CCL2 increases MMP-9 expression and cell motility in human chondrosarcoma cells via the Ras/Raf/MEK/ERK/NF-κB signaling pathway

Chih-Hsin Tang[1,2*] and Chia-Chun Tsai[2]

[1]Department of Pharmacology, School of Medicine, China Medical University, Taichung, Taiwan

[2]Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan

*: **Author for Correspondence:**

Chih-Hsin, Tang PhD

Department of Pharmacology, School of Medicine, China Medical University

No. 91, Hsueh-Shih Road, Taichung, Taiwan

Tel: (886) 4-22052121 Ext. 7726. Fax: (886) 4-22333641.

E-mail: chtang@mail.cmu.edu.tw

Abstract

Chondrosarcoma is a type of highly malignant tumor with a potent capacity to invade locally and cause distant metastasis. Chondrosarcoma shows a predilection for metastasis to the lungs. Chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1), belongs to the CC chemokine family that is associated with the disease status and outcomes of cancers. However, the effect of CCL2 on migration activity in human chondrosarcoma cells is mostly unknown. Here we found that CCL2 increased the migration and expression of matrix metalloproteinase (MMP)-9 in human chondrosarcoma cells. CCL2-mediated migration and MMP-9 up-regulation were attenuated by CCR2, Ras, Raf-1, and MEK inhibitor. Activation of the Ras, Raf-1, MEK, ERK, and NF-κB signaling pathway after CCL2 treatment was demonstrated, and CCL2-induced expression of MMP-9 and migration activity were inhibited by the specific inhibitor, and mutant of Ras, Raf-1, MEK, ERK, and NF-κB cascades. Taken together, our results indicated that CCL2 enhances the migration of chondrosarcoma cells by increasing MMP-9 expression through the CCR2 receptor, Ras, Raf-1, MEK, ERK, and NF-κB signal transduction pathway.

Running title: CCL2 induces the migration of chondrosarcoma

Key words: CCL2; Migration; Chondrosarcoma; Ras; Raf-1

1. Introduction

Chondrosarcoma is the second most common malignancy of bone and it has a poor response to chemotherapy or radiation treatment currently-used, making the management of chondrosarcomas a complicated challenge [1]. Clinically, surgical resection remains the primary mode of therapy for chondrosarcoma. In the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis and therefore, it is important to explore novel and adequate remedies [2]. Since chondrosarcoma is a type of highly malignant tumor with a potent capacity to invade locally and metastasize distantly [2, 3], an approach that decreases its ability to invade and metastasize may facilitate the development of effective adjuvant therapy.

Decades of scrutiny into the molecular bases of cancer have largely focused on what causes oncogenic transformation and the incipient emergence of tumors [4]. The invasion of tumor cells is a complex, multistage process. To facilitate cell motility, invading cells need to change cell-cell adhesion properties, rearrange the extracellular matrix (ECM) environment, suppress anoikis and reorganize their cytoskeletons [5]. Matrix metalloproteinases (MMPs) have important roles in these processes because their proteolytic activities assist in degradation of ECM and basement membrane [6-8]. MMPs, cytokines, growth factors, and chemokines have been shown to regulate tumor cell invasion through autocrine or paracrine pathways [5]. Previous studies demonstrated the expression of MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 in human chondrosarcoma cells [9]. Of them, MMP-9 has been found to play a role in the ECM degradation associated with cancer migration [10].

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 [11]. Chemokine ligand 2 (CCL2), also known as monocyte

chemoattractant protein-1 (MCP-1), belongs to the CC chemokine family. Beside its function as a chemoattractant for monocytes, memory T-cells and natural killer cells, CCL2 has also been reported to be implicated in the regulation of cancer cell growth, angiogenesis and metastasis of different tumors such as prostate cancer, breast, and colon [12-14].

