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CCN6 enhances ICAM-1 expression and cell motility in human chondrosarcoma cells

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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. CCN6 is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of matricellular proteins. However, the effects of CCN6 on human chondrosarcoma cells are largely unknown. In this study, we found that CCN6 increased the migration and the expression of ICAM-1 in human chondrosarcoma cells. αvβ3 and αvβ5 integrin monoclonal antibody and mitogen-activated protein kinase (MEK) inhibitors (PD98059 and U0126) inhibited the CCN6-induced increase of the migration and ICAM-1 up-regulation of chondrosarcoma cells. CCN6 stimulation increased the phosphorylation of focal adhesion kinase (FAK), MEK and extracellular signal-regulated kinase (ERK). In addition, activator protein-1 (AP-1) inhibitors suppressed the cell migration and ICAM-1 expression enhanced by CCN6. Moreover, CCN6 increased AP-1 luciferase activity and binding of c-Jun to the AP-1 element on the ICAM-1 promoter. Taken together, our results indicate that CCN6 enhances the migration of chondrosarcoma cells by increasing ICAM-1 expression through the αvβ3 and αvβ5 integrin receptor, FAK, MEK, ERK, c-Jun and AP-1 signal transduction pathway.

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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 (Terek et al., 1998). 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 develop a novel and effective therapy (Yuan et al., 2005).

Tumor invasion and metastasis are the critical steps in determining the aggressive phenotype of human cancers. Mortality in cancer patients principally results from metastatic spread of cancer cells to distant organs (Gupta and Massague, 2006). To facilitate cell motility, invading cells need to change the cell-cell adhesion properties, rearrange the extracellular matrix environment, suppress anoikis and reorganize their cytoskeletons (Desgrosellier and Cheresh). Cell adhesion molecules belonging to the integrin, cadherin, and immunoglobulin superfamily have been implicated in tumor progression (Makrilia et al., 2009). Intercellular adhesion molecule-1 (ICAM-1, also called CD54), a member of the immunoglobulin supergene family, is an inducible surface glycoprotein that mediates adhesion-dependent cell-to-cell interactions (Lawson and Wolf, 2009; Zimmerman and Blanco, 2008). The extracellular domain of ICAM-1 is essential for the transendothelial migration of leukocytes from the capillary bed into the tissue (Duperray et al., 1997), and ICAM-1 may also facilitate movement (or retention) of cells through the extracellular matrix (Duperray et al., 1997). It has been reported that ICAM-1 plays an important role in lung cancer cell invasion (Huang et al., 2004). ICAM-1 antibody or antisense ICAM-1 cDNA has also been reported to rescue the invasiveness of breast cancer cells (Rosette et al., 2005). Therefore, ICAM-1 might play a critical role in tumorigenesis, and its disruption may prevent metastasis.

CCN6 is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of matricellular proteins, with developmental functions (Holbourn et al., 2008;

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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. They have been proposed to connect signaling pathways and to facilitate cross-talks between epithelium and stroma (Holbourn et al., 2008). A previous study has shown that CCN affects cell migration through binding to cell surface integrin receptors (Leask and Abraham, 2006).

CCN6 is a tumor suppressor gene found to be down-regulated in the most aggressive form of locally advanced breast cancer, inflammatory breast cancer, as well as noninflammatory breast cancers with lymph node metastasis (van Golen et al., 1999). Immunohistochemical studies of human breast tissue samples have shown that whereas normal epithelium expresses CCN6 protein, CCN6 is reduced or lost in 60% of invasive carcinomas (Huang et al., 2008). The high frequency of reduction or loss of CCN6 in breast cancer suggests a potential role in initiation and/or progression of human breast cancer. Previous studies have shown that CCN6 reduces cell migration and invasion in breast cancer cells (Huang et al., 2008; Kleer et al., 2004). However, the effect of CCN6 on migration activity in human chondrosarcoma cells is mostly unknown. Here, we found that CCN6 increases the migration and ICAM-1 up-regulation of human chondrosarcoma cells. In addition, integrin receptor, focal adhesion kinase (FAK), MAPK kinase (MEK), ERK and AP-1 signaling pathways were involved.

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MATERIALS AND METHODS

Materials:

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, FAK, MEK, p-MEK, ERK2, p-ERK, c-Jun, p-c-Jun and the small interfering RNAs (siRNAs) against ICAM-1, c-Jun and control (for experiments using targeted siRNA transfection; each consisting of a scrambled sequence that would not lead to the specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies specific for FAK phosphorylated at Tyr^{397} was purchased from Cell Signaling and Neuroscience (Danvers, MA). Mouse monoclonal antibody specific for ICAM-1 was purchased from R&D Systems (Minneapolis, MN, USA). Mouse monoclonal antibodies specific for αvβ3(MAB1976Z), αvβ5(MAB1961Z) and α5β1(MAB1969) integrin were purchased from Cayman Chemical (Ann Arbor, MI). PD98059, U0126 and curcumin were purchased from Calbiochem (San Diego, CA). Tanshinone IIA was purchased from BIOMOL (Butler Pike, PA). The phosphorylation site mutant of FAK(Y397F) was a gift from Dr. J.A. Girault (Institut du Fera' Moulin, Paris, France). The MEK1 mutant was a gift from from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The ERK2 (K52R) dominant-negative mutant was a gift from Dr. M. Cobb (South-Western Medical Center, Dallas, TX). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell cultures

The human chondrosarcoma cell line (JJ012) was kindly provided by 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°C in a humidified atmosphere of 5% CO_2.

