CCN3 increases cell motility and MMP-13 expression in human chondrosarcoma through integrin dependent pathway

Huey-En, Tzeng[1,2], Jui-Ching Chen[3], Chun-Hao Tsai[4], Chien-Chung Kuo[4], Horng-Chaung Hsu[4], Wen-Lee Hwang[1], Yi-Chin Fong[4,5] and Chih-Hsin Tang[6,3*]

[1]Division of Hematology/Oncology, Taichung Veterans General Hospital, Taichung, Taiwan

[2]Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan

[3]Graduate Institute of Basic Medical Science, China Medical University and Hospital, Taichung, Taiwan

[4]Department of Orthopaedic Surgery, China Medical University Hospital, Taichung, Taiwan

[5]School of Chinese Medicine, China Medical University, Taichung, Taiwan

[6]Department of Pharmacology, School of Medicine, China Medical University and Hospital, Taichung Taiwan

***: Address correspondence to:**

Chih-Hsin Tang, PhD

Department of Pharmacology, School of Medicine, China Medical University

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

Tel: 886-4-22052121-7726; Fax: 886-4-22053764

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. CCN3, also called Nephroblastoma overexpressed gene (NOV), was regulation of proliferation and differentiation of cancer cells. However, the effect of CCN3 on migration activity in human chondrosarcoma cells is mostly unknown. Here we found that CCN3 increased the migration and expression of matrix metalloproteinase (MMP)-13 in human chondrosarcoma cells (JJ012 cells). αvβ3 or αvβ5 monoclonal antibody, phosphatidylinositol 3-kinase (PI3K) inhibitors (Ly294002 and wortmannin) and Akt inhibitor inhibited the CCN3-induced increase of the migration and MMP-13 up-regulation of chondrosarcoma cells. CCN3 stimulation increased the phosphorylation of focal adhesion kinase (FAK), PI3K and Akt. In addition, NF-κB inhibitors also suppressed the cell migration and MMP-13 expression enhanced by CCN3. Moreover, CCN3 increased NF-κB luciferase activity and binding of p65 to the NF-κB element on the MMP-13 promoter. Taken together, our results indicated that CCN3 enhances the migration of chondrosarcoma cells by increasing MMP-13 expression through the αvβ3/αvβ5 integrin receptor, FAK, PI3K, Akt, p65 and NF-κB signal transduction pathway.

Running title: CCN3 promotes migration of chondrosarcoma

Key words: CCN3; MMP-13; Chondrosarcoma; Migration; FAK.

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 (Terek et al., 1998). 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 (Yuan et al., 2005). Since chondrosarcoma is a type of highly malignant tumor with a potent capacity to invade locally and metastasize distantly (Yuan et al., 2005), an approach that decreases its ability to invade and metastasize may facilitate the development of effective adjuvant therapy.

Tumor metastatic cascade consists of multiple successive steps, including adhesion of tumor cells at primary site, invasion into intravascular space, dissemination to distant sites, adhesion of tumor cells to vascular endothelium of distant tissues, extravasation and invasion into surrounding tissues, and finally formation of secondary tumor colonies (Joyce and Pollard, 2009). 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 (Zucker et al., 2000). Matrix metalloproteinases (MMPs) have important roles in these processes because their proteolytic activities assist in degradation of ECM and basement membrane (Egeblad and Werb, 2002; Kerkela and Saarialho-Kere, 2003). MMPs, cytokines, growth factors and chemokines have been shown to regulate tumor cell invasion through autocrine or paracrine pathways (Zucker et al., 2000). Previous studies demonstrated the expression of MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 in human chondrosarcoma cells (Hou et al., 2009; Tan et al., 2009b).

CCN3 is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of matricellular proteins, with developmental functions (Holbourn et al., 2008; Perbal, 2001). Recent studies have shown that the CCN protein family members also

play important roles in tumorigenesis, including cancer cell proliferation, survival, adhesion, and invasion (Kleer et al., 2004; Kleer et al., 2002). CCN proteins are mostly secreted and extracellular matrix associated and have been proposed to connect signaling pathways and facilitate cross talk between epithelium and stroma (Holbourn et al., 2008).

