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Matrix metalloproteinase-9 is up-regulated by CCL19/CCR7 interaction via PI3K/Akt pathway and is involved in CCL19-driven BMSCs migration

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ABSTRACT

C–C chemokine receptor 7 (CCR7) and its ligands CCL19 contributes to the directional migration of certain cancer cell lines, but its role in the migration of BMSCs remains vague. The aim of this study was to determine the possible interaction between CCL19-induced conditions and matrix metalloproteinases-9 (MMP9) expression in BMSCs. Cell migration using Transwell assay indicated that activation of CCR7 by its specific ligand, exogenous chemokine ligand 19 (CCL19), was associated with a significant linear increase. Western blot and real-time PCR indicated that CCL19/CCR7 significantly upregulated expression of MMP9, which is related to metastasis-associated genes. The CCL19/CCR7 interaction significantly enhanced phosphorylation of Akt, as measured by Western blot. P-Akt and MMP9 protein expression exhibited a time-dependent pattern, and the peak was at 48 h. LY294002 significantly abolished the effects of exogenous CCL19. These results suggest that CCL19/CCR7 contributes to the migration of BMSCs by upregulating MMP9 potentially via the PI3K/Akt pathway.

1. Introduction

Bone marrow mesenchymal stem cells (BMSCs) have multiple differentiation potentials with self-renewal capabilities and can be differentiated into many tissue-specific lineages, including osteoblasts, chondroblasts, adipocytes and others [1]. For example, BMSCs successfully enhances recovery of motor function after lacunar stroke in rats [2]. Therefore, it is promising to exploit the broad differentiation potential of BMSCs for the therapeutic treatment of many human diseases. However, it has been suggested that the repair efficiency is limited [3]. The main reason could be that only a small proportion of the implanted BMSCs migrate to the injured tissues and play the role there.

Chemokines and chemokine receptors have an important role in the control of cellular proliferation and migration. Chemokines are small, secreted molecules that signal through G-protein-linked receptors. Chemokines can be made and secreted by many different cell types, including tumor cells, tumor-infiltrating immune cells and BMSCs. Chemokine receptor triggering results in the onset of complex intracellular signaling cascades leading to cell polarization and migration towards the chemokine source [4].

In addition, BMSCs have been shown to express CCR1, CCR7, and CCR9 and three CXC chemokine receptors (CXCR4, CXCR5, and CXCR6), someone suggested that several chemokine axes may operate in BMSCS biology and may be important parameters in the validation of cultured BMSCs intended for cell therapy [5].

The chemokine receptor CCR7 and its ligand CCL19 are thought to play a central role in regulating migration of mature dendritic cells (DCs) [6], and there is some evidence that CCR7 and its ligands CCL19 are involved in the directional migration of various cells [7,8], Phosphatidylinositol 3-kinase (PI3K) is one of the most important signaling pathway affected by CCL19/CCR7 axes [9]. Although PI3K and its downstream target, AKT/protein kinase B (PKB), have been shown to play a critical role in promoting maturation, migration, and survival of various cells [10,11], CCL19/CCR7 axis in BMSCs migration via PI3K/Akt has not been addressed until the present study.

To investigate whether CCL19 activation enhances BMSCs migration, we treated BMSCs in vitro with CCL19. In addition, we examined mechanisms by which MMP9 is taken up in BMSCs, and assessed the involvement of PI3K/Akt in CCL19-mediated cell migration.

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2. Materials and methods

2.1. Cell culture

The cell strain of Sprague-Dawle (SD) Rat BMSCs was purchased from the Cyagen Corporation as previously described [12]. The BMSCs lots were positive for CD90, CD29 and CD44, for negative expression of CD11, CD34 and CD45, and for their ability to differentiate into the osteogenic, chondrogenic and adipogenic lineage which were tested by the providing company. Cultivation of BMSCs in our experiments was performed using DMEM/F12 (Hyclone, CA, USA) containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (Gibco). BMSCs cultured to passage 5 or below were used for the following experiments.