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Migration assay

The migration assay was performed using Transwell (Costar, NY; pore size, 8-μm) in 24-well dishes. For invasion assay, filters were precoated with 25 μl Matrigel basement membrane matrix (BD Biosciences, Bedford, MA) for 30 min. The following procedures were the same for both migration and invasion assays. Before the migration assay was performed, cells were pretreated for 30 min with different concentrations of inhibitors, including the PD98059, U0126, curcumin or vehicle control (0.1% DMSO). Approximately 1×10^4 cells in 100 μl of serum-free medium were placed in the upper chamber, and 300 μl of the same medium containing CCN6 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 migrating cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of the CCN6 treatment (corrected migrating cell number = counted migrating cell number/percentage of viable cells) (Tang et al., 2008).

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.

Patients and specimen preparation

Upon approval by the local ethics committee, specimens of tumor tissue or normal

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cartilage tissue were obtained from patients, who had been pathologically diagnosed with chondrosarcoma or knee osteoarthritis (the articular cartilage was collected) and had undergone surgical resection at China Medical University Hospital. Tissue specimens were ground and then sonicated in a lysis buffer. The protein level was analyzed using Western blot analysis.

Immunohistochemistry

The 5-μm sections of paraffin-embedded tissue on glass slides were rehydrated and incubated in 3% hydrogen peroxide to block the endogenous peroxidase activity. After trypsinization, sections were blocked by incubation in 3% bovine serum albumin in PBS. The primary antibody monoclonal mouse anti-human CCN6 antibody was applied to the slides at a dilution of 1:50 and incubated at 4°C overnight. After three washes in PBS, the samples were treated with goat anti-mouse IgG biotin-labeled secondary antibodies at a dilution of 1:50. Bound antibodies were detected with an ABC kit (Vector Laboratories). The slides were stained with chromogen diaminobenzidine, washed, counterstained with Delafield's hematoxylin, dehydrated, treated with xylene, and mounted.

Quantitative real-time PCR

Total RNA was extracted from oral cancer cells using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using oligo(dT) primer. The quantitative real-time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). A volume of 100 ng 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 (β-actin was used as internal control) (Applied Biosystems, CA). qPCR assays were carried out in triplicate on a 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

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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).

Flow cytometric analysis

Human chondrosarcoma cells were plated in six-well dishes. The cells were then washed with PBS and detached with trypsin at 37°C. Cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After being rinsed in PBS, the cells were incubated with mouse anti-human antibody against integrin (1:100) for 1 hr at 4°C. Cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:100; Leinco Tec. Inc., St. Louis, MO, USA) for 45 min and analyzed by flow cytometry using FACS Calibur and CellQuest software (BD Biosciences).

Western blot analysis

The cellular lysates were prepared as described previously (Tang et al., 2008). 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 FAK, p-FAK, ERK or p-ERK (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).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was performed as described previously (Huang and Chen, 2005). DNA immunoprecipitated by anti-c-Jun Ab was purified. The DNA was then extracted with phenol-chloroform. The purified DNA pellet was

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subjected to PCR. PCR products were then resolved by 1.5% agarose gel electrophoresis and visualized by UV light. The primers 5'-AGACCTTAGCGCGGTGTAGA-3' and 5'-AGTAGCAGAGGAGCTCAGCG-3' were utilized to amplify across the ICAM-1 promoter region (–346 to –24) (Huang and Chen, 2005).

Transfection and reporter gene assay

Human chondrosarcoma cells were co-transfected with 0.8 μg AP-1-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 hr transfection, the cells were then incubated with the indicated agents. After a further 24 hr 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 by monitoring the co-transfected β-galactosidase expression vector.

Statistics

For statistical evaluation, Mann-Whitney *U* test was used for non-Gaussian parameters and Student's *t* test was used for Gaussian parameters (including Bonferroni correction). Differences were considered significant if the *P* value was <0.05

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RESULTS

CCN6-directed chondrosarcoma cell migration involves ICAM-1 up-regulation

Previous studies have shown that CCN6 reduces cell migration and invasion in breast cancer cells (Huang et al., 2008; Kleer et al., 2004). However, the effect of CCN6 in migration of chondrosarcoma is mostly unknown. CCN6-triggered migration in chondrosarcoma cells was examined by using the Transwell assay (Yang et al., 2009). CCN6 directed human chondrosarcoma cell (JJ012 cells) migration (Fig. 1A). It was also found that the invasive ability of chondrosarcoma cells through the Matrigel basement membrane matrix was increased by CCN6 stimulation (Fig. 1B). Furthermore, CCN6 also increased wound healing activity in human chondrosarcoma cells (Fig. 1C). We also examined human chondrosarcoma tissues for the expression of the CCN6 using Western blot and immunohistochemistry analysis. Expression of protein levels of CCN6 in human chondrosarcoma tissues was significantly higher than that detected in normal cartilage (Fig. 1D&E). Thus, expression of CCN6 was associated with a metastatic phenotype of chondrosarcoma cells. Previous studies have demonstrated significant expression of ICAM-1 in human cancer cells (Huang et al., 2004). We therefore, hypothesized that ICAM-1 may be involved in CCN6-directed migration of human chondrosarcoma. Treatment of cells with CCN6 increased ICAM-1 protein and mRNA expression in a time-dependent manner (Fig. 1F). Transfection of cells with ICAM-1 siRNA inhibited ICAM-1 expression (Fig. 1G). Furthermore, transfection of cells with ICAM-1 siRNA markedly inhibited the CCN6-induced cell migration and ICAM-1 expression (Fig. 1H&I). These data suggest that CCN6-induced cancer migration was enabled by ICAM-1 up-regulation.

