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Involvement of matrix metalloproteinase-3 in CCL5/CCR5 pathway of chondrosarcomas metastasis

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ABSTRACT

CCL5 (previously called RANTES) was originally recognized as a product of activated T cells, and plays a crucial role in the migration and metastasis of human cancer cells. It has been reported that the effect of CCL5 is mediated via CCR receptors. We found that human chondrosarcoma tissues had significant expression of the CCL5 and CCR5, which was higher than that in normal cartilage. We also found CCL5 increased the migration and matrix metalloproteinases-3 (MMP)-3 expression in human chondrosarcoma cells (JJ012 cells). In addition, MMP-3 small interfering RNA and inhibitor inhibited the CCL5-induced cell migration. Activations of phosphatidylinositol 3-kinase (PI3K), Akt and NF-κB pathways after CCL5 treatment was demonstrated, and CCL5-induced expression of MMP-3 and migration activity was inhibited by the specific inhibitor of PI3K, Akt and NF-κB cascades. Taken together, these results indicate that CCL5 and CCR5 interaction enhanced migration of chondrosarcoma cells through the increase of MMP-3 production.

1. Introduction

Chondrosarcoma is a malignant primary bone tumor with a poor response to currently used chemotherapy or radiation treatment, making the management of chondrosarcomas a complicated challenge [1]. Clinically, surgical resection remains the primary mode of therapy for chondrosarcoma. Due to the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis and therefore, it is important to explore a novel and adequate remedy [2].

Chemotactic cytokines (chemokines) induce direct migration of leukocytes along a chemical gradient of ligand(s). Their production is stimulated by proinflammatory cytokines, growth factors and, in general, by pathogenic stimuli arising in inflammatory tissues. In diseased tissues, different tumor cell types trigger a complex chemokine network that influences the quality and quantity of immune-cell infiltration and, consequently, malignant cell proliferation, survival, spread, and angiogenetic response [3]. Regulated upon activation normal T cell expressed and secreted (RANTES, CCL5), was originally recognized as a product of activated T cells [4]. Now widely established as an inflammatory chemokine, CCL5 is known to mediate chemotactic activity in T cells, monocytes, dendritic cells, natural killer cells, eosinophils and basophils [5–7]. CCL5 is associated with chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and cancer [8,9]. An association between CCL5 expression and cancer has been reported in melanoma, lung, prostate and pancreatic cancers [10–12]. The most striking findings thus far have been with breast cancer [10,11]. Several investigations have reported that CCL5 was detected in samples from patients with breast cancer and that expression levels correlated with disease progression [10,11].

The invasion of tumor cells is a complex, multistage process. To facilitate the cell motility, invading cells need to change the cell–cell adhesion properties, rearrange the extracellular matrix (ECM) environment, suppress anoikis and reorganize their cytoskeletons [13]. Matrix metalloproteinases (MMPs) have important roles in these processes because their proteolytic activities assist in degradation of ECM and basement membrane [14,15]. MMPs, cytokines, growth factors and chemokines have been shown to regulate tumor cell invasion through autocrine or paracrine pathways [13]. MMP-3 (stromelysin-1) is secreted as an inactive

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soluble pro-form which can be activated by a variety of proteases [16]. Like most MMPs, MMP-3 is not expressed in normal tissue, but is rapidly induced in cases of tissue repair or during remodeling processes [17]. It has been also reported that MMP-3 plays a critical role in ECM turnover and cell–cell interactions, as well as tumor metastasis [18].

Previous studies have shown that CCL5 modulates cell migration and invasion in human cancer cells [12,19,20]. Interaction of CCL5 with its specific receptor CCRs on the surface of cancer cells has been reported to induce cancer invasion [9,19,20]. However, the effect of CCL5 and CCR receptor on MMPs expression and migration activity in human chondrosarcoma cells is mostly unknown. Here we found a phenomenon whereby CCL5 and CCR5 interaction increased the migration and expression of MMP-3 in human chondrosarcoma cells. In addition, phosphatidylinositol 3-kinase (PI3K), Akt, IKKα/β and NF-κB signaling pathways were involved in.

2. Materials and methods

2.1. Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p-Akt, Akt, p85α, IKKα/β, IκB, p-IκBα, MMP-3 (sc-21732), α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ly294002, Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-((R)-2-O-methyl-3-O-octadecylcarbonate)), TPCK and PDTC were purchased from Calbiochem (San Diego, CA, USA). UK 370106 was purchased from TOCRIS (Bristol, UK). Mouse monoclonal antibody specific for CCR5 and Met-RANTES were purchased from R&D Systems (Minneapolis, MN, USA). Rabbit polyclonal antibody specific for phosphor-p85 (Tyr^{458}), phosphor-IKKα/β ($Ser^{180/181}$) and phosphor-p65 (Ser^{536}) were purchased from Cell Signaling (Danvers, MA, USA). The recombinant human CCL5 was purchased