2.2. Immunofluorescent detection of CCR7 in rat BMSCs

CCR7 expression and localization was performed using the immunofluorescence method as described [13]. The cells were fixed in 4% paraformaldehyde for 10 min on ice and then cell membranes were ruptured with 0.2% Triton at room temperature for 20 min, and nonspecific antigen-binding sites were blocked by 5% BSA for 30 min. The cells were then incubated overnight at 4 °C with anti-CCR7 antibodies (Abcam, USA, 1: 200). On day 2, slides were incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:1000, Santa Cruz) as well as with 0.5 μg/ml bisbenzimide dye (Hoechst No. 33342) for nuclear counterstaining. The cell morphology was analyzed with an inverted microscope (Nikon TE2000-S Eclipse) at ×400 magnification.

2.3. Cell migration assay

Cell migration was performed by the Transwell assay as previously described [14] using the Costar Transwell chamber system (24 well, 8 μm pore size, Corning life science, Lowell, MA). After different concentrations of rat recombinant CCL19 (Minneapolis, MN, USA) were added to the lower chamber, 1×10^5 BMSCs suspended in 100 μL DMEM/F12, were loaded into the upper wells and allowed to migrate for 24 h. For migratory inhibition experiments, cells were preincubated with LY294002 (Sigma, St. Louis, MO, USA) at 20 μM for 1 h. The respective inhibitor of LY294002 were also added to the medium in the upper and lower compartments in the same concentrations. After incubation at 37 °C and 5% CO_2 for 24 h, nonmigratory cells on the upper membrane were removed with a cotton swab, and stained with 1% crystal violet (Sigma) for 30 min, the number of cells that had migrated through to the underside of the insert membranes was calculated by counting at least five random separate fields (200-fold magnification).

2.4. Western blot analysis

Semiquantitative analysis of proteins was performed by Western blotting as described previously [15]. After treatment with CCL19 (100 ng/ml) for 24 h, cells were extracted with RIPA lysis buffer including protease inhibitors. Aliquots, each containing 40 μg protein, were separated by 10% SDS–polyacrylamide gel. The proteins were then transferred to nitrocellulose membrane and incubated overnight at 4 °C with following antibodies: rabbit anti-rat P-Akt, Akt (Santa Cruz, CA) at 1:1000, and MMP9 (Proteintech, USA) monoclonal antibody was used at 1: 200 dilution as primary dilution; and anti-β-actin antibody (Santa Cruz, USA) was used at 1:1000 as primary dilution overnight at 4 °C, followed by HRP-conjugated secondary antibodies (Abcam, MA, USA) at 1: 5000 for 2 h at room temperature. Signals were detected by using an ECL Western blotting kit, and proteins were visualized using the enhanced chemiluminescence plus system.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from BMSCs with TRIZOL reagent according to the manufacturer's instructions (Takara Bio Inc., Tokyo, Japan). Forward and reverse primers were based on published reports [16].β-actin F: 5′-ATATCGCTGCGCTCGTCGTC-3′, R:5′-CCTTGGGTCAGGTTTAGAG-3′, the β-actin amplicon length was 174 bp. MMP9 F: 5′-CTGCGTATTTCCATTCAT-3′, R: 5′-CCTT GGGTCAGGTTTAGAG-3′. The MMP9 amplicon length was 496 bp. The following conditions were used: 95 °C for 30 s, 95 °C for 5 s for 40 cycles and 60 °C for 30 s. The reliability of PCR results was supported by analyzing the dissociation curve. Real-time PCR data were calculated using the $2^{-\Delta\Delta CT}$ method on the SDS 2.4 software package.

2.6. Transfection of BMSCs with small interfering RNA (siRNA)

RNA interference (RNAi) technology was used to generate specific knockdowns of CCR7 transcription in BMSCs. BMSCs were plated onto 6 cm^2 cell culture dishes and grown to 30–50% confluence after 24 h of incubation and then the lentiviral transduction was performed. The CCR7 siRNA-specific targeting sequence was 5′-GCTTCTGCCAAGATGAGGTCA-3′, and scrambled oligonucleotides (5′-TTCTCCGAACGTGTCACGTTTC-3′) (Genepharma, Shanghai, China) was used as a negative control (NC). The CCR7 mimics and the scrambled oligonucleotide were diluted in DMEM/F12 at a final multiplicity of infection (MOI) of 100. Efficiencies of siCCR7 and non-silencing control siRNA were tested using Western blot.