CCN6-directed chondrosarcoma cell migration through αvβ3 and avβ5 integrin

Previous studies have shown that CCN affects cell migration through binding to cell surface integrin receptors (Tan et al., 2009a; Tan et al., 2009b). We therefore, hypothesized that integrin-signaling pathway may be involved in CCN6-directed chondrosarcoma cell migration. Pretreatment of cells for 30 min with anti-αvβ3 and

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αvβ5 monoclonal antibody (mAb) markedly inhibited the CCN6-induced cancer migration (Fig. 2A). Combination of αvβ3 and αvβ5 mAb induced an inhibitory effect on cell motility (Fig. 2A). Moreover, CCN6 up-regulated the mRNA expression of αv, β3 and β5 but not α5 and β1 integrin using qPCR analysis (Fig. 2B), suggesting that the amplification loop strengthens the CCN6-integrin-signaling pathway. Stimulation of cells with CCN6 also increased cell surface αv, β3 and β5 integrin expression (Fig. 2C). However, pretreatment of cells with αvβ3 and αvβ5 but not α5β1 mAb reduced CCN6-mediated ICAM-1 expression (Fig. 2D). These data suggest that CCN6-induced cancer migration may occur via activation of the αvβ3 and αvβ5 integrin.

FAK, MEK and ERK-signaling pathways are involved in the CCN6-mediated cell migration of chondrosarcoma cells

FAK has been shown to be capable of regulating integrin-mediated signaling (Chen et al., 2009). Phosphorylation of tyrosine 397 of FAK has been used as a marker of FAK activity. As shown in Fig 3A, FAK phosphorylation increased in a time-dependent manner in response to CCN6 stimulation. Transfection of cells with FAK(Y397F) mutant reduced the CCN6-mediated cell migration (Fig. 3B). In addition, transfection of cells with FAK siRNA also reduced CCN6-increased cell migration (Fig. 3B). In addition, FAK mutant and siRNA inhibited CCN6-enhanced ICAM-1 expression (Fig. 3C). Pretreatment of cells with αvβ3 and αvβ5 mAb reduced CCN-6-increased FAK phosphorylation (Fig 3D). Therefore, integrin-dependent FAK activation mediated CCN6-induced cell motility. The MEK/ERK signaling pathway can be activated by a variety of growth factors, such as insulin and growth factors (Yang et al., 2008). We then examined whether CCN6 stimulation also enhances the activation of the MEK/ERK pathway. Stimulation of JJ012 cells with CCN6 led to a significant increase of phosphorylation of MEK1/2 by Western blot analysis (Fig. 4A). CCN6-induced migration of JJ012 cells were greatly reduced by treatment with MEK inhibitors PD98059 and U0126 (Fig. 4B). The MEK inhibitors PD98095 and U0126 also inhibited the CCN6-increased ICAM-1 expression (Fig. 4C). In addition,

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treatment of cells with CCN6 increased phosphorylation of ERK (Fig. 4A). Transfection of cells with MEK and ERK2 mutant reduced the CCN6-mediated cell migration and ICAM-1 expression (Fig. 4D&E). Taken together, these results indicate that the FAK/MEK and ERK pathways are involved in CCN6-induced migration activity and ICAM-1 up-regulation in human chondrosarcoma cells.

Involvement of AP-1 in CCN6-induced cell migration and ICAM-1 expression

The promoter region of human ICAM-1 contains AP-1, NF-κB, CCAAT/enhancer-binding protein and SP binding sites (van de Stolpe and van der Saag, 1996). It has been reported that AP-1 plays a critical role in ICAM-1 expression (Roebuck, 1999). To examine the role of AP-1 binding site in CCN6-mediated ICAM-1 expression, the AP-1 inhibitors (curcumin and tanshinone IIA) were used. Pretreatment of cells with curcumin and tanshinone IIA reduced CCN6-induced cell migration and ICAM-1 expression (Fig. 5A&B). The AP-1 binding site between -284 and -279 has been shown to be important for the activation of the ICAM-1 gene (van de Stolpe and van der Saag, 1996). AP-1 activation was further evaluated by analyzing the phosphorylation c-Jun, as well as by the chromatin immunoprecipitation assay. Treatment of cells with CCN6 resulted in a marked phosphorylation of c-Jun (Fig. 5C). Transfection of cells with c-Jun siRNA suppressed the expression of c-Jun (Fig. 5D; upper panel). CCN6-induced cell migration and ICAM-1 expression was also inhibited by c-Jun siRNA but not by control siRNA (Fig. 5D&E). Next, we investigated whether c-Jun binds to the AP-1 element on the ICAM-1 promoter after CCN6 stimulation. The *in vivo* recruitment of c-Jun to the ICAM-1 promoter (–346 to –24) was assessed by the chromatin immunoprecipitation assay (Huang and Chen, 2005). *In vivo* binding of c-Jun to the AP-1 element of the ICAM-1 promoter occurred after CCN6 stimulation (Fig. 6B). The c-Jun phosphorylation and the binding of c-Jun to the AP-1 element by CCN6 were attenuated by the PD98059 and U0126 (Fig. 6A&B). To directly determine AP-1 activation after CCN6 treatment, cells were transiently transfected with AP-1-luciferase as an indicator of AP-1 activation. As shown in Fig. 6C, CCN6