Ras has been found to couple with multiple effector systems to activate distinct physiological and pathological responses such as cell proliferation and proinflammatory mediator release [15]. An important class of Ras effectors is the MAPK family. The classic Ras-mediated pathway involves the binding of Raf-1 and subsequent phosphorylation of Raf-1 at Ser^{338} by many kinases [16, 17], which in turn activates ERK [17], and consequently phosphorylates many target proteins including transcription factors and protein kinases [16]. A role for Ras in cancer migration has been implied in many cell types [18, 19]. However, the role of the Ras/Raf-1/MEK/ERK pathway in CCL2-mediated cancer migration has not been investigated in chondrosarcoma. Here we show that CCL2 increases migration and up-regulates MMP-9 expression in human chondrosarcoma cells. In addition, CCR2 receptor, Ras, Raf-1, MEK, ERK, and NF-κB signaling pathways were involved.

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-MEK, MEK, p-ERK, ERK, Raf-1, p65, MMP-9, β-actin, MMP-9 siRNA, Ras siRNA, Raf-1 siRNA, and control siRNA, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). U0126, PD98059, TPCK and PDTC were purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal antibody specific for phosphor-Raf-1 (Ser^{338}) and phosphor-p65 (Ser^{536}) were purchased from Cell Signaling (Danvers, MA, USA). Ras

activity assay kit was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The recombinant human CCL2 was purchased from PeproTech (Rocky Hill, NJ, USA). The MEK1 dominant-negative mutant was a gift from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The ERK2 dominant-negative mutant was a gift from Dr. M. Cobb (South-Western Medical Center, Dallas, TX, USA). 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 purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Cell culture

The human chondrosarcoma cell line (JJ012) was kindly provided from the laboratory of Dr. Sean P Scully (University of Miami School of Medicine, Miami, FL, USA). The cells were cultured in Dulbecco's modified Eagle's medium/α-minimum essential medium supplemented with 10% fetal bovine serum and maintained at 37℃ in a humidified atmosphere of 5% CO_2.

2.3 Migration assay

The migration assay was performed by using Transwell (Costar, NY, USA; pore size, 8-μm) in 24-well dishes. Before migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the MMP-9 inhibitor, RS102895, manumycin A, GW5074, U0126, PD98059, PDTC, TPCK or vehicle control (0.1% DMSO) (These inhibitors in experimental concentration did not affect cell viability in human chondrosarcoma cells). 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 CCL2 was placed in the lower chamber. The plates were incubated for 24 h at 37℃ 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. [20, 21].

2.4 Wound-healing migration assay

For the wound-healing migration assay, cells were seeded on 12-well plates at a density of 1 X 10^5 cells/well in culture medium. At 24 h after seeding, the confluent monolayer of culture was scratched with a fine pipette tip, and migration was visualized by microscope and magnification. The rate of wound closure was observed at the indicated time.

2.5 Western blot analysis

The cellular lysates were prepared as described previously [22]. 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 p-MEK, MEK, p-ERK, ERK, IκBα, p-IκB or IKKα/β (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).

2.6 Zymography analysis

The supernatants of JJ012 cells were mixed with sample buffer without reducing agent or heating. The sample was loaded into a gelatin (1 mg/ml) containing SDS–polyacrylamide gel and underwent electrophoresis with constant voltage. Afterwards, the gel was washed with 2.5% Triton X-100 to remove SDS, rinsed with 50 mM Tris-HCl, pH 7.5, and then incubated overnight at room temperature with the

developing buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% thimerosal, 1% Triton X-100).

2.7 Quantitative real time PCR

Total RNA was extracted from chondrosarcomas by using a TRIzol kit (MDBio Inc., Taipei, Taiwan). Two μg of total RNA was reverse transcribed into cDNA by using oligo(dT) primer. The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA, USA). Two μl of total cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems, Foster City, 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 ℃ followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ 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).

2.8 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.