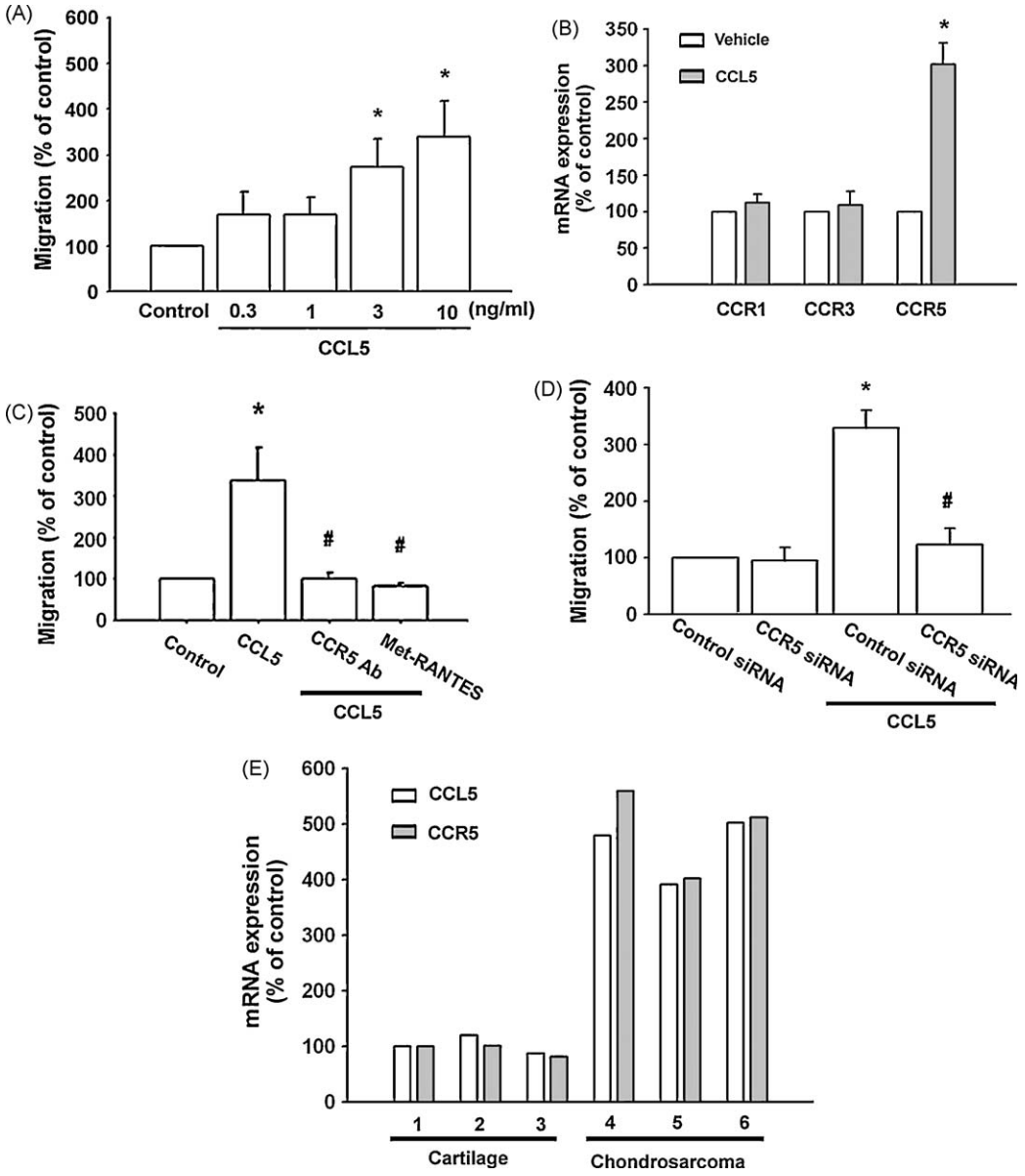

Fig. 1. CCL5/CCR5 axis-directed migration of human chondrosarcoma cells. (A) JJ012 cells were incubated with various concentrations of CCL5, and *in vitro* migration activity measured with the Transwell after 24 h. (B) JJ012 cells were incubated with CCL5 (3 ng/ml) for 24 h, and the mRNA expression of CCR1, CCR3 and CCR5 was examined by qPCR. Cells were pretreated for 30 min with CCR5 Ab, Met-RANTES (C) or transfected with CCR5 siRNA (D) for 24 h. Then they were followed by stimulation with CCL5 (3 ng/ml), and *in vitro* migration was measured with the Transwell after 24 h. (E) Total RNA were extracted from normal cartilage (lines 1–3) or from chondrosarcoma patients (lines 4–6) and subjected to qPCR analysis for CCL5 and CCR5. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with CCL5-treated group.

from PeproTech (Rocky Hill, NJ, USA). The p85α (Δp85; deletion of 35 amino acids from residues 479–513 of p85) and Akt (Akt K179A) dominant-negative mutants were gifts from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The IKKα (KM) and IKKβ (KM) mutants were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). The NF-κB-luciferase plasmid was purchased from Stratagene (La Jolla, CA, USA). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA, USA). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The human chondrosarcoma cell line (JJ012; Grade II chondrosarcoma cell line) was kindly provided from the laboratory of Dr. Sean P. Scully (University of Miami School of Medicine, Miami, FL, USA). The JJ012 cells were cultured in DMEM/α-MEM supplemented with 10% fetal bovine serum and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Migration assay

The migration assay was performed using Transwell (Costar, NY, USA; pore size, 8-μm) in 24-well dishes. Before performing the migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the Ly294002 (10 μM), Akt inhibitor (10 μM), PDTC (10 μM), TPCK (3 μM) or vehicle control (0.1% DMSO). The concentrations of inhibitors did not affect cell death of JJ012 cells as shown by a cell viability assay (data not shown). Approximately 1×10^4 cells in 200 μl of serum-free medium were placed in the upper chamber, and 300 μl of the same medium containing CCL5 was placed in the lower chamber. The plates were incubated for 24 h at 37 °C in 5% CO_2, then cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times. The number of invading cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of CCL5 treatment (corrected invading cell number = - counted invading cell number/percentage of viable cells) [21].