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treatment of JJ012 cells for 24 hr resulted in increased AP-1-luciferase activity. In addition, αvβ3 mAb, αvβ5 mAb, PD98059, U0126, curcumin and tanshinone IIA antagonized the CCN6-induced AP-1-luciferase activity (Fig. 6C). Co-transfection of cells with FAK, MEK and ERK mutant also reduced CCN6-increased AP-1-luciferase activity (Fig. 6D). These inhibitors, mutants and siRNAs did not affect basal cell viability, migration and ICAM-1 expression (Supplemental data Fig. 1S). Taken together, these data suggest that activation of FAK, MEK, ERK and c-Jun are required for CCN6-induced AP-1 activation in human chondrosarcoma cells.

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DISCUSSION

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which have seen dramatic increases in long-term survival with the advent of systemic chemotherapy, chondrosarcoma continues to have a poor prognosis due to the 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. However, 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 an effective adjuvant therapy for prevention of chondrosarcoma metastasis. We hypothesized that CCN6 would help to direct the metastasis of chondrosarcoma cells. We found that CCN6 increased the migration of chondrosarcoma cells. One of the mechanisms underlying CCN6-directed migration was transcriptional up-regulation of ICAM-1 and activation of αvβ3/αvβ5 integrin, FAK, MEK, ERK and AP-1 pathways.

CCN6 is a tumor suppressor gene found to be down-regulated in the most aggressive form of locally advanced breast cancer (van Golen et al., 1999). Previous studies have shown that CCN6 reduces cell migration and invasion in breast cancer cells (Huang et al., 2008; Kleer et al., 2004). In this study, we found that CCN6 increased migration of human chondrosarcoma cells. We compared the CCN6 and ICAM-1 expression in chondrosarcoma (JJ012 and SW1353 cells) and breast cancer cells (MCF-7). JJ012 and MCF-7 expressed higher levels of CCN6 and ICAM-1 than SW1353 cells (Supplemental data Fig. 2S). Therefore, these results suggest that the role of CCN6 in different types of cancer may vary considerably, depending on the tissue involved. The mechanism by which the cell or tissue context is able to determine the action of the CCN6 protein warrants further investigation.

Integrins link the extracellular matrix to intracellular cytoskeletal structures and signaling molecules and are implicated in the regulation of a number of cellular processes, including adhesion, signaling, motility, survival, gene expression, growth

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and differentiation (Humphries, 2000). A previous study has shown that CCN affects cell migration through binding to cell surface integrin receptors (Leask and Abraham, 2006). Here, we used integrin antibody to determine the role of integrin and found that αvβ3 and αvβ5 mAb inhibited CCN6-induced cell migration, indicating the possible involvement of αvβ3 and αvβ5 integrin activation in CCN6-induced migration in chondrosarcoma cells. In addition, αvβ3 and αvβ5 mAb also reduced CCN6-enhanced ICAM-1 expression. However, ICAM-1 siRNA did not affect CCN6-increased αv, β3 and β5 mRNA expression (data not shown). Therefore, ICAM-1 is actually contributing to increased integrin-dependent motility to CCN6. These data suggest that CCN6-induced cancer migration may occur via activation of the αvβ3 and αvβ5 integrin.

FAK, a potential candidate-signaling molecule, has been shown to be capable of regulating integrin-mediated signaling (Tan et al., 2009a). Our results demonstrate that CCN6 increased phosphorylation of tyrosine 397 of FAK. Furthermore, the FAK(Y397F) mutant and FAK siRNA antagonized the CCN6-mediated potentiation of migration activity, suggesting that FAK activation is an obligatory event in CCN6-induced migration in these cells. We also found that αvβ3 and αvβ5 mAb reduced CCN6-enhanced FAK phopshorylation. Therefore, FAK is a downstream molecule of αvβ3 and αvβ5 integrin receptors. MEK/ERK also plays a critical role in integrin signaling (Kapur et al., 2003). We found that PD98059 and U0126 (MEK inhibitors) also inhibited CCN6-induced migration. Stimulation of cells with CCN6 increased phosphorylation of MEK and ERK. The MEK inhibitors or MEK and ERK2 mutant also reduced the CCN6-mediated migration and ICAM-1 expression. These results indicate that CCN6 might act through the FAK, MEK and ERK pathways to induce migration and ICAM-1 activation in human chondrosarcoma cells.

There are several binding sites for a number of transcription factors including AP-1, NF-κB, CCAAT/enhancer-binding protein and SP binding sites on human ICAM-1 promoter (van de Stolpe and van der Saag, 1996). The results of this study show that AP-1 activation contributes to CCN6-induced migration and ICAM-1 production in