CCN3 is widely expressed, especially in the nervous and musculoskeletal systems as well as in the blood vessels. The functions of CCN3 protein among these different tissues might, however, be very different. Although CCN3 was originally described as antiproliferative (Joliot et al., 1992) and its expression was associated with differentiation and growth arrest in Wilm's tumour, chondrosarcomas and rhabdomyosarcomas (Chevalier et al., 1998; Yu et al., 2003), more recent data correlate CCN3 with increased proliferative index of 3T3 fibroblast and tissue samples of prostate and renal carcinomas (Liu et al., 1999; Maillard et al., 2001). In osteosarcoma, it is inversely associated with expression of liver/bone/kidney alkaline phosphatase isoform early marker of osteoblastic differentiation (Manara et al., 2002; Stein et al., 1990). In Ewing's sarcoma, expression of CCN3 was only sporadically observed, but it was associated with a significantly higher risk of developing lung and bone metastasis (Manara et al., 2002).

Previous studies have shown that CCN1 (Cyr61) and CCN2 (CTGF) modulates cell migration and invasion in human chondrosarcoma cells (Tan et al., 2009a; Tan et al., 2009b). However, the effect of CCN3 on migration activity in human chondrosarcoma cells is mostly unknown. Here we show that CCN3 increases migration and upregulate MMP-13 expression in human chondrosarcoma cells. In addition, αvβ3/αvβ5 integrin receptor, focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), Akt and NF-κB signaling pathways were involved.

Materials and Methods

Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, p-p85, p85, p-Akt, Akt, MMP-13, control siRNA, FAK siRNA, CCN3 shRNA plasmid and control shRNA plasmid were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies specific for αvβ3 and αvβ5 integrin was purchased from Chemicon (Temecula, CA). Ly294002, TPCK, PDTC, and Akt inhibitor [1L-6-hydroxymethyl-chiro-inositol-2-((*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate)] were purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal antibody specific for phospho-FAK (Tyr^{397}), phospho-p85 (Tyr^{458}), phospho-IKKα/β ($Ser^{180/181}$), and phospho-p65 (Ser^{536}) were purchased from Cell Signaling (Danvers, MA, USA). The phosphorylation site mutant of FAK (Y397F) was a gift from Dr. J.A. Girault (Institut du Fer à Moulin, Moulin, France). The p85α (Δp85; deletion of residues 479–513 of p85), Akt (Akt K179A) 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). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

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

Migration assay

The migration assay was performed using Transwell 24-well dishes with a pore

size of 8 μm (Costar, NY, USA). Before performing the migration assay, cells were pretreated for 30 min with inhibitors including Ly294002, wortmannin, Akt inhibitor, PDTC, TPCK, or vehicle control (0.1% DMSO). These concentrations of inhibitors did not affect cell death of JJ012 cells, as shown by cell viability assay (Supplementary Figure S2). 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 CCN3 was placed in the lower chamber. The cells were incubated for 24 h at 37℃ in 5% CO_2, then 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 CCN3 treatment (corrected invading cell number = counted invading cell number/percentage of viable cells) (Chuang et al., 2009).

Western blot analysis

The cellular lysates were prepared as described previously (Chiu et al., 2009). Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 hr at room temperature and then probed with rabbit anti-human antibodies against Akt, p-Akt or p-p65 (1:1000) for 1 hr at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 hr at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Zymography analysis

Supernatants collected from JJ012 cell cultures were mixed with sample buffer without reducing agent or heating. Samples were loaded onto an 10% SDS-PAGE gel containing 1 mg/ml gelatin and electrophoresed under constant voltage. Afterward, 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 developing buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% thimerosal, and 1% Triton X-100). Zymographic activity was revealed by staining with 1% Coomassie Blue (Chiu et al., 2008).

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. Cells were grown to 80% confluent in 12 well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. RPMI-1640 containing 20% FCS was added 4 hr later. After 24 hr transfection, the cells were then incubated with the indicated agents. After further 24 hr's incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) 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.

Quantitative real time PCR

Total RNA was extracted from chondrosarcoma 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). One hundred ng 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, CA). 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).

Immunofluorescence staining

Human chondrosarcoma cells were plated on 24-well culture plates with coverslips. Cells were treated with CCN3 and washed twice with ice-cold phosphate-buffered saline. Immunofluorescence staining using a primary anti-p65 monoclonal antibody was performed as described previously (Tang et al.).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (Tan et al., 2009b). 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’-TTTGGCTGTATTTTGTTTGGA-3’ and 5’-AAAATGCTGCTCAGGTCAGG-3’ were utilized to amplify across the human MMP-13 promoter region (-1138 to -947).