2.9 Ras activity assay

Ras activity was measuring by using a Ras activity assay kit. The assay was performed according to the manufacturer's instructions. Briefly, cells were washed twice with ice-cold PBS, lysed in magnesium lysis buffer (25mM HEPES (pH 7.5), 150mM NaCl, 5% Igepal CA-630 (Upstate Biotechnology; Lake Placid, NY, USA), 10 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and centrifuged. An equal volume of lysate was incubated with 5 μg of Ras-binding domain for Raf-1 (Raf-1 RBD) at 4°C overnight, and beads were washed three times with magnesium lysis buffer. Bound Ras proteins were then solubilized in 2 X Laemmli sample buffer and quantitatively detected by Western blotting (10% SDS–PAGE) using mouse monoclonal Ras with the ECL system, and by densitometry of corresponding bands using scientific imaging systems.

2.10 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously [23]. DNA immunoprecipitated by anti-p65 antibody was purified. The DNA was then extracted with phenol-chloroform. The purified DNA pellet was subjected to PCR. PCR products were then resolved by 1.5% agarose gel electrophoresis and visualized by UV.

The primers: 5'- TGTCCCTTTACTGCCCTGA-3'

and 5'-ACTCCAGGCTCTGTCCTCCTCTT-3' were utilized to amplify across the human MMP-9 promoter region (−657 to −484).

2.11 Immunofluorocytochemistry

Cells were cultured in 12-mm coverslips. After treatment with CCL2, cells were fixed with 4% paraformaldehyde at room temperature. Thirty minutes later, 4% nonfat 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.

2.12 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. Result

3.1 CCL2-directed chondrosarcoma cells migration through up-regulation of MMP-9

CCL2-triggered migration in chondrosarcoma cells was examined by using the Transwell assay [20]. CCL2 directed human chondrosarcoma cell migration (JJ012 cells) (Fig. 1A). In addition, CCL2 also dose-dependently increased wound healing migration activity in human chondrosarcoma cells (Fig. 1B). Previous study has shown a significant expression of MMP-1, -2, -3, -9 and -13 in human chondrosarcoma cells [9]. We therefore, hypothesized that any of these MMPs may be involved in CCL2-directed chondrosarcoma migration. Treatment of cells with CCL2 induced the expression of MMP-9 but not other MMPs by using qPCR analysis (Fig. 2A). In addition, CCL2 also slightly increased MMP-2 mRNA expression (Fig. 2A). MMP-9 but not MMP-2 expression was increased in the supernatant, and its enzyme

activity was up-regulated (Fig. 2B). Furthermore, CCL2 further increased protein expression of MMP-9 in JJ012 cells (Fig. 2C). Pretreatment of cells with MMP-9 inhibitor blocked CCL2-induced cell migration and wound healing activity (Fig. 2D&E). JJ012 cells were transfected with MMP-9 or control siRNA for 24 h, and the Western blot analysis showed that the expression of protein levels of MMP-9 was suppressed by transfection with MMP-9 siRNA (Fig. 2D). Transfection of cells with MMP-9 siRNA reduced the CCL2-increased cell migration (Fig. 2D). Therefore, CCL2 increased cell migration through up-regulation of MMP-9 in chondrosarcoma cells.

3.2 CCL2/CCR2 axis directed migration of human chondrosarcoma cells

Previous study has shown CCL2 affects cell migration through binding to cell surface CCR2 or CCR4 receptor [24, 25]. Pretreatment of cells with CCR2 inhibitor RS102895 but not CCR4 inhibitor C021 abrogated the CCL2-induced increases in cell migration (Fig. 3A). Therefore, CCR2 but not CCR4 is mediated CCL2-induced cell migration. In addition, CCR2 inhibitor also reduced CCL2-incrased wound healing activity and MMP-9 expression (Fig. 3B-D). Therefore, the CCL2 induced cell migration through CCR2 receptor in human chondrosarcoma cells.