2.7. Statistical analyses

Data are presented as mean ± SD. Statistically significant differences were evaluated using the Student's *t* test when two groups were compared and with One-way ANOVA when three or more groups were compared. One-way ANOVA test followed by Bonferroni's multiple comparison tests was applied with SPSS version 19.0. A P value <0.05 was considered statistically significant.

3. Results

3.1. Immunofluorescence

As shown in Fig. 1A, almost 100% of BMSCs expressed CCL19 receptor CCR7. The results confirmed that CCR7 was highly expressed in BMSCs, and suggested that CCR7 might play a role in the migration of the BMSCs.

3.2. CCL19/CCR7 promotes migration of BMSCs in vitro

According to the experiment of transwell, few migrated cells were found in control group (7.11 ± 1.17); at 100 ng/mL concentrations CCL19 significantly promoted cell migration, compared with 50 ng/mL concentrations (19.11 ± 6.70 versus 38.33 ± 5.85, $P < 0.01$) (Fig. 1B), specially, the number of migrated cells significantly increased at the dose of 100 and 200 ng/mL compared to control (Fig. 1B), while there were no significant difference between 100 ng/mL and 200 ng/mL concentrations. (38.33 ± 5.85 versus 41.10 ± 2.33, $P > 0.05$). Therefore, at 100 ng/mL concentration CCL19 was used in the following experiment.

3.3. Downregulation of CCR7 attenuated BMSCs migration

To investigate the role of CCR7 in the functioning of BMSCs, CCR7 activation and inhibition were induced with exogenous CCL19 and with CCR7 siRNA, respectively. After transfection with