Establishment of stably transfected cells

CCN3 shRNA or control shRNA plasmids are transfected into cancer cells with Lipofetamine 2000 transfection reagent. Twenty-four hours after transfection, stable transfectants are selected in puromycin (Life Technologies) at a concentration of 10 μg/mL. Thereafter, the selection medium is replaced every 3 days. After 2 weeks of selection in puromycin, clones of resistant cells are isolated.

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 is significant if the *p* value is <0.05.

Results

CCN3 increased chondrosarcoma cell migration and MMP-13 expression

Previous studies have shown that CCN3 regulates cell migration and invasion in human cancer cells (Benini et al., 2005; Sin et al., 2008). However, the effect of CCN3 in migration of chondrosarcoma is mostly unknown. CCN3-triggered migration in chondrosarcoma cells was examined by using the Transwell assay with correction of CCN3-induced proliferation effects on human chondrosarcoma cells. CCN3 directed human chondrosarcoma cells (JJ012 cells) migration (Fig. 1A). On the other hand, stimulation of chondrosarcoma with CCN3 did not affect cell viability (Fig. 1B). Previous studies have shown a significant expression of MMP-1, -2, -3, -9, and -13 in human chondrosarcoma (Hou et al., 2009; Tan et al., 2009b). We therefore hypothesized that any of these MMPs may be involved in CCN3-induced chondrosarcoma migration. Treatment of cells with CCN3 increased transcriptional expression of MMP-13 but not MMP-1, -3 and -9 as measured by qPCR (Fig. 1C). In addition, CCN3 slightly increased MMP-2 mRNA expression in chondrosarcoma (Fig.

1C). Furthermore, CCN3 further increased protein expression of MMP-13 in JJ012 cells (Fig. 1D). MMP-13 expression was also increased in the supernatant, and its enzyme activity was up-regulated (Fig. 1D). In siRNA experiments, MMP-13 protein levels were reduced in cells transfected with MMP-13 siRNA compared to control siRNA (Fig. 1E), and transfection of cells with MMP-13 siRNA also reduced CCN3-induced cell migration (Fig. 1E). Together, these results suggest that CCN3 increased cell migration and MMP-13 expression in human chondrosarcoma cells.

CCN3-directed chondrosarcoma cell migration through αvβ3 and αvβ5 integrin

Previously study has shown that CCN affects cells migration through binding to cell surface integrin receptors (Tan et al., 2009b). We therefore, hypothesized that integrin-signaling pathway may be involved in CCN3-directed chondrosarcoma cells migration. Pretreatment of cells for 30 min with anti-αvβ5 and αvβ3 but not α5β1 monoclonal antibody (mAb) markedly inhibited the CCN3-induced cancer migration (Fig. 2A). On the other hand, pretreatment of cells with αvβ5 and αvβ3 but not α5β1 mAb reduced CCN3-mediated MMP-13 expression (Fig. 2B). These data suggest that CCN3-induced cancer migration may occur via activation of the αvβ5 and αvβ3 integrin receptor.

FAK, PI3K and Akt signaling pathways are involved in the CCN3-mediated MMP-13 upregulation and migration of chondrosarcoma

FAK has been shown to be capable of regulating integrin-mediated signaling (Giancotti and Ruoslahti, 1999). Phosphorylation of tyrosine 397 of FAK has been used as a marker of FAK activity. As shown in Fig. 2C, FAK phosphorylation increased in a time-dependent manner in response to CCN3 stimulation. Transfection of cells with FAK(Y397F) mutant reduced the CCN3-mediated cells migration and MMP-13 expression (Fig. 2D&E). On the other hand, transfection of cells with FAK siRNA also reduced the CCN3-induced migration and MMP-13 expression (Fig. 2D&E). Phosphorylation of tyrosine 397 of FAK may provide a binding site for the

Src homology 2 domain of the p85 subunit of PI3K (Giancotti and Ruoslahti, 1999). Because activation of integrin by CCN3 stimulation potentiates FAK phosphorylation, we then examined whether CCN3 stimulation also enhances the association of FAK with PI3K. Treatment of cells with CCN3 led to a significant increase of phosphorylation of p85 subunit of PI3K (Fig. 3A). To explore whether PI3K is involved in CCN3-induced cells migration, PI3K inhibitor Ly294002 and wortmannin were used. As shown in Fig. 3B&C, pretreatment of cells with Ly294002 or wortmannin inhibited CCN3-induced migration activity and MMP-13 expression of chondrosarcoma. Transfection of cells with p85 mutant also antagonized the potentiating effect of CCN3 (Fig. 3B&C). We then directly measured the Akt phosphorylation in response to CCN3 stimulation. Figure 3A shows that CCN3 increased Akt phosphorylation in a time-dependent manner. Furthermore, Akt inhibitor or mutant also antagonized CCN3-induced cell migration and MMP-13 expression (Fig. 3B-E). Taken together, these results indicate that the integrin/FAK/PI3K and Akt pathway is involved in CCN3-induced migration and MMP-13 expression of human chondrosarcoma cells.