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chondrosarcoma cells. The AP-1 sequence binds to members of the Jun and Fos families of transcription factors. These nuclear proteins interact with the AP-1 site as Jun homodimers or Jun-Fos heterodimers formed by protein dimerization through their leucine zipper motifs. The results of this study show that CCN6 induced c-Jun phosphorylation. In addition, c-Jun siRNA abolished the CCN6-induced cell migration in chondrosarcoma cells. Furthermore, CCN6 also increased the binding of c-Jun to the AP-1 element on the ICAM-1 promoter, as shown by chromatin immunoprecipitation assay. The c-Jun phosphorylation and the binding of c-Jun to the AP-1 element were attenuated by PD98059 and U0126. However, PD98059, U0126, curcumin and tanshione IIA or FAK, MEK and ERK mutant reduced CCN6-mediated AP-1 promoter activity. These results indicate that CCN6 might act through the FAK, MEK, ERK, c-Jun, and AP-1 pathway to induce ICAM-1 activation in human chondrosarcoma cells. In this study, we used qPCR to examine ICAM-1 mRNA expression. mRNA levels are not sufficient because it has been shown that ICAM-1 is translationally regulated as well (Hu et al.). We also used flow cytometry to confirm the cell surface ICAM-1 expression. CCN6 increased cell surface ICAM-1 expression, and integrin mAb, PD98059, U0126, curcumin and tanshione IIA reduced CCN6-increased cell surface ICAM-1 expression (Supplemental data Fig. 3S). Therefore, the same signaling pathway is involved in CCN6-mediated cell surface ICAM-1 expression.

In conclusion, we present a novel mechanism of CCN6-directed migration of chondrosarcoma cells via up-regulation of ICAM-1 production. CCN6 increases cell migration and ICAM-1 expression by activation of αvβ3/αvβ5 integrin, FAK, MEK, ERK, c-Jun and AP-1-dependent pathway (Fig. 6E).

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FIGURE LEGENDS

Fig. 1 CCN6-directed migration activity of human chondrosarcoma cells involves up-regulation of ICAM-1.

(A&B) JJ012 cells were incubated with CCN6 for 24 hr, and *in vitro* migration and invasion was measured with the Transwell after 24 hr (n=5). (C) Cells were treated with CCN6 for 24 hr, the wound-scratching assay was performed. (D) Total proteins were extracted from chondrosarcoma patients and normal cartilage, and subjected to Western blot analysis for CCN6. (E) Immunohistochemistry of CCN6 expression in normal cartilage and chondrosarcoma tissue. (F) JJ012 cells were incubated with CCN6 (50 ng/ml) for indicated time intervals, the protein and mRNA expression of ICAM-1 were examined by Western blot and qPCR analysis (n=4). (G) Cells were transfected with ICAM-1 siRNA for 24 hr, the ICAM-1 expression was examined by Western blot. (H&I) Cells were transfected with ICAM-1 siRNA for 24 hr followed by stimulation with CCN6 (50 ng/ml), and *in vitro* migration and ICAM-1 expression was measured with the Transwell and Western blot. Results are expressed as the mean ± S.D. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCN6-treated group.

Fig. 2 Integrin receptors are involved in CCN6-mediated migration of human chondrosarcoma cells.

(A) JJ012 cells were pretreated for 30 min with αvβ3 mAb (1 μg/ml) or αvβ5 mAb (1 μg/ml) followed by stimulation with CCN6 (50 ng/ml), and in vitro migration was measured with the Transwell after 24 hr. (B) JJ012 cells were incubated with CCN6 (50 ng/ml) for 24 hr, and mRNA expression of αv, α5, β1, β3 and β5 integrin were examined by qPCR. (C) JJ012 cells were incubated with CCN6 (50 ng/ml) for 24 hr, and cell surface αv, β3 and β5 integrin were examined by flow cytometry. (D) JJ012 cells were pretreated for 30 min with αvβ3, αvβ5 or α5β1 mAb followed by stimulation with

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CCN6 (50 ng/ml), and the mRNA expression of ICAM-1 was measured with qPCR after 24 hr. Results are expressed as the mean ± S.D. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCN6-treated group.

Fig. 3 FAK is involved in CCN6-induced migration and ICAM-1 production.

(A) Cells were incubated with CCN6 (50 ng/ml) for indicated time intervals, and FAK phosphorylation was examined by Western blot. (B; upper panel) Cells were transfected with FAK siRNA for 24 hr, the FAK expression was examined by Western blot. (B) Cells were transfected with FAK siRNA or FAK mutant for 24 hr followed by stimulation with CCN6 (50 ng/ml), and *in vitro* migration measured with the Transwell after 24 hr. (C) Cells were transfected with FAK siRNA or FAK mutant for 24 hr followed by stimulation with CCN6 (50 ng/ml), and mRNA expression of ICAM-1 were examined by qPCR. (D) JJ012 cells were pretreated with αvβ3 or αvβ5 mAb for 30 min. Then they were followed by stimulation with CCN6 (50 ng/ml) for 30 min, and FAK phosphorylation was examined. Results are expressed as the mean ± S.D. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCN6-treated group.

Fig. 4 MEK/ERK is involved in CCN6-induced migration and ICAM-1 up-regulation in human chondrosarcoma cells.

(A) JJ012 cells were incubated with CCN6 (50 ng/ml) for indicated time intervals, and MEK and ERK phosphorylation were examined by Western blot analysis (n=5). (B&C) JJ012 cells were pretreated for 30 min with U0126 and PD98059 followed by stimulation with CCN6 (50 ng/ml), and in vitro migration and ICAM-1 expression were measured with the Transwell and qPCR after 24 hr. (D&E) JJ012 cells were transfected with dominant negative (DN) mutant of MEK1 or ERK for 24 hr followed by stimulation with CCN6 (50 ng/ml), and in vitro migration and ICAM-1 expression were

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measured with the Transwell and qPCR after 24 hr. Results are expressed as the mean ± S.D. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCN6-treated group

Fig. 5 AP-1 is involved in the potentiation of ICAM-1 expression by CCN6.