2.4. siRNA transfection

ON-TARGET smart pool CCR5 siRNA (L-181467-00) and ON-TARGET plus siCONTROL non-targeting poll (D-001810-10) were

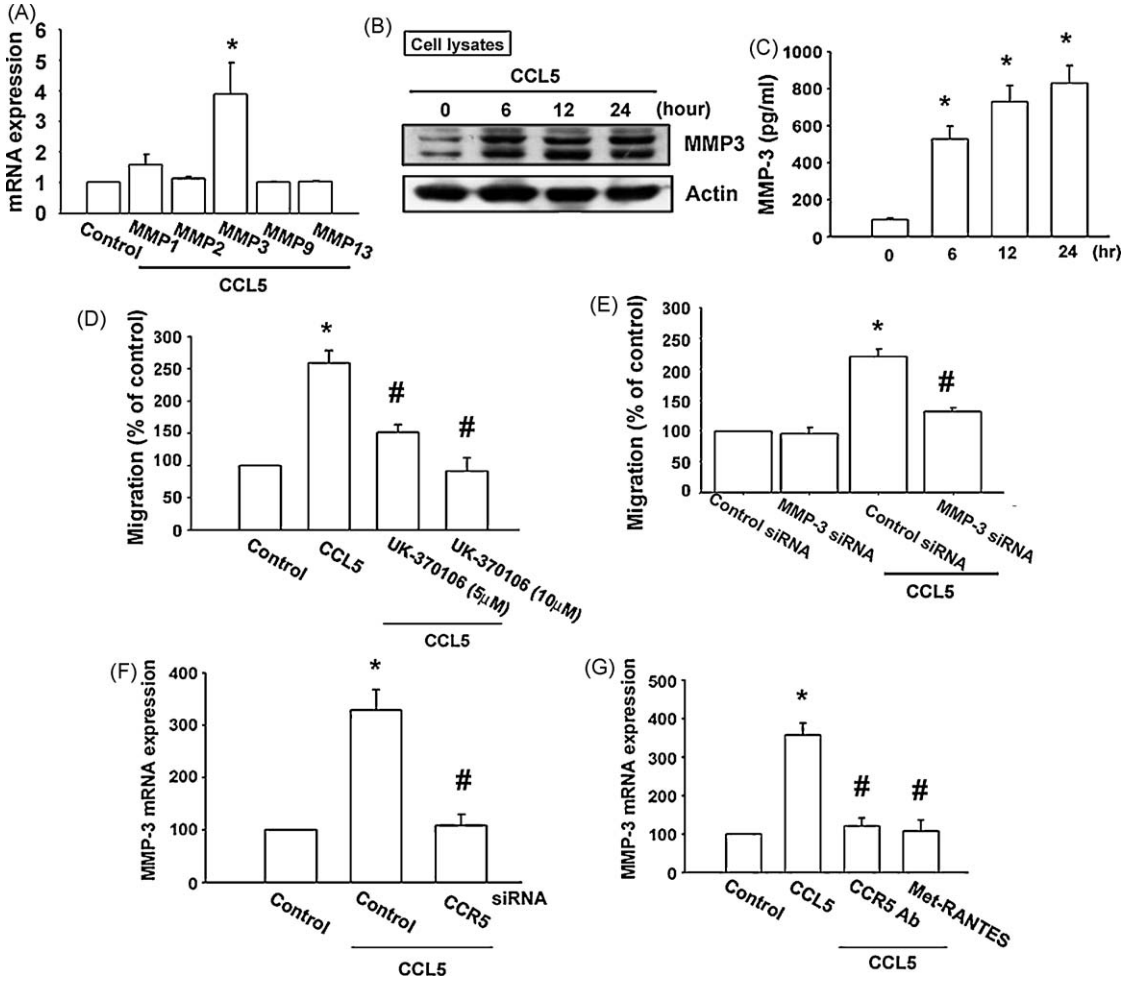

Fig. 2. CCL5/CCR5-directed migration of human chondrosarcoma cells involves up-regulation of MMP-3. (A) Cells were incubated with CCL5 (3 ng/ml) for 24 h, and the mRNA level of MMP-1, -2, -3, -9 and -13 was determined using qPCR. Cells were incubated with CCL5 (3 ng/ml) for indicated time intervals, and the protein level of MMP-3 was determined using Western blot (B) and ELISA (C). Cells were pretreated for 30 min with UK-370106 (D) or transfected with MMP-3 siRNA (E) for 24 h. Then they were followed by stimulation with CCL5 (3 ng/ml), and *in vitro* migration was measured with the Transwell after 24 h. Cells were pretreated for 30 min with CCR5 Ab, Met-RANTES (G) or transfected with CCR5 siRNA (F) for 24 h, and mRNA expression of MMP-3 was examined by qPCR. Results are expressed as the mean ± S.E. $^*p < 0.05$ compared with control; $^\#p < 0.05$ compared with CCL5-treated group.

purchased from Dharmacon (Lafayette, CO, USA). The siRNA against human MMP-3 (sc-29399) and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transfected with siRNAs (100 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions [22].

2.5. ELISA assay

Human chondrosarcoma cells (2×10^4) were cultured in 24-well culture plates. Cells were incubated with CCL5 for various time intervals (6, 12 and 24 h) at 37 °C. After incubation, the medium was removed and stored at −80 °C until assay. MMP-3 in the medium was assayed using the MMP-3 enzyme immunoassay kits, according to the procedure described by the manufacturer (Biocompare; San Jose, CA, USA).

2.6. Western blot analysis

The cellular lysates were prepared as described previously [23]. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against IκBα, p-IκB, IKKα/β or p-Akt (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA). The activities of Akt were determined using kit from Cell Signaling Technology according to the manufacturer's instructions.