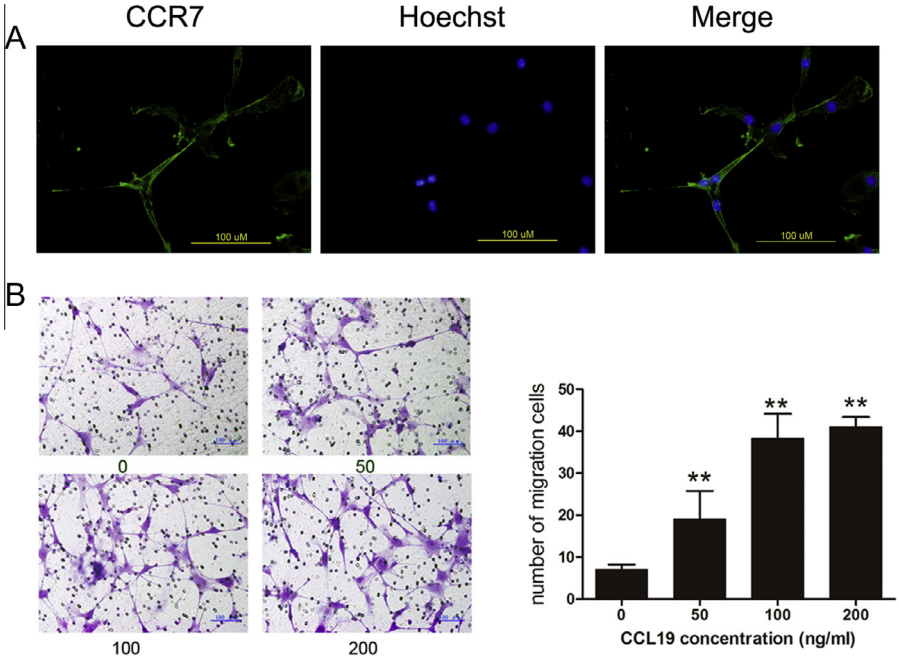

Fig. 1. Expression of CCR7 and in vitro migration of BMSCs. (A) Immunofluorescence stain of the BMSCs, which were stained with anti-CCR7 antibodies, demonstrated the existence of CCR7 protein (green) in the rat BMSCs, Hoechst No. 33342 staining was used to identify the nuclei (blue). (magnification = ×400). Scale bar = 100 μm. (B) Representative images of transmigrated BMSCs expressing CCR7 in response to CCL19 at concentrations of 0, 50, 100, or 200 ng/mL in transwell assay. Average number of cells migrated in transwell migration assay counted in 200× magnification field. Results are mean ± SD of five different fields from 3 independent experiments. $^{**}P < 0.01$, compared with control cells (CCL19 = 0). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

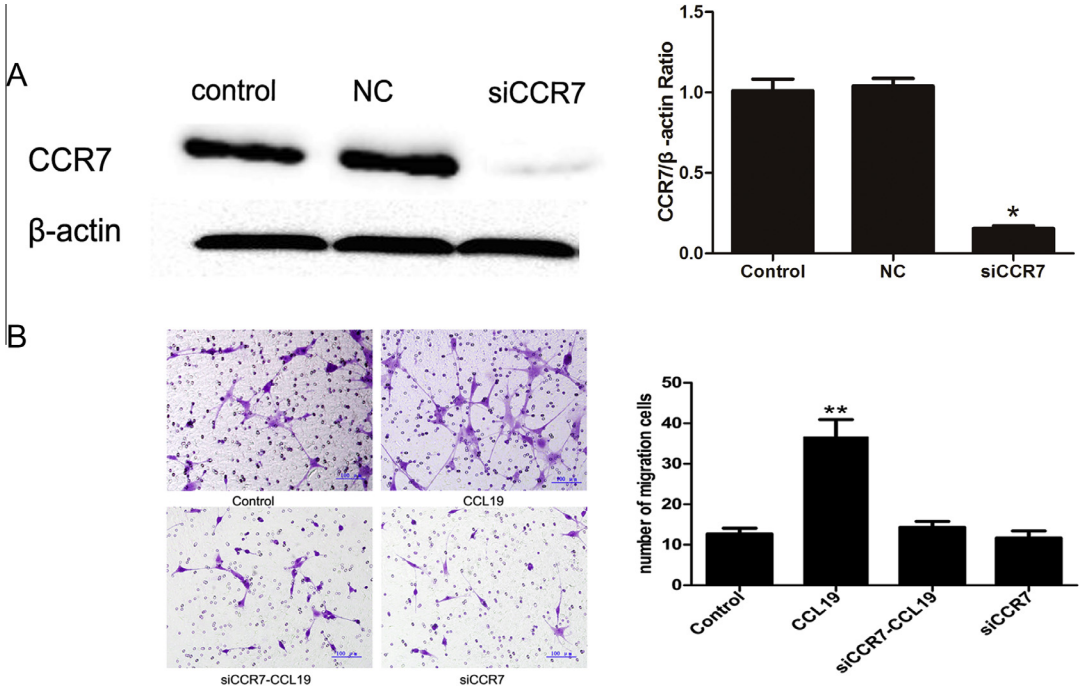

Fig. 2. CCR7 knock-downs in BMSCs: (A) BMSCs were transfected with control siRNA or CCR7 siRNA (siCCR7). After transfection, the expression of CCR7 protein was evaluated using Western blot and compared to untransfected BMSCs. Each bar represents the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control cells. (B) BMSCs were transfected with CCR7 siRNA and treated with CCL19 (100 ng/mL) for 24 h, average number of cells migrated in transwell migration assay counted in 200× magnification field. Results are mean ± SD of five different fields from 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control cells.

CCR7 siRNA (siCCR7) or control siRNA, the expression of CCR7 was evaluated using Western blot. As determined by Western blot 48 h after transfection, we achieved knock-down efficiencies of 90% for CCR7 when compared with control siRNA (Fig. 2A).