Cells were pretreated for 30 min with curcumin or tanshinone IIA followed by stimulation with CCN6 (50 ng/ml) for 24 hr, and *in vitro* migration (A) and ICAM-1 expression (B) were measured with the Transwell and qPCR after 24 hr (n=5). (C) Cells were incubated with CCN6 (50 ng/ml) for indicated time intervals, and c-Jun phosphorylation was determined by Western blot. (D; upper panel) Cells were transfected with c-Jun or control siRNA for 24 h, the protein levels of c-Jun was determined by using Western blot analysis. (D; lower panel) Cells were transfected with c-Jun or control siRNA for 24 h, and then stimulated with CCN6 (50 ng/ml) for 24 h. The *in vitro* migration measured with the Transwell after 24 hr. (E) Cells were transfected with c-Jun or control siRNA for 24 h followed by stimulation with CCN6 (50 ng/ml) for 24 hr, and ICAM-1 expression was measured with and qPCR. Results are expressed as the mean ± S.D. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCN6-treated group

Fig. 6 MEK/ERK pathway is involved in CCN6-mediated AP-1 activation and ICAM-1 expression.

(A) JJ012 cells were pretreated with PD98059 and U0126 for 30 min. Then they were followed by stimulation with CCN6 (50 ng/ml) for 120 min, and c-Jun phosphorylation was examined. (n=5) (B) Cells were pretreated with PD98059 and U0126 then stimulated with CCN6 (50 ng/ml) for 120 min, and the chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with anti-c-Jun. One percentage of the precipitated chromatin was assayed to verify equal loading (input). (C) Cells were

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pretreated with αvβ3 mAb, αvβ5 mAb, PD98059, U0126, curcumin and tanshinone IIA for 30 min or transfected with mutant of FAK, MEK or ERK (D) before exposure to CCN6 (50 ng/ml). AP-1 luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.D. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with control. #, $p < 0.05$ compared with CCN6-treated group. (E) Schematic presentation of the signaling pathways involved in CCN6-induced migration and ICAM-1 expression of chondrosarcoma. CCN6 and integrin receptor interaction activates FAK, MEK and ERK pathways, which in turn induces AP-1 activation, which leads to ICAM-1 expression and increases the migration of human chondrosarcoma.

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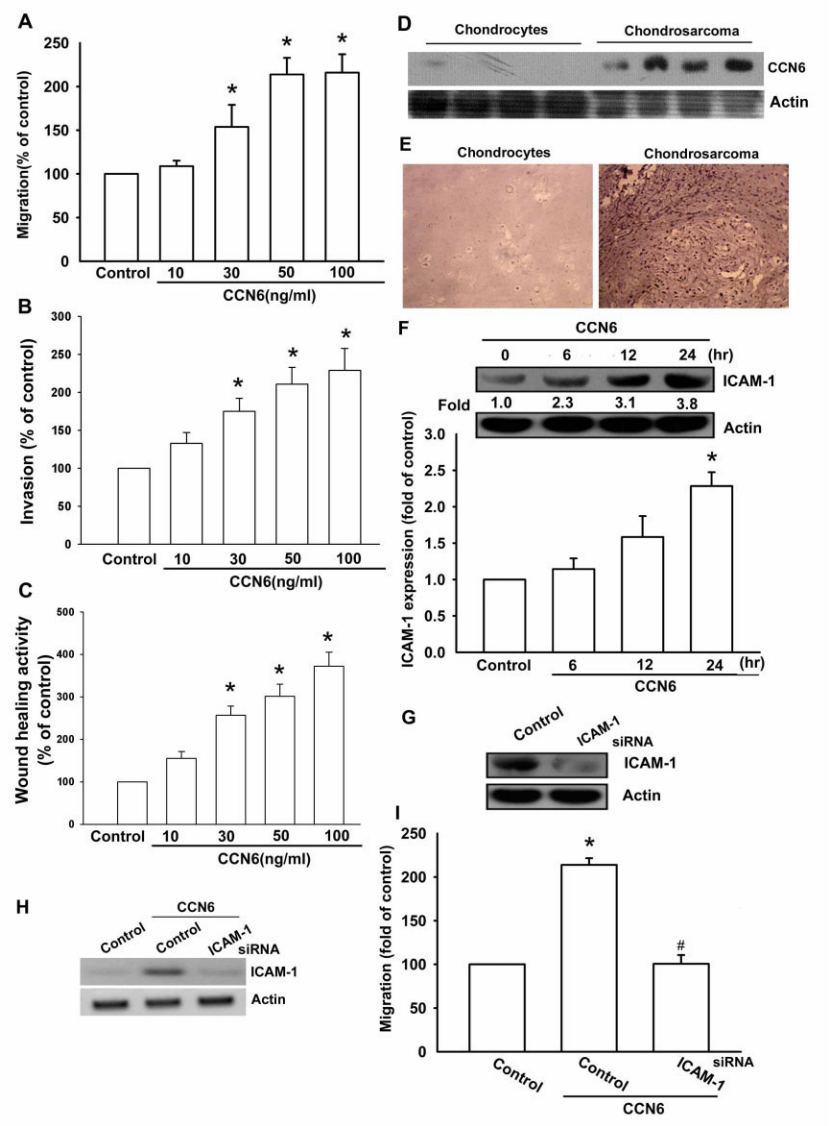