Involvement of NF-κB in CCN3-induced cell migration and MMP-13 expression

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human chondrosarcoma cells (Liu et al.; Tang et al.). To examine whether NF-κB activation is involved in the signal transduction pathway caused by CCN3 that leads to cell migration and MMP-13 expression, the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was used. Fig. 4A-B show that PDTC inhibited the enhancement of cell migration and MMP-13 expression induced by CCN3. Furthermore, pretreatment of chondrosarcoma cells with an IκB protease inhibitor [L-1-tosylamido-2-phenylenylethyl chloromethyl ketone (TPCK)] or NF-κB inhibition peptide (IP) antagonized the potentiating action of cell migration and MMP-13 expression (Fig. 4A&B). We further examined the upstream molecules involved in CCN3-induced NF-κB activation. Stimulation of cells with

CCN3 induced IKKα/β phosphorylation in a time-dependent manner (Fig. 4C). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the CCN3-induced cell migration and MMP-13 expression (Fig. 4D&E). These data suggest that IKKα/β activation is involved in CCN3-induced the migration activity of human chondrosarcoma cells. Treatment with chondrosarcoma cells with CCN3 also caused IκBα phosphorylation in a time-dependent manner (Fig. 4C). Previous studies showed that p65 Ser^{536} phosphorylation increases NF-κB transactivation (Madrid et al., 2001), and the antibody specific against phosphorylated p65 Ser^{536} was used to examine p65 phosphorylation. Treatment of chondrosarcoma cells with CCN3 for various time intervals resulted in p65 Ser^{536} phosphorylation (Fig. 4C). It has been reported that the NF-κB binding site between -1005 and -1014 is important for the activation of the MMP-13 gene (Muddasani et al., 2007). We next investigated whether p65 binds to the NF-κB element on the MMP-13 promoter (-1138 to -947) after CCN3 stimulation. The *in vivo* recruitment of p65 to the MMP-13 promoter was assessed by the chromatin immunoprecipitation assay. *In vivo* binding of p65 to the NF-κB element of the MMP-13 promoter occurred after CCN3 stimulation (Fig. 5A). Binding of p65 to the NF-κB element and p65 translocation to nucleus by CCN3 was attenuated by FAK siRNA, Ly294002, Akt inhibitor, αvβ3 mAb and αvβ3 mAb (Fig. 5A&B). To directly determine NF-κB activation after CCN3 treatment, chondrosarcoma cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 5C, CCN3 treatment of chondrosarcoma cells for 24 h caused increase in κB-luciferase activity. CCN3-induced increase in κB-luciferase activity was also inhibited by treatment with Ly294002, wortmannin, Akt inhibitor, PDTC, TPCK and NF-κB IP (Fig. 5C). Co-transfection with FAK, p85, Akt, IKKα and IKKβ mutant also reduced CCN3-increased NF-κB luciferase activity (Fig. 5D) Taken together, these data suggest that activation of αvβ3/αvβ5 integrin receptor, FAK, PI3K and Akt are required for CCN3-induced NF-κB activation in human chondrosarcoma cells.

Decrease cell motility in CCN3-shRNA over-expression clone

To further confirm the CCN3 mediated cell migration and MMP-13 expression in human chondrosarcoma cells, the CCN3-shRNA expression cells was established. The CCN3 expression level in stable transfectants was compared using Western blotting. The expression of CCN3 was dramatically inhibited by CCN3-shRNA orientation in JJ012/CCN3-shRNA cells (Fig. 6C). We next to characterize the cellular growth rate of control cells and transfectants, by performing the MTT assay 1-6 days after cell seeding. No appreciable difference in cell growth ability was evident among these cells (Supplementary Figure S1), suggesting that CCN3 does not have any mitogenic effect in human chondrosarcoma cells. Furthermore, the migratory ability of these transfectants was analyzed using a Transwell migration assay. Knockdown of CCN3 expression inhibited the migratory ability by approximately 70% in JJ012 cells (Fig. 6A). In addition, knockdown CCN3 also reduced MMP-13 expression in JJ012 cells (Fig. 6C). On the other hand, CCN3-shRNA also reduced migration, CCN3 and MMP-13 expression in SW1353/CCN3-shRNA cells (Fig. 6B&D). Therefore, human chondrosarcoma cells with a higher tendency to migrate expressed more CCN3 and MMP-13.

