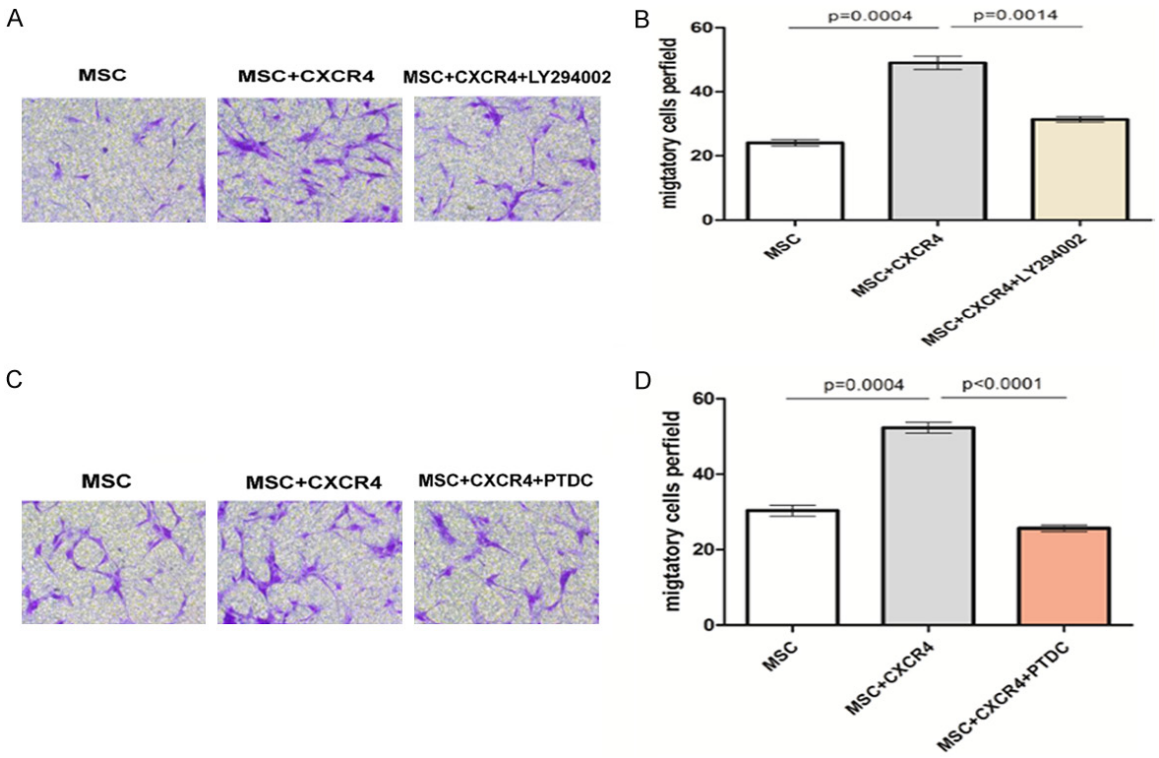

Figure 5. CXCR4 regulates MSCs migration via PI3K/AKT/NF-κB signaling. A, C. Transwell assay showed the migration behavior of MSCs, CXCR4 overexpressed MSCs, CXCR4 overexpressed MSCs treated with LY294002, CXCR4 overexpressed MSCs treated with PDTC. B, D. The quantification of migrated MSCs among the MSCs, CXCR4 overexpressed MSCs, CXCR4 overexpressed MSCs treated with LY294002, CXCR4 overexpressed MSCs treated with PDTC.

0.05 level. Significance levels were set at: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

Construction of CXCR4 overexpressed MSCs

As shown in **Figure 1B**, the RT-PCR results showed that the relative expression of CXCR4 genes in CXCR4 overexpressed MSCs was obviously higher than in MSCs ($P < 0.01$). Also a similarly results can been seen in western blotting, the expression of CXCR4 in CXCR4 overexpressed MSCs was significant higher than in MSCs.

CXCR4 promotes MSCs invasion and proliferation

After we constructed CXCR4 overexpressed MSCs, we explored the effects of CXCR4 on MSCs invasion. As shown in **Figure 2A** and **2B**, the invasion behavior in CXCR4 overexpressed MSCs was obvious higher than in MSCs ($P < 0.001$). After that we used SPION to label MSCs and CXCR4 overexpressed MSCs. There was no significant difference in cell invasion of SPION labeled MSCs and MSCs ($P > 0.05$), which suggests SPION can not affect MSCs invasion. Moreover, the invasion behavior in CXCR4 overexpressed SPION labeled MSCs was dramatically higher than in SPION labeled MSCs ($P < 0.001$). The results were similar with that in MSCs. Furthermore, we also explored the effects of CXCR4 on proliferation of MSCs. As shown in **Figure 2C**, CXCR4 was able to promote MSCs and CXCR4 overexpressed SPION labeled MSCs proliferation from day 1 to day 4 ($P < 0.05$), and SPION did not affect MSCs proliferation.