In a next step we used RNAi to elucidate the role of constitutively expressed CCR7 in the migratory capability of BMSCs. For this purpose, BMSCs carrying specific CCR7 knock-downs were applied in the Transwell assay and analyzed for their migratory

potential. We found that the CCL19/CCR7 interaction significantly promoted cell migration, whereas siCCR7 significantly abrogated the action of CCL19 (Fig. 2B). siCCR7 alone had no significant effect on cell migration, compared with control cells. These results indicate that CCR7 is essential for the migration of BMSCs.

3.4. CCL19/CCR7 induced MMP 9 expression and Akt phosphorylation in BMSCs

Next, we investigated whether the cytokines CCL19 influenced the expression of MMP-9 in BMSCs. As shown in Fig. 3A, the CCL19/CCR7 interaction significantly upregulated the expression of P-Akt and MMP9 at 12, 24 and 48 h, whereas there was no significant impact on the expression of Akt, induction with CCL19 for 24 h and 48 h increased over two and threefold in the P-Akt protein expression, and increased MMP9 protein expression by three and fivefold compared with control, respectively. In concordance with the protein data, as shown in Fig. 3B compared with control cells, the CCL19/CCR7 interaction significantly upregulated the mRNA levels of MMP9. After transfection with CCR7 siRNA, as shown in Fig. 3C and D, siCCR7 significantly abrogated the effects of CCL19, whereas siCCR7 alone had no significant effect on MMP9 expression.