2.7. Patients and specimen preparation

After approval by the local ethics committee, specimens of tumor tissue were obtained from patients, who had been pathologically diagnosed with chondrosarcoma and had undergone surgical resection at the China Medical University Hospital. The histologic grade of chondrosarcoma was checked. Cytologically, increased cellularity and cytological atypia are the most important features, and these characteristics are used to determine the grade of the chondrosarcoma [24]. The high grade patients (grade II or grade III) have been used in this study.

2.8. Quantitative real-time PCR

The quantitative real-time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA, USA). One hundred nanograms of total cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes.

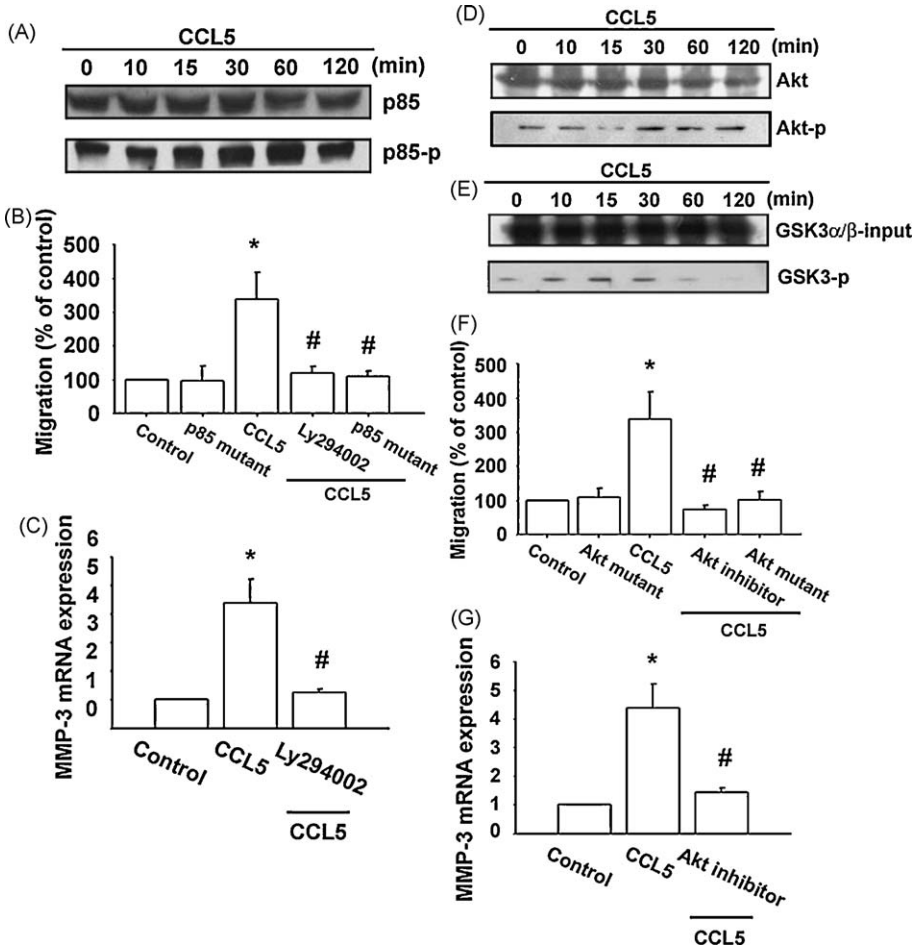

Fig. 3. PI3K/Akt pathway is involved in CCL5-mediated migration and MMP-3 up-regulation in human chondrosarcoma cells. (A and D) Cells were incubated with CCL5 (3 ng/ml) for indicated time intervals, and p-p85 and p-Akt expression was determined by Western blot analysis. (E) Cells were incubated with CCL5 (3 ng/ml) for indicated time intervals, and Akt kinase assay was determined by Akt kinase assay kit. (B and F) Cells were pretreated for 30 min with Ly294002 and Akt inhibitor or transfected with p85 and Akt mutant for 24 h. Then they were followed by stimulation with CCL5 (3 ng/ml), and *in vitro* migration was measured with the Transwell after 24 h. (C and G) Cells were pretreated for 30 min with Ly294002 and Akt inhibitor, followed by stimulation with CCL5 (3 ng/ml) for 24 h, and mRNA expression of MMP-3 was examined by qPCR. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with CCL5-treated group.

Sequences for all target gene primers and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems, CA, USA). qPCR assays were carried out in triplicate on an StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

2.9. Immunofluorocytochemistry

Cells were cultured in 12-mm coverslips. After treatment with CCL5, cells were fixed with 4% paraformaldehyde at room temperature. Thirty minutes later, 4% non-fat milk in PBS containing 0.5% Triton X-100 was added to the cells. The cells were then incubated with rabbit anti-p65 (1:100) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:500; Leinco Technology Inc., St. Louis, MO, USA) for 1 h, respectively. The FITC was detected using a Zeiss fluorescence microscope.

2.10. Transfection and reporter gene assay

Human chondrosarcoma cells were co-transfected with 0.8 μg κB-luciferase plasmid, 0.4 μg β-galactosidase expression vector. JJ012 cells were grown to 80% confluence in 12 well plates and were transfected on the following day with Lipofectamine 2000 (LF2000; Invitrogen, Carlsbad, CA, USA). DNA and LF2000 were premixed for 20 min and then applied to cells. After 24 h transfection, the cells were then incubated with the indicated agents. After a further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI, USA) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector.

2.11. Statistics

The values given are means ± S.E.M. The significance of difference between the experimental groups and controls was assessed by Student's t test. The difference was significant if the p value was <0.05.