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 (Fong et al., 2007). 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 (Fong et al., 2007). Therefore, it is important to

develop effective adjuvant therapy for preventing chondrosarcoma metastasis. Previous studies have shown that CCN1 (Cyr61) and CCN2 (CTGF) modulates cell migration and invasion in human chondrosarcoma cells (Tan et al., 2009a; Tan et al., 2009b). However, the effect of CCN3 on migration activity in human chondrosarcoma cells is mostly unknown. We found that CCN3 increased the migration of chondrosarcoma cells. Therefore, CCN family plays an important role in metastasis of chondrosarcoma cells. The discovery of CCN family blocker helps us to therapy of metastasis of chondrosarcoma in the future. One of the mechanisms underlying CCN3 directed migration was transcriptional up-regulation of MMP-13 and activation of αvβ3/αvβ5 integrin, FAK, PI3K, Akt and NF-κB pathways.

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 (Hou et al., 2009). It has been reported that MMP plays important role in CCN3-induced metastasis in human cancer cells (Benini et al., 2005). In this study, we found that CCN3 induced MMP-13 expression and secretion in human chondrosarcoma cells without significantly changing the expression of MMP-1, -2, -3 and -9 mRNAs. In addition, the inhibition of CCN3-enhanced MMP-13 protein expression with siRNA significantly suppressed CCN3-induced migration. Therefore, MMP-13 may be the CCN3-responsive mediator, and it causes the degradation of ECM may lead to subsequent cancer migration and metastasis.

FAK, a potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated signaling (Giancotti and Ruoslahti, 1999). We demonstrate that CCN3 increased phosphorylation of tyrosine 397 of FAK. Furthermore, the FAK(Y397F) mutant and FAK siRNA antagonized the CCN3-mediated potentiation of migration activity, suggesting that FAK activation is an obligatory event in CCN3-induced migration in these cells. Phosphorylation of tyrosine 397 of FAK may provide a binding site for the Src homology 2 domain of the p85 subunit of PI3K (Giancotti and Ruoslahti, 1999). Our results demonstrate that

pretreatment of chondrosarcoma with PI3K and Akt inhibitor antagonized the increase of cell migration and MMP-13 expression under CCN3 stimulation. This was further confirmed by the result that the dominant negative mutant of PI3K and Akt inhibited the enhancement of cell migration and MMP-13 expression under CCN-3 stimulation. Here we also found that the cytoplasmic kinase PI3K and Akt were activated by CCN3 stimulation in chondrosarcoma. These effects implicate the involvement of FAK, PI3K and Akt activation in CCN3-mediated induction of cell migration and MMP-13 expression.

A variety of growth factors stimulate the expression of MMP-13 via signal-transduction pathways that converge to activate NF-κB complex of transcription factors (Yeh et al., 2009). In this study, NF-κB inhibitors reduced the CCN3-mediated cell migration and MMP-13 activation in chondrosarcoma cells. Stimulation of cells with CCN3 increased p65 translocation into nucleus and p65 binding to NF-κB site on MMP-13 promoter. On the other hand, FAK, siRNA, Ly294002 and Akt inhibitor reduced CCN3-mediated p65 translocation into nucleus and p65 binding to NF-κB site on MMP-13 promoter. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that CCN3-induced an increase in NF-κB activity. In this study, we found that CCN3-induced NF-κB activity was inhibited by Ly294002, wortmannin, Akt inhibitor, PDTC, TPCK and NF-κB IP or FAK, p85, Akt, IKKα and IKKβ mutant. These results indicate that integrin, FAK, PI3K and Akt signaling pathways are involved in CCN3-mediated NF-κB transactivation.

Due to the prognosis of patients with chondrosarcoma distant metastasis is generally considered as very poor. Thus, preventing human chondrosarcoma metastasis is an important issue nowadays. Our study presents that CCN3 increases the activity of MMP-13 via αvβ3/αvβ5 integrin, FAK, PI3K, Akt, and NF-κB-dependent pathway and increasing migration of human chondrosarcoma cells (Fig. 6C). Furthermore, the discovery of CCN3-mediated pathway helps us to understand the mechanism of human chondrosarcoma metastasis and may help us to

develop effective therapy in the future.