CXCR4 enhances invasion related genes expression

We have demonstrated that CXCR4 was able to promote MSCs invasion, following we further explored the effects of CXCR4 on the expression of invasion related genes in MSCs. As

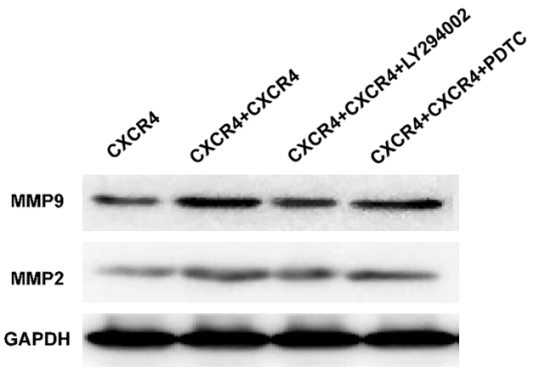

Figure 6. The expression of MMP9 and MMP2 in MSCs, CXCR4 overexpressed MSCs, CXCR4 overexpressed MSCs treated with LY294002, CXCR4 overexpressed MSCs treated with PDTC.

shown in **Figure 3**, the RT-PCR results showed that the expression of MMP9, MMP2, MMP13, MMP7, MMP10, MMP8, MMP1 were significantly increased in CXCR4 overexpressed MSCs or SPION labeled MSCs when compared with MSCs and SPION labeled MSCs ($P < 0.001$).

PI3K/AKT/NF-κB signaling involves in the process of CXCR4 regulating MSCs invasion

After we demonstrated CXCR4 was able to enhance MSCs invasion, we further explored the possible mechanism. As shown in **Figure 4**, we used PI3K/AKT signaling inhibitor LY294002 and NF-κB inhibitor PDTC to treat MSCs respectively, we found only the expression of MMP9 and MMP2 were reduced in LY294002 and PDTC treated groups ($P < 0.01$). Then, we further verified whether PI3K/AKT/NF-κB signaling can regulate MSCs invasion. As shown in **Figure 5A** and **5B**, the migrated MSCs was significantly decreased in CXCR4 overexpressed MSCs treated with LY294002 group when compared with CXCR4 overexpressed MSCs group ($P < 0.01$). Similarly, the migrated MSCs was significantly decreased in CXCR4 overexpressed MSCs treated with PTDC group when compared with CXCR4 overexpressed MSCs group ($P < 0.001$) (**Figure 5C** and **5D**). Furthermore, the western blot results showed that the expression of MMP9 and MMP2 were obviously decreased in LY294002 and PDTC treated groups (**Figure 6**).

Discussion

Osteosarcoma is the most frequent primary bone tumor in young adults and adolescents. Pulmonary metastasis occurs in ~40-50% of the osteosarcoma patients [16], who present an overall 5-year survival rate of only 28% [17]. What's worse, currently there is no cure for these patients. The purpose of this study is trying to find out a safe and effective therapeutic method. We try to use BMSCs load with SPION to migrate into cancerous parts, then using alternating magnetic field to produce the high temperature to kill cancer cells in vitro. Before we do that, the first thing we should figure it out is to enhance the invasion ability of BMSCs. We try to transfer some genes into BMSCs to increase invasion ability of BMSCs.

It is well known that the human chemokine system has more than 40 chemokines and 18 chemokine receptors [18]. Chemokine receptors are defined by their ability to induce the directional migration of cells toward a gradient of a chemotactic cytokine. Chemokine receptors are a family of seven transmembrane G protein-coupled cell surface receptors (GPCR) that are classified into four groups (CXC, CC, C, and CX3C) based on the position of the first two cysteines [19, 20]. Among these receptors, C-X-C chemokine receptor type 4 (CXCR4) is one of the best studied chemokine receptors, CXCR-4 also known as fusin or CD184 (cluster of differentiation 184) is a protein that in humans is encoded by the CXCR4 gene [21]. It was primarily knew as a co-receptor for HIV entry [22], and it also can mediate the metastasis of a variety of cancers. It is overexpressed in more than 23 different types of human cancers including kidney, lung, brain, prostate, breast, pancreas, ovarian, melanomas, osteosarcoma [23, 24] and contributes to the tumor growth, angiogenesis, metastasis, and therapeutic resistance.

Luster et al. reported that chemokine receptors are coupled to heterotrimeric G proteins and induce cell movement towards a concentration gradient of the cognate chemokine ligand [25]. And Müller et al. demonstrated that CXCR4 signaling plays a crucial role in metastasis of breast cancer by inducing chemotactic and invasive responses [26]. The involvement of CXCR4 in metastasis is not limited to breast cancer, as CXCR4 is expressed in several other tumor cell lines [27, 28], including osteosarcoma [29]. In this study, we found the proliferation of CXCR4 overexpressed MSCs was faster

than normal MSCs, and the invasion behavior of CXCR4 overexpressed MSCs was significantly enhanced. These results were consistent with others study. Moreover the proliferation and invasion behaviors of CXCR4 overexpressed MSCs were not affected by SPION label. In our future study, we will use the CXCR4 overexpressed SPION labeled MSCs to invade the cancerous parts, then using alternating magnetic field to produce the high temperature to kill cancer cells in vitro, we will show these results in our future work.

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Disclosure of conflict of interest

None.

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