Figure 1

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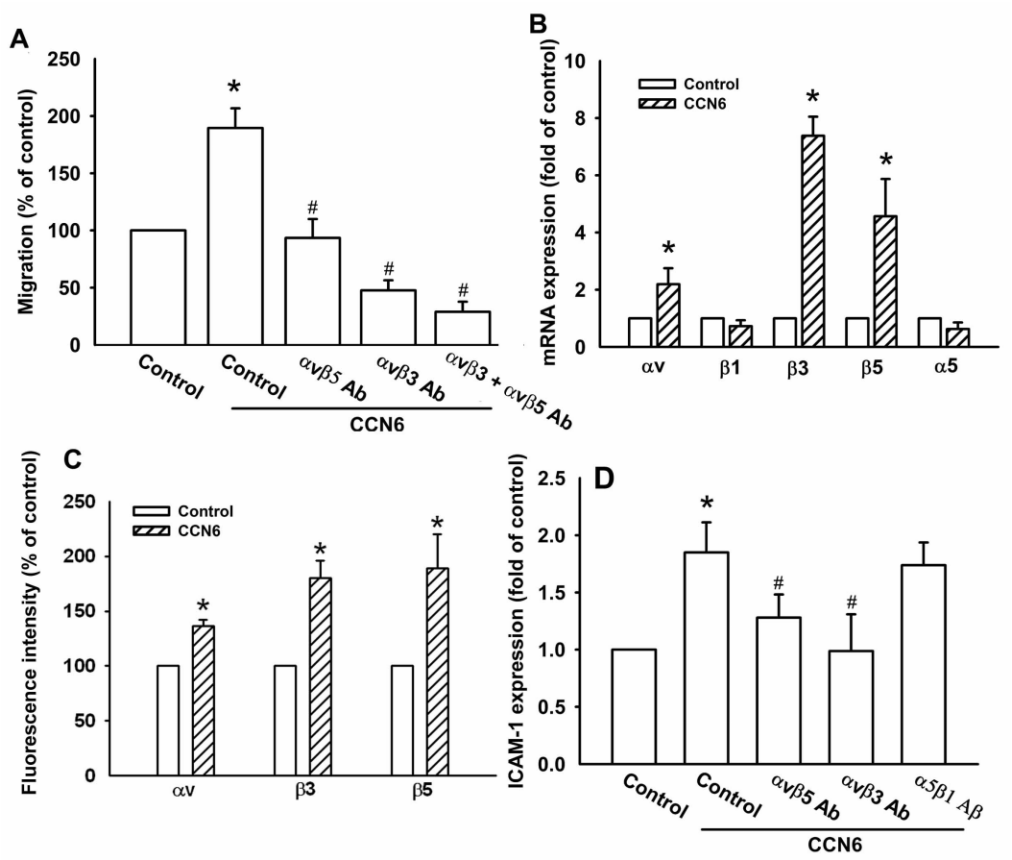

Figure 2

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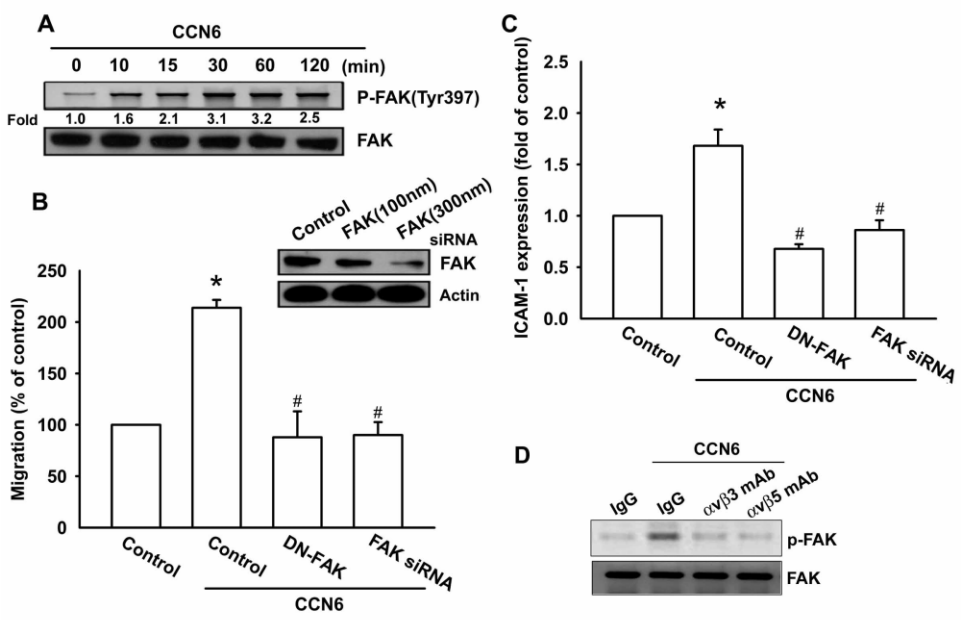