Acknowledgments

This work was supported by grants from China Medical University Hospital (DMR-99-088) and Taiwan Department of Health, China Medical University Hospital Cancer Research Center of Excellence (DOH100-TD-C-111-005). We thank Dr. W.M. Fu for providing p85 and Akt mutant; Dr. J.A. Girault for providing FAK mutant. Dr. H. Nakano for providing IKKα and IKKβ mutant.

References

Benini S, Perbal B, Zambelli D, Colombo MP, Manara MC, Serra M, Parenza M, Martinez V, Picci P, Scotlandi K. 2005. In Ewing's sarcoma CCN3(NOV) inhibits proliferation while promoting migration and invasion of the same cell type. Oncogene 24(27):4349-4361.

Chevalier G, Yeger H, Martinerie C, Laurent M, Alami J, Schofield PN, Perbal B. 1998. novH: differential expression in developing kidney and Wilm's tumors. Am J Pathol 152(6):1563-1575.

Chiu YC, Huang TH, Fu WM, Yang RS, Tang CH. 2008. Ultrasound stimulates MMP-13 expression through p38 and JNK pathway in osteoblasts. J Cell Physiol 215(2):356-365.

Chiu YC, Lin CY, Chen CP, Huang KC, Tong KM, Tzeng CY, Lee TS, Hsu HC, Tang CH. 2009. Peptidoglycan enhances IL-6 production in human synovial fibroblasts via TLR2 receptor, focal adhesion kinase, Akt, and AP-1-dependent pathway. J Immunol 183(4):2785-2792.

Chuang JY, Yang WH, Chen HT, Huang CY, Tan TW, Lin YT, Hsu CJ, Fong YC, Tang CH. 2009. CCL5/CCR5 axis promotes the motility of human oral cancer cells. J Cell Physiol 220(2):418-426.

Egeblad M, Werb Z. 2002. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2(3):161-174.

Fong YC, Yang WH, Hsu SF, Hsu HC, Tseng KF, Hsu CJ, Lee CY, Scully SP. 2007. 2-methoxyestradiol induces apoptosis and cell cycle arrest in human chondrosarcoma cells. J Orthop Res 25(8):1106-1114.

Giancotti FG, Ruoslahti E. 1999. Integrin signaling. Science 285(5430):1028-1032.

Holbourn KP, Acharya KR, Perbal B. 2008. The CCN family of proteins: structure-function relationships. Trends Biochem Sci 33(10):461-473.

Hou CH, Hsiao YC, Fong YC, Tang CH. 2009. Bone morphogenetic protein-2 enhances the motility of chondrosarcoma cells via activation of matrix metalloproteinase-13. Bone 44(2):233-242.

Joliot V, Martinerie C, Dambrine G, Plassiart G, Brisac M, Crochet J, Perbal B. 1992. Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas. Mol Cell Biol 12(1):10-21.

Joyce JA, Pollard JW. 2009. Microenvironmental regulation of metastasis. Nat Rev Cancer 9(4):239-252.

Kerkela E, Saarialho-Kere U. 2003. Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer. Exp Dermatol 12(2):109-125.

Kleer CG, Zhang Y, Pan Q, Merajver SD. 2004. WISP3 (CCN6) is a secreted

tumor-suppressor protein that modulates IGF signaling in inflammatory breast cancer. Neoplasia 6(2):179-185.

Kleer CG, Zhang Y, Pan Q, van Golen KL, Wu ZF, Livant D, Merajver SD. 2002. WISP3 is a novel tumor suppressor gene of inflammatory breast cancer. Oncogene 21(20):3172-3180.

Liu C, Liu XJ, Crowe PD, Kelner GS, Fan J, Barry G, Manu F, Ling N, De Souza EB, Maki RA. 1999. Nephroblastoma overexpressed gene (NOV) codes for a growth factor that induces protein tyrosine phosphorylation. Gene 238(2):471-478.

Liu JF, Fong YC, Chang CS, Huang CY, Chen HT, Yang WH, Hsu CJ, Jeng LB, Chen CY, Tang CH. Cyclooxygenase-2 enhances alpha2beta1 integrin expression and cell migration via EP1 dependent signaling pathway in human chondrosarcoma cells. Mol Cancer 9:43.