3. Results

3.1. CCL5/CCR5 axis-directed migration of chondrosarcoma cells

CCL5 has been reported to stimulate directional migration and invasion of human cancer cells [3,20]. CCL5-triggered migration in chondrosarcoma cells was examined using the Transwell assay with correction of CCL5-induced proliferation effects on human chondrosarcoma cells [21]. CCL5-directed human chondrosarcoma cell (JJ012 cell) migration (Fig. 1A). Interaction of CCL5 with its specific receptor CCR on the surface of cancer cells has been reported to induce cancer invasion [10,19]. Stimulation of cells with CCL5 increased the mRNA expression of CCR5 but not CCR1 and CCR3 (Fig. 1B), suggesting that the amplification loop strengthens the CCL5–CCR5-signaling pathway. Pretreatment of cells with CCR5 mAb or CCR5 receptor inhibitor (Met-RANTES) reduced CCL5-increased cell migration (Fig. 1C). In addition, transfection of cells with CCR5 siRNA reduced CCR5 protein expression (data not shown). However, transfection of cells with CCR5 siRNA antagonized CCL5-increased migration activity (Fig. 1D). Next, we examined human chondrosarcoma patients for the expression of the CCL5 and CCR5 using qPCR. Expression of mRNA levels of CCL5 and CCR5 in chondrosarcoma patients (Fig. 1E, lines 4–6) were significantly higher than those in normal cartilage (Fig. 1E, lines 1–3). Therefore, CCL5 and CCR5 interaction is very important in migration activity in chondrosarcoma cells.

3.2. Involvement of MMP-3 in the CCL5/CCR5-directed migration of chondrosarcoma

Previous studies have shown a significant expression of MMP-1, -2, -3, -9 and -13 in human chondrosarcoma cells [25,26]. We therefore, hypothesized that any of these human chondrosarcoma cells-associated MMPs may be involved in CCL5/CCR5-directed chondrosarcoma cell migration. qPCR analysis showed that CCL5 significantly increased the expression of MMP-3 mRNA but not MMP-1, -2, -9 and -13 in JJ012 cells (Fig. 2A). On the other hand, CCL5 also did not affect the enzyme activity of MMP-1, -2, -9 and -13 (Supplemental data Fig. S1). Furthermore, CCL5 further increased protein expression of MMP-3 in JJ012 cells in a time-dependent manner by using Western blot and ELISA assay (Fig. 2B and C). To further explore whether MMP-3 might play a crucial role in CCL5-mediated migration activity, the selective MMP-3 inhibitor, UK-370106 [27], was used. It was demonstrated that treatment with UK-370106 antagonized the CCL5-increased cell migration (Fig. 2D). In addition, MMP-3 siRNA also reduced CCL5-mediated migration activity (Fig. 2E). Furthermore, MMP-3 mRNA expression was abolished by CCR5 siRNA or CCR5 Ab or Met-RANTES (Fig. 2F and G), confirming the involvement of CCL5 and CCR5 interaction in MMP-3 regulation.

3.3. PI3K and Akt signaling pathways are involved in CCL5/CCR5-mediated MMP-3 up-regulation and cell migration of chondrosarcoma cells

PI3K/Akt can be activated by a variety of growth factors, such as insulin, nerve growth factors, and CCL5 [20,28]. We examined whether CCL5 stimulation also enhanced PI3K activation. Stimulation of JJ012 cells led to a significant increase in phosphorylation of p85 (Fig. 3A). CCL5-induced migration and MMP-3 expression of JJ012 cells were greatly reduced by treatment with Ly294002 (10 μM), a specific PI3K inhibitor (Fig. 3B and C). In addition, transfection of cells with p85α mutant also inhibited CCL5-induced migration of chondrosarcoma cells (Fig. 3C). Ser473 residue phosphorylation of Akt by a PI3K-dependent signaling pathway causes enzymatic activation [29]. To examine the crucial role of PI3K/Akt in cancer migration and MMP-3 up-regulation, we next determined Akt Ser473 phosphorylation in response to CCL5 treatment. As shown in Fig. 3D, treatment of JJ012 cells with CCL5 resulted in time-dependent phosphorylation of Akt Ser473. In addition, CCL5 also increased Akt activity by determining phosphorylation of one of its substrates GSK3 (Fig. 3E). Pretreatment of cells with Akt inhibitor (10 μM) antagonized CCL5-induced migration and MMP-3 expression of JJ012 cells (Fig. 3F and G). In addition, the Akt mutant also reduced CCL5-mediated cell migration (Fig. 3G). Based on these results, it appears that CCL5/CCR5 axis acts through the PI3K and Akt-dependent signaling pathway to enhance MMP-3 expression and cell migration in human chondrosarcoma cells.

3.4. NF-κB signaling pathway is involved in CCL5/CCR5-mediated MMP-3 up-regulation and migration activity

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human chondrosarcoma cells [30]. To examine whether NF-κB activation is involved in CCL5-induced cancer migration, an NF-κB inhibitor, PDTC, was used. Fig. 4A shows that JJ012 cells pretreated with PDTC (10 μM) and inhibited CCL5-induced chondrosarcoma cell migration. Furthermore, JJ012 cells pretreated with TPCK (3 μM), an IκB protease inhibitor, also reduced CCL5-induced cancer cell migration (Fig. 4A). In addition, treatment of cells with PDTC or TPCK also antagonized CCL5-induced expression of MMP-3 (Fig. 4B). We further examined the upstream molecules involved in CCL5-induced NF-κB activation. Stimulation of cells with CCL5-induced IKKα/β phosphorylation in a time-dependent manner (Fig. 4C). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited CCL5-induced cancer cell migration and MMP-3 expression (Fig. 4D and E). These data suggest that IKKα/β activation is involved in CCL5-induced migration activity of human chondrosarcoma cells. Treatment of chondrosarcoma cells with CCL5 also caused IκBα phosphorylation in a time-dependent manner (Fig. 4C). Previous studies showed that p65 Ser536 phosphorylation increased NF-κB transactivation, and the specific antibody against phosphorylated p65 Ser536 was used to examine p65 phosphorylation [31]. Treatment of JJ012 cells with CCL5 for various time intervals resulted in p65 Ser536 phosphorylation (Fig. 4C). To directly determine NF-κB activation after CCL5 treatment, chondrosarcoma cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 4F, CCL5 (3 ng/ml) treatment of chondrosarcoma cells for 24 h caused increase in κB-luciferase activity. These results indicated that NF-κB activation is important for CCL5-induced cancer cell migration and the expression of MMP-3.