3.3 Ras and Raf-1 signaling pathways are involved in the CCL2-mediated cell migration of human chondrosarcoma

It has been demonstrated that Ras mediated cell motility in cancer cells [15]. To explore whether Ras might mediate CCL2-induced cancer migration, manumycin A, a Ras inhibitor was used. As shown in Fig. 4A-D, pretreatment of cells with manumycin A inhibited CCL2-induced cell migration and MMP-9 expression. Transfection of cells with Ras siRNA also reduced CCL2-increased migration activity and MMP-9 expression (Fig. 4A&C). Next, we directly measured the Ras activity in response to CCL2. Fig. 4E shows that exposure of JJ012 cells to CCL2 induced an increase in Ras activity in a time-dependent manner, as assessed by immunoblotting samples for Ras

immunoprecipitated from lysates using Raf-1 RBD. To examine whether Raf-1, a target protein for Ras, might play a crucial role in CCL2-induced cancer migration, the Raf-1 inhibitor, GW5074 was used. As shown in 4A-D, pretreatment of cells with GW5074 inhibited CCL2-induced migration and MMP-9 activity. Furthermore, Raf-1 siRNA also blocked CCL2-increased migration and MMP-9 expression (Fig. 4A&C). Raf-1 is associated with Ras-GTP, and then by additional modifications such as phosphorylation at Ser^{338}, becomes the active form [26]. The activated Raf-1 then triggers sequential activation of downstream molecules. Thus, phosphorylation of Raf-1 at Ser^{338} is a critical step in Raf-1 activation. Next, we further examined Raf-1 Ser^{338} phosphorylation by CCL2 stimulation. Stimulation of cells with CCL2 increased Raf-1 Ser^{338} phosphorylation (Fig. 4F). In addition, CCL2-induced Ras activity or Raf-1 Ser^{338} phosphorylation was inhibited by treatment with RS102895 or manumycin A (Fig. 4G&H). The results indicate that Raf-1 is a downstream molecule of Ras and is involved in CCL2-mediated migration and MMP-9 expression.

3.4 The signaling pathways of MEK and ERK are involved in the potentiating action of CCL2 stimulation

We next wished to determine whether CCL2 is able to activate MEK/ERK, a critical downstream target of Raf-1 [27], which has been shown to induce gene expression [28]. Stimulation of cells with CCL2 induced MEK and ERK phosphorylation (Fig. 5A). CCL2-induced the cell migration were greatly reduced by treatment with MEK inhibitors PD98059 and U0126 (Fig. 5B&C). The MEK inhibitors also inhibited the CCL2-increased MMP-9 expression (Fig. 5D&E). Transfection of cells with MEK1 or ERK2 mutant reduced the CCL2-mediated cell migration and MMP-9 expression (Fig. 5B&D). Furthermore, CCL2-induced MEK phosphorylation was markedly inhibited if cells were pretreated for 30 min with RS102895, manumycin A, and GW5074 (Fig. 5F). Taken together, these results indicate that the CCR2/Ras/Raf-1/MEK/ERK pathway is involved in CCL2-induced migration activity and MMP-9 up-regulation in human chondrosarcoma cells.

3.5 Involvement of NF-κB in CCL2-induced cell migration and MMP-9 expression

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human chondrosarcoma cells [29]. To examine whether NF-κB activation is involved in the signal transduction pathway leading to migration and MMP-9 expression caused by CCL2, the NF-κB inhibitor PDTC was used. Figure 6A-D shows that PDTC inhibited the enhancement of migration and MMP-9 production induced by CCL2. Furthermore, pretreatment of cells with an IκB protease inhibitor [L-1-tosylamido-2-phenylenylethyl chloromethyl ketone; TPCK] also antagonized the potentiating action of CCL2 (Fig. 6A-D). These results indicated that NF-κB activation is important for CCL2-induced cancer cell migration and the expression of MMP-9. We further examined the upstream molecules involved in CCL2-induced NF-κB activation. Stimulation of cells with CCL2 induced IKKα/β phosphorylation in a time-dependent manner (Fig. 6E). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the CCL2-induced cell migration and MMP-9 expression (Fig. 6A&C). These data suggest that IKKα/β activation is involved in CCL2-induced the migration activity of human chondrosarcoma cells. Previous study showed that p65 Ser^{536} phosphorylation increases NF-κB transactivation [30], and the antibody specific against phosphorylated p65 Ser^{536} was used to examine p65 phosphorylation. Treatment of chondrosarcoma cells with CCL2 for various time intervals resulted in IκB and p65 Ser^{536} phosphorylation (Fig. 6E). Pretreatment of cells with RS102895, manumycin A, GW5974, or PD98059 reduced CCL2-induced p65 phosphorylation (Fig. 6F).