Madrid LV, Mayo MW, Reuther JY, Baldwin AS, Jr. 2001. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. J Biol Chem 276(22):18934-18940.

Maillard M, Cadot B, Ball RY, Sethia K, Edwards DR, Perbal B, Tatoud R. 2001. Differential expression of the ccn3 (nov) proto-oncogene in human prostate cell lines and tissues. Mol Pathol 54(4):275-280.

Manara MC, Perbal B, Benini S, Strammiello R, Cerisano V, Perdichizzi S, Serra M, Astolfi A, Bertoni F, Alami J, Yeger H, Picci P, Scotlandi K. 2002. The expression of ccn3(nov) gene in musculoskeletal tumors. Am J Pathol 160(3):849-859.

Muddasani P, Norman JC, Ellman M, van Wijnen AJ, Im HJ. 2007. Basic fibroblast growth factor activates the MAPK and NFkappaB pathways that converge on Elk-1 to control production of matrix metalloproteinase-13 by human adult articular chondrocytes. J Biol Chem 282(43):31409-31421.

Perbal B. 2001. NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. Mol Pathol 54(2):57-79.

Sin WC, Bechberger JF, Rushlow WJ, Naus CC. 2008. Dose-dependent differential upregulation of CCN1/Cyr61 and CCN3/NOV by the gap junction protein Connexin43 in glioma cells. J Cell Biochem 103(6):1772-1782.

Stein GS, Lian JB, Owen TA. 1990. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J 4(13):3111-3123.

Tan TW, Lai CH, Huang CY, Yang WH, Chen HT, Hsu HC, Fong YC, Tang CH. 2009a. CTGF enhances migration and MMP-13 up-regulation via alphavbeta3

integrin, FAK, ERK, and NF-kappaB-dependent pathway in human chondrosarcoma cells. J Cell Biochem 107(2):345-356.

Tan TW, Yang WH, Lin YT, Hsu SF, Li TM, Kao ST, Chen WC, Fong YC, Tang CH. 2009b. Cyr61 increases migration and MMP-13 expression via alphavbeta3 integrin, FAK, ERK and AP-1-dependent pathway in human chondrosarcoma cells. Carcinogenesis 30(2):258-268.

Tang CH, Yamamoto A, Lin YT, Fong YC, Tan TW. Involvement of matrix metalloproteinase-3 in CCL5/CCR5 pathway of chondrosarcomas metastasis. Biochem Pharmacol 79(2):209-217.

Terek RM, Schwartz GK, Devaney K, Glantz L, Mak S, Healey JH, Albino AP. 1998. Chemotherapy and P-glycoprotein expression in chondrosarcoma. J Orthop Res 16(5):585-590.

Yeh WL, Lu DY, Lee MJ, Fu WM. 2009. Leptin induces migration and invasion of glioma cells through MMP-13 production. Glia 57(4):454-464.

Yu C, Le AT, Yeger H, Perbal B, Alman BA. 2003. NOV (CCN3) regulation in the growth plate and CCN family member expression in cartilage neoplasia. J Pathol 201(4):609-615.

Yuan J, Dutton CM, Scully SP. 2005. RNAi mediated MMP-1 silencing inhibits human chondrosarcoma invasion. J Orthop Res 23(6):1467-1474.

Zucker S, Cao J, Chen WT. 2000. Critical appraisal of the use of matrix metalloproteinase inhibitors in cancer treatment. Oncogene 19(56):6642-6650.

Figure legends

Fig. 1 CCN3-directed migration activity of human chondrosarcoma cells involves upregulation of MMP-13.

(A) Cells were incubated with CCN3 for 24 hr, and *in vitro* migration was measured with the Transwell after 24 hr. (B) Cells were incubated with CCN3 for 24 hr, and cell viability was measured by MTT assay. (C) Cells were incubated with CCN3 (30 ng/ml) for 24 hr, the mRNA level of MMP-1, -2, -3, -9 and -13 was determined using qPCR. (D) Cells were incubated with CCN3 (30 ng/ml) for indicated time intervals. The cultured medium and cell lysates were then collected. The protein level of MMP-13 in cell lysates and enzyme activity of MMP-13 in supernatant were examined by western blot and zymography. (E) Cells were transfected with MMP-13 siRNA for 24 hr followed by stimulation with CCN3, and *in vitro* migration was measured after 24 hr. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with CCN3-treated group.

Fig. 2 Involvement of integrin signaling pathway in response to CCN3 in chondrosarcoma cells.