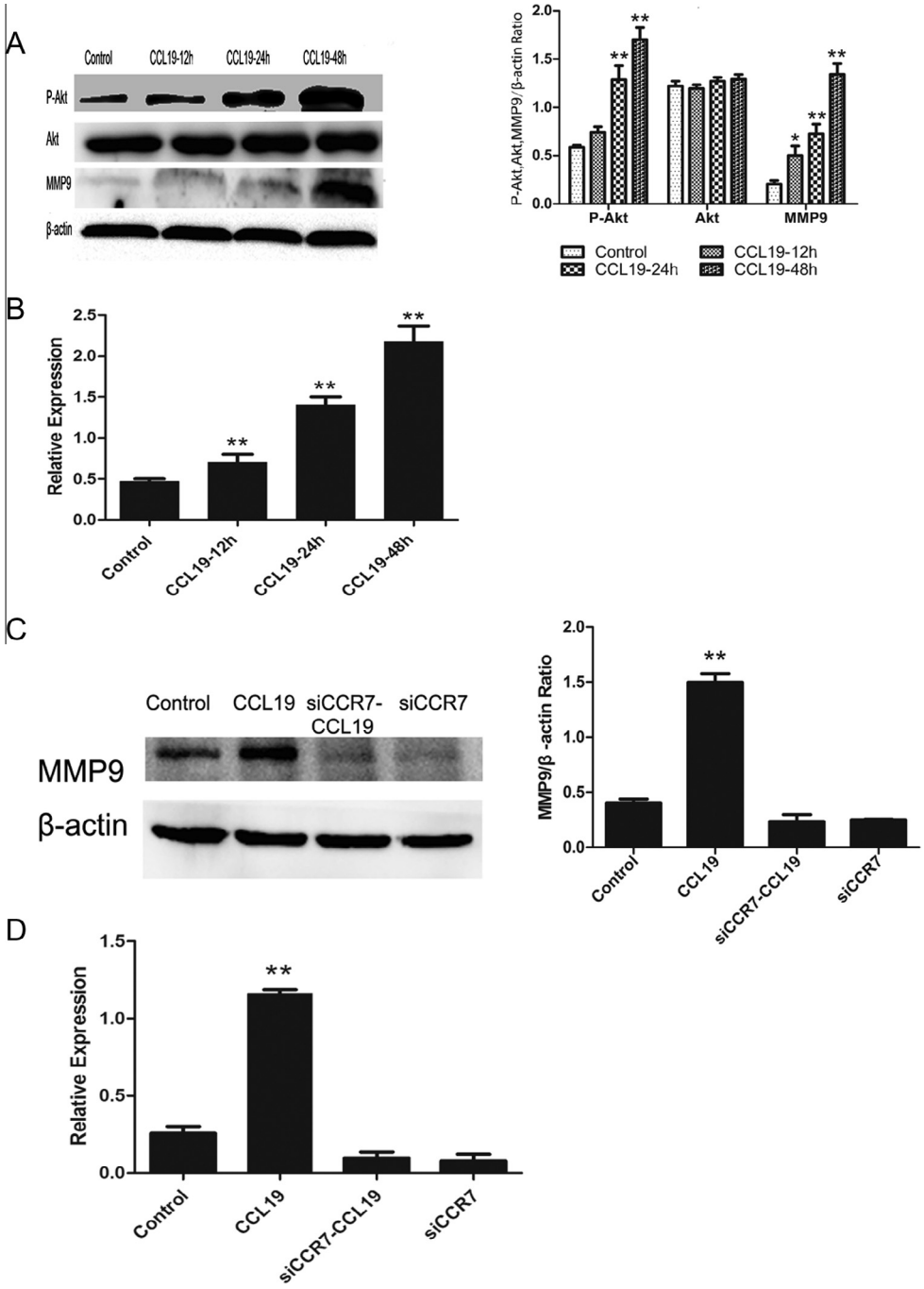

Fig. 3. Activation of Akt and MMP9 induced by CCL19. BMSCs were treated with CCL19 (100 ng/mL) for 12, 24, or 48 h, the protein levels of total Akt, P-Akt and MMP9 were measured by Western blot (A) and the mRNA levels of MMP9 were estimated using real-time PCR (B). The results are mean ± SD of expression relative to β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$ versus corresponding control BMSCs were transfected with CCR7 siRNA and treated with CCL19 (100 ng/mL) for 24 h, and the protein (C) and mRNA (D) levels of MMP9 were estimated using Western blot and real-time PCR. Each bar represents the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus corresponding control.

These results indicate that CCL19/CCR7 upregulated MMP9 expression, siCCR7 abrogated the effects of CCL19.

3.5. LY294002 attenuated MMP9 expression and BMSCs migration

To verify whether LY294002, the specific inhibitor of PI3K/Akt, can abolish the effects of CCL19/CCR7 on the expression of P-Akt and MMP9 of BMSCs, the protein levels were measured by Western blot. As shown in Fig. 4A, after cells were pre-incubated with LY294002 for 1 h, the protein levels were measured by Western blot. LY294002 abolished the influence of CCL19/CCR7 on the expression of P-Akt and MMP9, the interaction between P-Akt and MMP9 was salient. In addition, LY294002 alone had a significant inhibitory effect on the expression of P-Akt and MMP9. The interaction between P-Akt and MMP9 was weakened in response to LY294002 exposure.

Furthermore, we performed cell migration assays adding CCL19 (100 ng/mL) as chemoattractant into the lower compartment of the Transwell chamber in the presence or absence of LY294002 (20 μM). As shown in Fig. 4B, according to the experiment of transwell, migrating cells achieved the most at 100 ng/ml CCL19 in BMSCs, and the amount was decreased by 80% after cells were pre-incubated with LY294002. This indicates that blocking PI3K/Akt with LY294002 in BMSCs significantly attenuated CCL19-induced BMSC migration.

These data indicated that the CCL19/CCR7 axis, along with MMP9, regulates BMSC migration through PI3K/Akt pathway.

4. Discussion

BMSCs have an important role in many physiological and pathological procedures. Culturing BMSCs is now a well-established procedure and has been used for various studies, especially in cell proliferation, migration and differentiation studies in vitro [17]. But the migratory behavior of BMSCs is a critical clinical problem. In this study, we outline the roles of CCL19 in BMSCs migration and provide mechanistic explanations. CCL19 directly recruits the BMSCs and enhances the chemotaxis of BMSCs to CCL19 via activating PI3K/Akt pathways.