We next investigated whether p65 binds to the NF-κB element on the MMP-9 promoter after CCL2 stimulation. The *in vivo* recruitment of p65 to the MMP-9 promoter (−657 to −484) was assessed by the chromatin immunoprecipitation assay. *In vivo* binding of p65 to the NF-κB element of the MMP-9 promoter occurred after CCL2 stimulation (Fig. 7A). Binding of p65 to the NF-κB element by CCL2 was

attenuated by RS102895, manumycin A, GW5074, or PD98059 (Fig. 7A). Pretreatment of cells with RS102895, manumycin A, GW5074, or PD98059 also reduced CCL2-induced accumulation of p65 into the nucleus (Fig. 7B). To directly determine NF-κB activation after CCL2 treatment, chondrosarcoma cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 7C, CCL2 treatment of chondrosarcoma cells for 24 h caused increase in κB-luciferase activity. In addition, the CCL2-induced increase in κB-luciferase activity was also inhibited by treatment with RS102895, manumycin A, GW5074, PD98059, U0126, PDTC, or TPCK (Fig. 7C). Cotransfection of cells with Ras, or Raf-1 siRNA and MEK, ERK, IKKα, or IKKβ mutant blocked CCL2-enhanced κB-luciferase activity (Fig. 7D). Taken together, these data suggest that activation of CCR2 receptor, Ras, Raf-1, MEK, and ERK are required for CCL2-induced NF-κB activation in human 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 continues to have a poor prognosis due to absence of an effective adjuvant therapy [3]. 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 [3]. Therefore, it is important to develop effective adjuvant therapy for preventing chondrosarcoma metastasis. We hypothesized that CCL2 would help to direct the metastasis of chondrosarcoma cells. We found that CCL2 increased the migration of chondrosarcoma cells. One of the mechanisms underlying CCL2-directed migration was transcriptional up-regulation of MMP-9 and activation of CCR2 receptor, Ras,

Raf-1, MEK, ERK and NF-κB pathways. In addition, MMP-9 inhibitor, RS102895, manumycin A, GW5074, U0126, and PDTC reduced CCL2-mediated cell migration in other chondrosarcoma, SW1353 cells (Supplementary Fig. S1). On the other hand, MMP-9 inhibitor, RS102895, manumycin A, GW5074, U0126, and PDTC also abolished CCL2-increased MMP-9 expression in SW1353 cells (Supplementary Fig. S1). Therefore, the same signaling pathways of migration are involved in all chondrosarcoma cell lines.

The CC-chemokine regulated on activation, normal T-cell expression, and presumably secreted CCL2 mediates its biological activities through activation of G protein–coupled receptors, CCR2 or CCR4 [24, 25]. It have reported that CCL2 affects cell migration through binding to cell surface CCR2 or CCR4 receptor [24, 25]. In this study, we found that pretreatment of cells with CCR2 inhibitor but not CCR4 inhibitor blocked CCL2-increased cell migration. In addition, CCR2 inhibitor also reduced CCL2-induced MMP-9 expression. The results indicated that expression of CCL2/CCR2 axis was associated with an invasive and/or metastatic phenotype of human chondrosarcoma cells.