(A&B) Cells were pretreated with α5β1, αvβ3 and αvβ5 monoclonal antibody (10 μg/ml) for 30 min followed by stimulation with CCN3 (30 ng/ml). The *in vitro* migration activity and MMP-13 expression was examined by Transwell, western blot and qPCR. (B) Cells were incubated with CCN3 (30 ng/ml) for indicated time intervals, and p-FAK expression was determined by western blot. (D&E) Cells were transfected with mutant or siRNA of FAK for 24 hr followed by stimulation with CCN3 (30 ng/ml). The *in vitro* migration activity and MMP-13 expression was examined by Transwell and qPCR. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with CCN3-treated group.

Fig. 3 PI3K/Akt pathway is involved in CCN3-mediated migration in human chondrosarcoma cells.

(A) Cells were incubated with CCN3 (30 ng/ml) for indicated time intervals, and p-85 and p-Akt expression was determined by western blot. (B&C) Cells were pretreated with Ly294002 (10 μM), wortmannin (1 μM) and Akt inhbitor (10 μM) for 30 min followed by stimulation with CCN3 (30 ng/ml) for 24 hr. The *in vitro* migration activity and MMP-13 expression was examined by Transwell and qPCR. (D&E) Cells were transfected with dominant negative (DN) mutant of p85 or Akt for 24 hr followed by stimulation with CCN3 (30 ng/ml). The *in vitro* migration activity and MMP-13 expression was examined by Transwell and qPCR. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with CCN3-treated group.

Fig. 4 CCN3 induces cells migration and MMP-13 up-regulation through NF-κB.

(A&B) Cells were pretreated for 30 min with PDTC (10 μM), TPCK (3 μM) or NF-κB inhibitor peptide (IP; 10 μg/ml) followed by stimulation with CCN3(30 ng/ml) for 24 hr. The *in vitro* migration activity and MMP-13 expression was examined by Transwell and qPCR. (C) Cells were incubated with CCN3 (30 ng/ml) for indicated time intervals, and IKK, IκBα and p65 phophorylation was examined by western blot. (D&E) Cells were transfected with IKKα or IKKβ mutant for 24 hr followed by stimulation with CCN3 (30 ng/ml), and *in vitro* migration and mRNA expression of MMP-13 measured with the Transwell and qPCR. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with CCN3-treated group.

Fig. 5 FAK/PI3K/Akt pathway is involved in CCN3-mediated NF-κB activation and MMP-13 expression.

(A) Cells were pretreated with Ly294002 (10 μM), Akt inhibitor (10 μM), αvβ3 mAb (10 μg/ml) or αvβ5 mAb (10 μg/ml) then stimulated with CCN3 (30 ng/ml) for 120 min, and the chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with anti-p-65. One percentage of the precipitated chromatin was assayed to verify equal loading (input). (B) Cells were pretreated with Ly294002 (10 μM), wortmannin (1 μM) or Akt inhibitor (10 μM) then stimulated with CCN3 (30 ng/ml) for 120 min, and p65 translocation in nucleus was examined by immunofluorescence staining. Cells were pretreated with Ly294002 (10 μM), wortmannin (1 μM), Akt inhibitor (10 μM), PDTC (10 μM), TPCK (3 μM) or NF-κB IP (10 μg/ml) for 30 min (C) or transfected with mutant of FAK, p85, Akt, IKKα and IKKβ (D) before exposure to CCN3 (30 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. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with CCN3-treated group.

Fig. 6 Knockdown of CCN3 inhibited the migratory ability in chondrosarcoma cells.

(A&B) The *in vitro* migration activity of JJ012, JJ012/control-shRNA and JJ012/CCN3-shRNA, SW1353, SW1353/control-shRNA and SW1353/CCN3-shRNA cells was measured with the Transwell. (C&D) The protein levels of CCN3 and MMP-13 in JJ012, JJ012/control-shRNA, JJ012/CCN3-shRNA, SW1353, SW1353/control-shRNA and SW1353/CCN3-shRNA cells was examined by western blot analysis. Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with CCN3-treated group. (E) Schematic presentation of the signaling pathways involved in CCN3-induced migration and MMP-13 expression of chondrosarcoma cells. CCN3 activates αvβ3/αvβ5 integrin,

FAK, PI3K and Akt pathways, which in turn induces NF-κB activation, which lead to MMP-13 expression and increases the migration of human chondrosarcoma cells.