To clarify the biological effects of CCL19 on BMSCs migration, we exposed the cells to CCL19 at different concentrations, and examined the mRNA and protein expression of MMP9 and P-Akt. Our results showed that both the mRNA and protein levels of MMP9 and P-Akt were upregulated by CCL19 in a time-dependent manner. The peak were at 48 h. The P-Akt and MMP9 genes highly expressed in BMSCs under CCL19 stimulation, it indicated that CCL19 stimulation upregulated the expressions of invasion/metastasis associated gene, and further changed the migration ability of BMSCs. Inhibition of CCR7 expression in BMSCs significantly decreases BMSC migration.

Chemokines are important factors controlling cellular migration. CCR7, which mediates the survival and migration of immune cells to lymph nodes, has recently been associated with nodal metastasis of squamous cell carcinomas of the head and neck (SCCHN) [18]. Several studies have documented that the activation

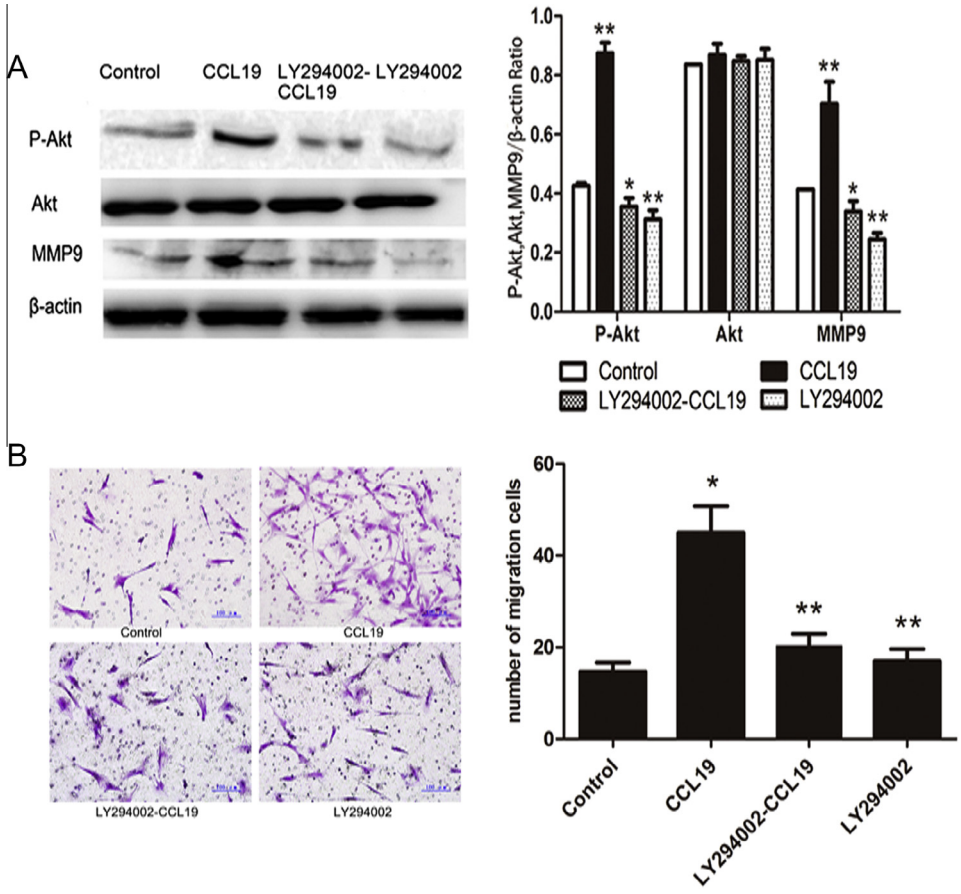

Fig. 4. Effect of PI3K/Akt inhibitors LY294002 on the expression of P-Akt, MMP9 and cell migration (A) The protein levels of P-Akt, Akt and MMP9 were measured in BMSCs pre-treated with LY294002 for 1 h followed by the challenge with 100 ng/ml CCL19 for 24 h, the protein levels of total Akt, P-Akt and MMP9 were measured by Western blot. The results are mean ± SD of expression relative to β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$ versus corresponding control .(B) BMSCs was pre-incubated with or without LY294002 for 1 h, followed by the stimulation with CCL19 at 100 ng/ml for 24 h. Average number of cells migrated in transwell migration assay counted in 200× magnification field. Results are mean ± SD of five different fields from 3 independent experiments. $^{*}P < 0.05$ compared with control, $^{**}P < 0.05$ compared with CCL19 group.

of CCR7 is responsible for promoting migration of certain cancer cell lines, others have reported that CCR7 may increase the phosphorylation of Akt, which are related to cell survival and migration [19–22].