Enzymatic degradation of ECM is one of the crucial steps in cancer invasion and metastasis. In human cancer cells, MMP-1, -2, -3, -9 and -13 have been found to correlate with malignant grade and metastasis [6, 31]. It has been reported that MMP plays important role in CCL2-induced migration in human cancer cells [32]. In this study, we found that CCL2 induced MMP-9 expression and secretion in human chondrosarcoma cells without significantly changing the expression of MMP-1, -3, and -13 mRNAs. In addition, treatment of cells with MMP-9 inhibitor reduced CCL2-induced cell migration. Furthermore, the inhibition of CCL2-enhanced MMP-9 protein expression with siRNA significantly suppressed CCL2-induced migration. Therefore, MMP-9 may be the CCL2-responsive mediator, and it causes the degradation of ECM may lead to subsequent cancer migration and metastasis.

Ras proteins are members of the superfamily of small GTPases claimed to play a key role in signaling pathways leading to cell proliferation, differentiation, and

transformation [33]. Several reports have indicated that Ras might play a critical role in the induction of cancer migration [15, 16]. In the present study, we show that manumycin A, a Ras farnesyl transferase inhibitor, inhibited the CCL2-induced increase in migration and MMP-9 expression. In addition, CCL2 stimulation also increased kinase activity of Ras. These results suggest that Ras activation might be involved in CCL2-mediated migration and MMP-9 expression.

Ras, an oncogenic protein, plays a critical role in the induction of genes expression [34]. Ras might activate a number of signal pathways, including the Raf-1/MEK/ERK pathway and the phosphatidylinositol 3-kinase/Akt/NF-κB pathway [34, 35]. In RAW 264.7 macrophages, LPS induces tumor necrosis factor gene expression through the Ras/Raf-1/MEK/ERK pathway [36]. In murine fibroblasts, oncogene- and growth factor-induced COX-2 transcription requires Ras-dependent Raf-2/MAPKK/ERK activation [37]. In this study, we first found that exposure of chondrosarcoma cells with CCL2 caused sequential activations of Ras, Raf-1, MEK, and ERK, and that manumycin A, GW5074, U0126, and PD98059 all inhibited CCL2-induced migration activation and MMP-9 expression. Furthermore, Ras or Raf-1 siRNA and MEK1 or ERK2 mutant also reduced CCL2-increase migration and MMP-9 expression. These results suggested that Ras/Raf-1/MEK/ERK signal pathway is important for CCL2-induced migration activity and MMP-9 expression.

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 CCL2 increases the activity of MMP-9 via the CCR2 receptor, Ras, Raf-1, MEK, ERK, and NF-κB-dependent pathway and to enhance migration of human chondrosarcoma cells. Furthermore, the discovery of CCL2-mediated signaling pathway helps us understand the mechanism of human chondrosarcoma metastasis and may lead us to develop effective therapy in the future.

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Figure legends

Fig. 1 CCL2 induced the migration activity of human chondrosarcoma cells

(A) JJ012 cells were incubated with CCL2 (25-100 ng/ml) for 24 h, and *in vitro* migration was measured with the Transwell after 24 h. (B) JJ012 cells were treated with CCL2 (25-100 ng/ml) for 24 h, the wound-scratching assay was performed. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control.

Fig. 2 CCL2-directed migration activity of human chondrosarcoma cells involves up-regulation of MMP-9.

(A) JJ012 cells were incubated with CCL2 for 24 h, the mRNA level of MMP-1, -2, -3, -9 and -13 was determined using qPCR. (B&C) JJ012 cells were incubated with CCL2 for indicated time intervals. The cultured medium and cell lysates were then collected. The protein level of MMP-9 in cell lysates and enzyme activity of MMP-2 and MMP-9 in supernatant were examined by Western blot and zymography. (D&E) JJ012 cells were pretreated with MMP-9 inhibitor (5 nM) for 30 min or transfected with MMP-9 siRNA for 24 h followed by stimulation with CCL2, and *in vitro* migration and wound healing activity was measured after 24 h. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCL2-treated group.

Fig. 3 CCL2 increased cell migration and MMP-9 expression through CCR2 receptor.