3.5. PI3K/Akt signaling pathway is involved in CCL5-mediated NF-κB activity and MMP-3 expression

To further investigate whether CCL5-induced p65 Ser536 phosphorylation and NF-κB activation occur through the PI3K/Akt

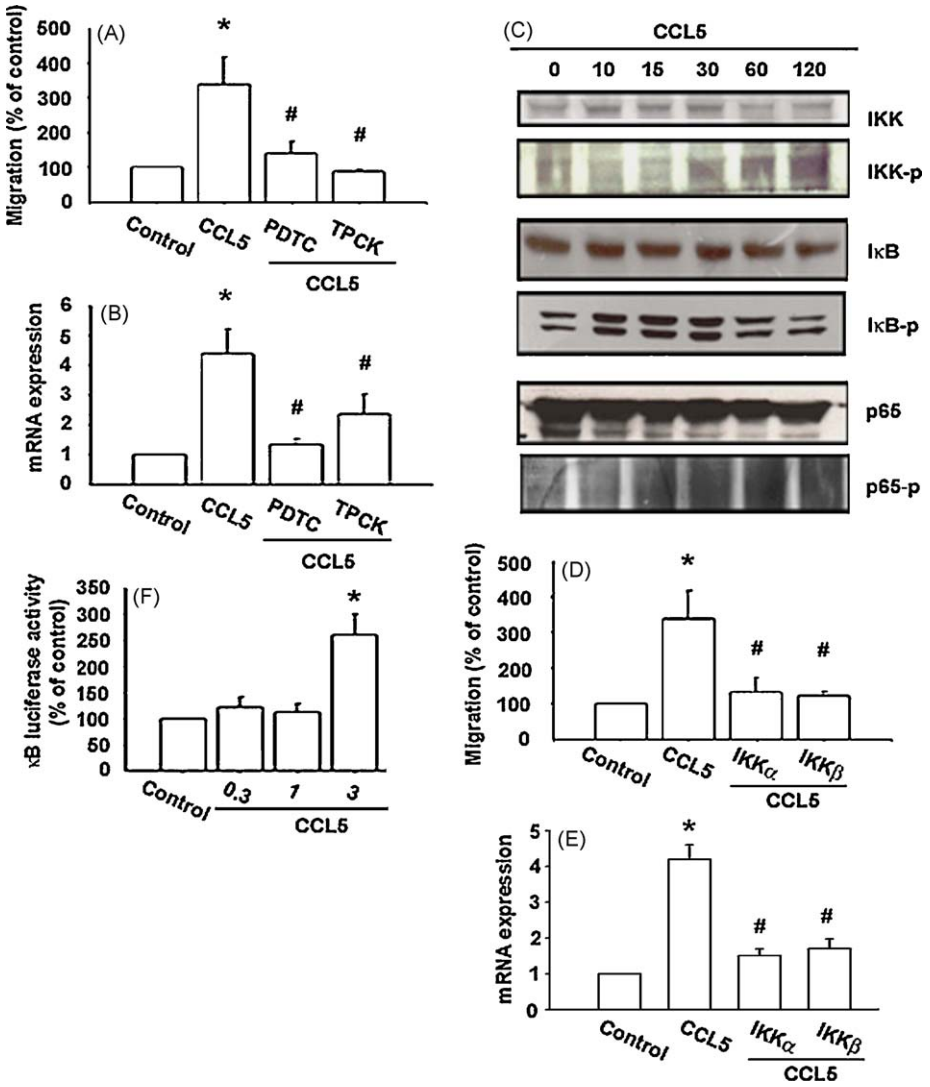

Fig. 4. CCL5 induces MMP-3 up-regulation through NF-κB activation. Cells were pretreated with PDTC (60 μM) or TPCK (3 μM) for 30 min before incubation with CCL5 for 24 h. Then they were followed by stimulation with CCL5 (3 ng/ml), and *in vitro* migration (A) and mRNA expression of MMP-3 (B) was measured with the Transwell and qPCR after 24 h. (C) Cells were incubated with CCL5 (3 ng/ml) for indicated time intervals, and p-IKK, p-IκBα and p-p65 expression was determined by Western blot analysis. (D) Cells were transfected with IKKα and IKKβ mutant for 24 h before incubation with CCL5 for 24 h. Then they were followed by stimulation with CCL5 (3 ng/ml), and *in vitro* migration (D) and mRNA expression of MMP-3 (E) was measured with the Transwell and qPCR after 24 h. (F) Cells were incubated with CCL5 (0.3–3 ng/ml) for 24 h, and NF-κB activity was examined. The results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with CCL5-treated group.

pathway, JJ012 cells were pretreated for 30 min with Ly294002, Akt inhibitor, PDTC and TPCK, which inhibited the CCL5-induced increase in κB-luciferase activity (Fig. 5A). In addition, co-transfection of cells with p85, Akt, IKKα or IKKβ mutant also reduced CCL5-mediated κB-luciferase activity (Fig. 5B). Stimulation of cells with CCL5 increased p65 translocation into nucleus by immunofluorescence staining (Fig. 5C). Ly294002 and Akt inhibitor also reduced CCL-5-mediated translocation of p65 (Fig. 5C). Moreover, the CCL5-induced increase in p65 Ser536 phosphorylation was also attenuated by Ly294002 and Akt inhibitor (Fig. 5D). Taken together, these data suggest that activation of PI3K/Akt pathway is required for CCL5-induced p65 Ser^{536} phosphorylation and NF-κB activation in chondrosarcoma cells.