In this study, we showed that expression of CCR7 induced by CCL19 promoted the expression of MMP9 as well as migration of BMSCs and that inhibited by LY294002 resulted in decreased MMP9 expression and BMSCs migration. These results suggested a possible role of CCR7 in mediating MMP9 expression. Interestingly, LY294002 exposed down-regulation of MMP9 expression in BMSCs. As such, 20 μM LY294002 resulted in an approximate 60% decrease in the expression of MMP9 in BMSCs. These effects were abolished by the PI3K/Akt inhibitor LY294002, which is a potent and reversible inhibitor of PI3k activity by inhibiting ATP binding site of the enzyme and casein kinase II. All of above indicated that the effects result from specific activation of CCL19/CCR7 axes. The same phenomenon was also reported in head and neck cancer cells [19].

Matrix metalloproteinases (MMPs) are a family of highly homologous zinc-dependent endopeptidases that digest components of the extracellular matrix (ECM) as well as non-matrix proteins [23]. Twenty-eight members of the MMPs have been identified. Each MMP interacts specifically with certain elements of the ECM, MMPs are implicated in a wide range of diverse pathological processes that include atherosclerosis, arthritis, cancer, and neurodegeneration [24,25]. Among these proteases, MMP-9 (gelatinase B) plays a more specific role in cell migration. The first research on the relationship between CCR7 and MMP9 was performed by Redondo and colleagues [26]. Redondo showed that MMP9 was regulated by the ligand of CCR7, and MMP-9 was involved in B-cell chronic lymphocytic leukemia nodal infiltration.

Our results showed that, CCL19 induction led to an obvious increase in phosphorylated Akt and MMP9 in rBMSCs. However, LY294002 inhibited PI3K/Akt expression in BMSCs decreases BMSCs migration. This show that CCL19-induced activation of PI3K/Akt pathway significantly enhanced the migration indexes of BMSCs and these effects were blocked by PI3K/Akt inhibitor, LY294002. Thus, the interaction of CCL19/CCR7 likely controls the migration of BMSCs, and suggest that PI3K/Akt pathway is involved in regulation of rBMSCs migration.

AKT is an important cellular kinase involved in cell differentiation and migration [27]. Increased PI3K/Akt activation reportedly changes the migratory activity and invasiveness of cells [28], Han et al. [29] confirm that the PI3K/Akt pathway contributed significantly to the process in the activation of MMP-9 secretion. While the effect of CCL19/CCR7 axes on PI3K/Akt activation was observed in cancer cells, the present study is the first to report the effects of CCL19/CCR7 axes on PI3K/Akt activation in BMSCs.

Several obstacles to clinical application of BMSCs, such as poor cell vitality in culture and inadequate numbers for transplantation, exist for the use of BMSCs in therapy [30]. Several methods such as pretreatment with growth factors or hypoxia were applied to modify the BMSCs before transplantation to overcome these limitations [31]. However, someone demonstrated that BMSCs combined with hepatocyte growth factor (HGF) has no superior to only BMSCs transplantation [32]. Chemokines including CCR7 may prove useful to enhance BMSC migration capacity during early events in transplantation.

In summary, to the best of our knowledge, this is the first report showing enhanced migration by CCL19 activation in BMSCs. This study suggests that activation of CCR7 with CCL19 can significantly promote migration of rat BMSCs in a dose-dependent manner involving increasing the expression of MMP9, possibly via the PI3K/Akt pathway. These results provide an important insight into the cellular mechanisms by which CCL19 influences cell migration.

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