(A) JJ012 cells were pretreated with RS102895 (400 nM) or C0214 (400 nM) for 30 min followed by stimulation with CCL2, and *in vitro* migration was measured after 24 h. (B-D) JJ012 cells were pretreated with RS102895 (400 nM) for 30 min followed by stimulation with CCL2, and wound healing activity and MMP-9 mRNA and protein expression was measured after 24 h.

Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCL2-treated group.

Fig. 4 CCL2 increased cell migration and MMP-9 expression through Ras and Raf-1 pathways

(A&C) Cells were pretreated with manumycin A (3 μM) and GW5074 (3 μM) for 30 min or cotransfected with Ras and Raf-1 siRNA for 24 h followed by stimulation with CCL2 for 24 h, and *in vitro* migration and MMP-9 expression was measured with the Transwell and qPCR. (B&D) Cells were pretreated with manumycin A and GW5074 for 30 min followed by stimulation with CCL2 for 24 h, and wound healing activity and MMP-9 protein expression was measured. (E&G) JJ012 cells were incubated with CCL2 for indicated time intervals or pretreated with RS102895 for 30 min followed by stimulation with CCL2 for 60 min, and then cell lysates were immunoprecipitated with an antibody specific for Raf-1 RBD. (F&H) JJ012 cells were incubated with CCL2 for indicated time intervals or pretreated with RS102895 or manumycin A for 30 min followed by stimulation with CCL2 for 60 min, and then p-Raf-1 expression was examined by Western blotting. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCL2-treated group.

Fig. 5 CCL2 increased cell migration and MMP-9 expression through MEK and ERK pathways

(A) JJ012 cells were incubated with CCL2 for indicated time intervals, and p-MEK and p-ERK expression was determined by Western blotting. (B&D) Cells were pretreated with PD98059 (30 μM) and U0126 (30 μM) for 30 min or cotransfected with MEK1 and ERK mutant for 24 h followed by stimulation with CCL2 for 24 h, and *in vitro* migration and MMP-9 expression was measured with the Transwell and qPCR. (C&E) Cells were

pretreated with PD98059 and U0126 for 30 min followed by stimulation with CCL2 for 24 h, and wound healing activity and MMP-9 protein expression was measured. (F) JJ012 cells were pretreated with RS102895, manumycin A, or GW5904 for 30 min followed by stimulation with CCL2, and then p-MEK expression was examined by Western blotting. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCL2-treated group.

Fig. 6 CCL2 induced cell migration and MMP-9 up-regulation through NF-κB.

(A) Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) or cotransfected with IKKα and IKKβ mutant for 24 h followed by stimulation with CCL2 for 24 h, and *in vitro* migration and MMP-9 expression was measured with the Transwell and qPCR. (B&D) Cells were pretreated for 30 min with PDTC or TPCK followed by stimulation with CCL2 for 24 h, and wound healing activity and MMP-9 protein expression was measured. (E) JJ012 cells were incubated with CCL2 for indicated time intervals, and p-IKK, p-IκBα, or p-p65 expression was determined by Western blotting. (F) JJ012 cells were pretreated with RS102895, manumycin A, GW5904, or PD98059 for 30 min followed by stimulation with CCL2, and then p-p65 expression was examined by Western blotting. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCL2-treated group.

Fig. 7 CCL2 induced NF-κB activation through CCR2/Ras/Raf-1/MEK/ERK pathway.

(A) JJ012 cells were pretreated with RS102895, manumycin A, GW5904, or PD98059 for 30 min then stimulated with CCL2 for 120 min, and the chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with anti-p65. One percentage of the precipitated

chromatin was assayed to verify equal loading (input). (B) JJ012 cells were pretreated with RS102895, manumycin A, GW5904, or PD98059 for 30 min then stimulated with CCL2 for 120 min, and p65 immunofluorescence staining was examined. JJ012 cells were pretreated with RS102895, manumycin A, GW5904, PD98059, PDTC, or TPCK for 30 min (C) or transfected with Ras and Raf-1 siRNA or MEK, ERK, IKKα and IKKβ mutant (D) before exposure to CCL2. 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. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCL2-treated group

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