Figure 3

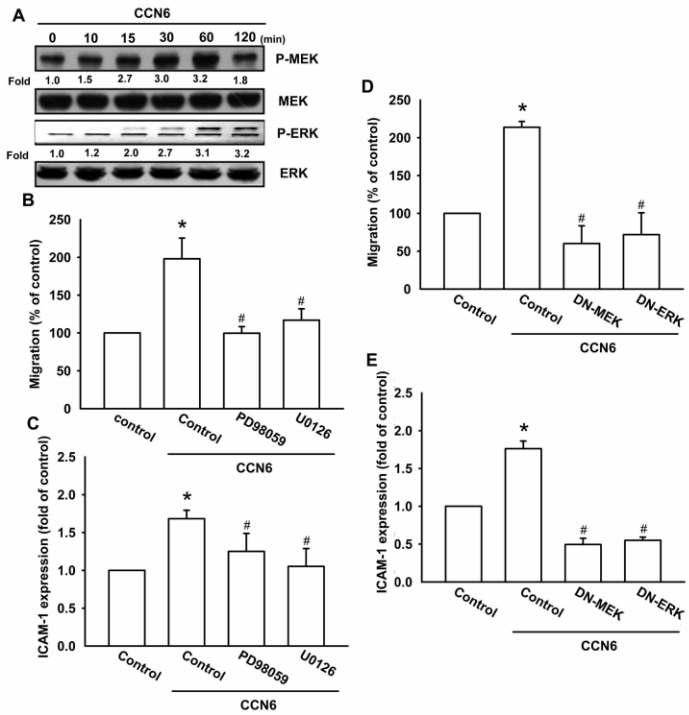

Figure 4

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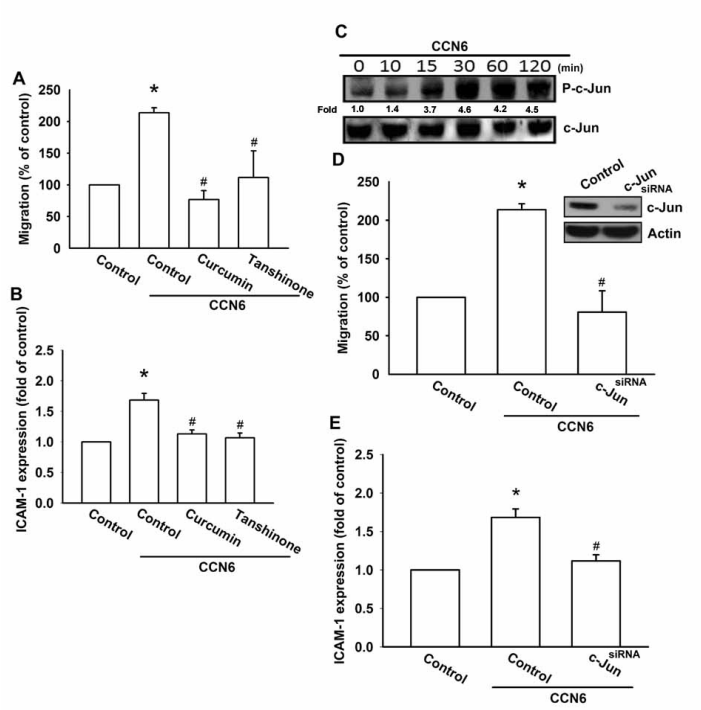

Figure 5

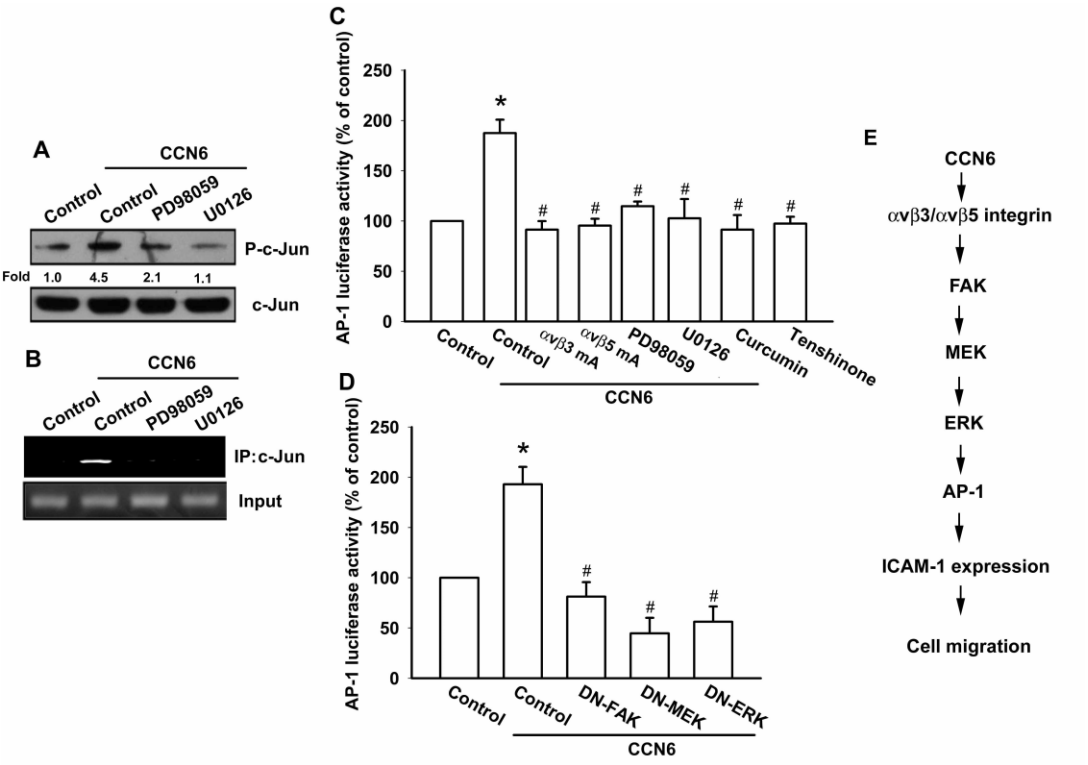

Figure 6