4. Discussion

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which cause dramatic increases in long-term survival with the advent of systemic chemotherapy, chondrosarcoma continue to have a poor prognosis due to absence of an effective adjuvant therapy [32]. The metastatic potential for conventional chondrosarcomas correlates well with the histologic grade of the tumor. But due to the relatively indolent growth rates of many low- and moderate-grade chondrosarcomas, ~15% of patients dying from metastatic disease do so >5 years after initial diagnosis [32]. Therefore, it is important to develop effective adjuvant therapy for preventing chondrosarcoma metastasis. We hypothesized that CCL5 and its CCR5 receptor would help to direct the metastasis of chondrosarcoma cells. We found that CCL5 increased the migration of chondrosarcoma cells. One of the mechanisms underlying CCL5-directed migration was transcriptional up-regulation of MMP-3 and activation of CCR5, PI3K, Akt and NF-κB pathways.

The CC-chemokine regulated on activation, normal T-cell expression, and presumably secreted CCL5/RANTES mediates its biological activities through activation of G protein-coupled receptors, CCR1, CCR3, or CCR5, and binds to glycosaminoglycans [33]. Stimulation of JJ012 cells with CCL5 increased the mRNA expression of CCR5 but not CCR1 and CCR3. Therefore, CCR5 is more important than CCR1 and CCR3 in CCL5-mediated cell functions. The expression of the CCL5 and CCR receptor isoforms in human chondrosarcoma cells are mostly unknown. Using qPCR analysis, we found that primary chondrosarcoma cells express both CCL5 and CCR5 receptor. In addition, the expression of mRNA levels of CCL5 and CCR5 in chondrosarcoma patients were significantly higher than those in normal cartilage. Our data provided the evidence that the expression of CCL5 and CCR5 are associated with a metastatic phenotype of chondrosarcoma cells. Moreover, CCR5 Ab, Met-RANTES or CCR5 siRNA reduced CCL5-mediated cell migration. Therefore, CCL5 and CCR5 interaction is mediated migration activity in human chondrosarcoma cells.

MMPs, a large family of zinc-dependent neutral proteinases, participate in normal physiological processes including development and tissue remodeling, as well as in pathological states such as the progression of metastasis and inflammation [34]. The same functions of MMPs that are beneficial in physiologic conditions turn into key mechanisms of disease pathogenesis in cancer and diseases due to overactive inflammation [35]. In human chondrosarcoma cells, MMP-1, -2, -3, -9 and -13 have been found to correlate with malignant grade and metastasis [25,26]. Our data show that CCL5 increased the MMP-3 but not MMP-1, -2, -9 and -13 expression. CCL5 also enhanced the protein expression of MMP-3 by using Western blot and ELISA assay. MMP-3 is synthesized by cancer cells and plays an important role in tumor cell invasion, metastasis and angiogenesis in human cancer cells [36,37]. In the present study, we found that MMP-3 siRNA and inhibitor reduced CCL5-enhanced migration activity. Therefore, MMP-3 may be the CCL5-responsive mediator, and it causes the degradation of

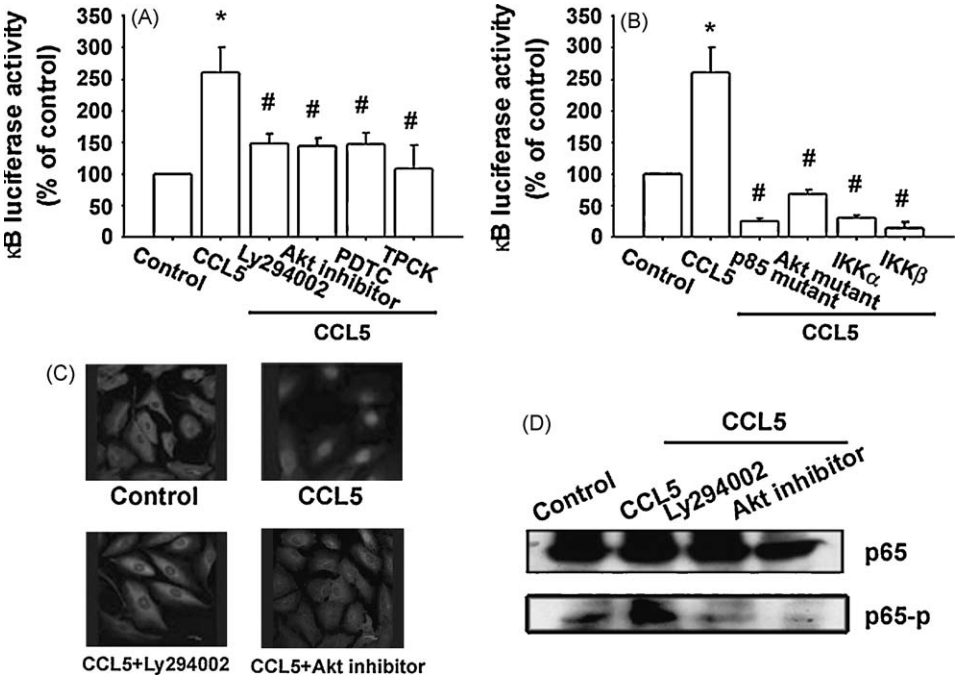

Fig. 5. PI3K/Akt pathway is involved in CCL5-mediated NF-κB activation and MMP-3 expression. (A and B) Cells were pretreated with Ly294002, Akt inhibitor, PDTC and TPCK for 30 min or transfected with mutant of p85, Akt, IKKα and IKKβ before exposure to CCL5 (3 ng/ml). NF-κB-luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. (C and D) Cells were pretreated with Ly294002, Akt inhibitor, PDTC and TPCK for 30 min. Then they were followed by stimulation with CCL5 (3 ng/ml) for 120 min, and p65 immunofluorescence staining and p65 phosphorylation was examined. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with CCL5-treated group.

extracellular matrix which may lead to subsequent cancer migration and metastasis.

A variety of growth factors stimulate the expression of MMPs via signal-transduction pathways that converge to activate NF-κB complex of transcription factors [38]. The PI3K/Akt pathway is a major cascade mediating activation of the NF-κB signaling pathway in human cancer cells [39]. Phosphorylation of the p85α subunit is required for activation of the p110 catalytic subunit of PI3K [40]. We found CCL5-enhanced the p85α subunit phosphorylation in human chondrosarcoma cells. Pretreatment of cells with PI3K inhibitors Ly294002 antagonized an increase in migration and MMP-3 expression by CCL5 stimulation. This was further confirmed by the result that the dominant-negative mutant of p85α inhibited the enhancement of migration by CCL5. Moreover, we also found that CCL5 activated Akt Ser473 phosphorylation and Akt kinase activity, while Akt inhibitor and Akt mutant inhibited CCL5-mediated cell migration. Our data indicates that PI3K/Akt could play an important role in the expression of MMP and migration of human chondrosarcoma cells. It has been reported that ERK is mediated CCL5-induced gene transcription [41]. Pretreatment of cells with ERK inhibitors (PD98059 or U0126) also reduced CCL5-mediated cell migration (Supplemental data Fig. S2). Therefore, ERK pathway may also involved in CCL5-induced cell motility. It has been reported that NF-κB element is important in MMP-3 transcription activity [42]. In this study, NF-κB inhibitors reduced the CCL5-mediated cell migration and MMP-3 activation in chondrosarcoma cells. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that CCL5-induced an increase in NF-κB activity. In this study, we found that CCL5-induced NF-κB activity was inhibited by Ly294002, Akt inhibitor, PDTC and TPCK or p85, Akt, IKKα and IKKβ mutant. On the other hand, Ly294002 and Akt inhibitor also reduced CCL5-increased p65 translocation and p65 phosphorylation. These results indicate that CCR5, PI3K and Akt signaling pathways are involved in CCL5-mediated NF-κB transactivation.

The prognosis of patients with chondrosarcoma distant metastasis is generally considered very poor; hence, preventing human chondrosarcoma metastasis is an important issue nowadays. Our study observes that CCL5 increases the activity of MMP-3 via the CCR5, PI3K, Akt, IKKα/β and NF-κB-dependent pathway and enhances migration of human chondrosarcoma cells (Fig. 6). Furthermore, the discovery of CCL5/CCR5-mediated signaling pathway helps us understand the mechanism of human chondrosarcoma metastasis and may lead us to develop effective therapy in the future.

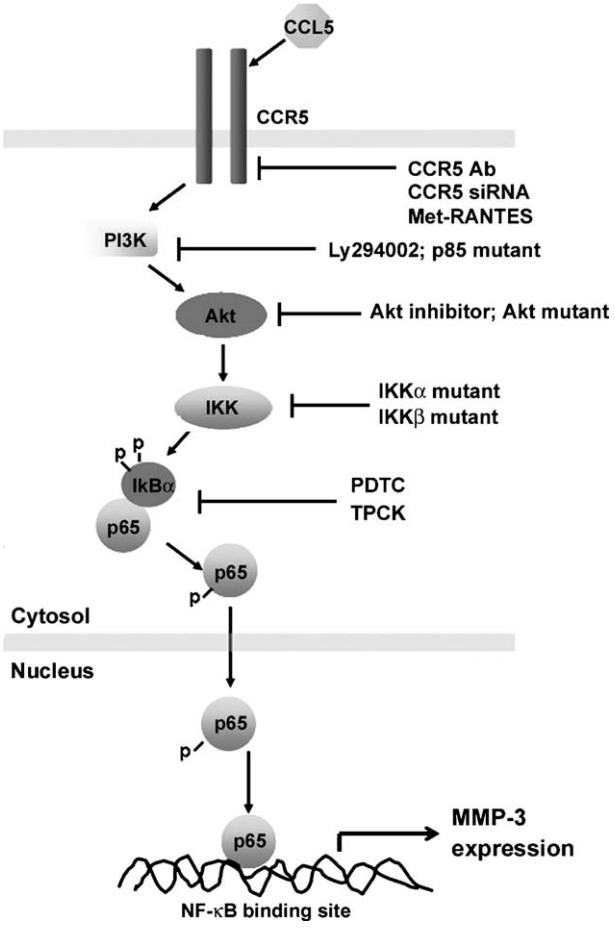

Fig. 6. Schematic presentation of the signaling pathways involved in CCL5-induced migration and MMP-3 expression of chondrosarcoma cells. CCL5 and CCR5 interaction activates PI3K and Akt pathways, which in turn induces NF-κB activation, which leads to MMP-3 expression and increases the migration of human chondrosarcoma cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.08.006.

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