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CTGF Enhances Migration and MMP-13 Up-Regulation Via αvβ3 Integrin, FAK, ERK, and NF-κB-Dependent Pathway in Human Chondrosarcoma Cells

Tzu-Wei Tan,[1] Chih-Ho Lai,[2] Chun-Yiu Huang,[3] Wei-Hung Yang,[3] Hsien-Te Chen,[4,5] Horng-Chaung Hsu,[4] Yi-Chin Fong,[4,6,7] and Chih-Hsin Tang[1,8*]

[1]*Department of Pharmacology, China Medical University, Taichung, Taiwan*
[2]*Department of Microbiology, China Medical University, Taichung, Taiwan*
[3]*Department of Orthopaedic, China Medical University Beigang Hospital, Yun-Lin County, Taiwan*
[4]*Department of Orthopaedic, China Medical University Hospital, Taichung, Taiwan*
[5]*Department of Materials Science and Engineering, Feng Chia University, Taichung, Taiwan*
[6]*School of Chinese Medicine, China Medical University, Taichung, Taiwan*
[7]*Graduate Institute of Chinese Medical Science, China Medical University, Taichung, Taiwan*
[8]*Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan*

ABSTRACT

Tumor malignancy is associated with several features such as proliferation ability and frequency of metastasis. Connective tissue growth factor (CTGF), a secreted protein that binds to integrins, modulates the invasive behavior of certain human cancer cells. However, the effect of CTGF on migration activity in human chondrosarcoma cells is mostly unknown. Here we found that CTGF increased the migration and expression of matrix metalloproteinase (MMP)-13 in human chondrosarcoma cells (JJ012 cells). RGD peptide, αvβ3 monoclonal antibody (mAb) and MAPK kinase (MEK) inhibitors (PD98059 and U0126) but not RAD peptide inhibited the CTGF-induced increase of the migration and MMP-13 up-regulation of chondrosarcoma cells. CTGF stimulation increased the phosphorylation of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK). In addition, treatment of JJ012 cells with NF-κB inhibitor (PDTC) or IκB protease inhibitor (TPCK) inhibited CTGF-induced cell migration and MMP-13 up-regulation. Stimulation of JJ012 cells with CTGF also induced IκB kinase α/β (IKK α/β) phosphorylation, IκBα phosphorylation, p65 Ser^{536} phosphorylation, and κB-luciferase activity. The CTGF-mediated increases in κB-luciferase activities were inhibited by RGD, PD98059, U0126 or FAK, and ERK2 mutant. Taken together, our results indicated that CTGF enhances the migration of chondrosarcoma cells by increasing MMP-13 expression through the αvβ3 integrin, FAK, ERK, and NF-κB signal transduction pathway. J. Cell. Biochem. 107: 345–356, 2009.

KEY WORDS: CTGF; CHONDROSARCOMA; MMP-13; INTEGRIN; MIGRATION

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 explore a novel and adequate remedy [Yuan et al., 2005].

Since chondrosarcoma is a type of highly malignant tumor with a potent capacity to invade locally and cause distant metastasis [Berend et al., 1998], an approach decreasing the ability of invasion and metastasis may facilitate the development of effective adjuvant therapy. The invasion of tumor cells is a complex, multistage process.

Tzu-Wei Tan and Chun-Yiu Huang contributed equally to this work.

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*Correspondence to: Prof. Chih-Hsin Tang, Department of Pharmacology, College of Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung, Taiwan. E-mail: chtang@mail.cmu.edu.tw

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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 [Woodhouse et al., 1997]. Matrix metalloproteinases (MMPs) have important roles in these processes because their proteolytic activities assist in the degradation of ECM and basement membrane [Egeblad and Werb, 2002; Kerkelä and Saarialho-Kere, 2003]. MMPs, cytokines, growth factors, and chemokines have been shown to regulate tumor cell invasion through autocrine or paracrine pathways [Woodhouse et al., 1997]. Among the MMPs, MMP-13 (collagenase-3) is considered to be of particular interest due to its role in cartilage degradation. MMP-13 actively degrades type II collagen, the major type of collagen in cartilage [Vincenti and Brinckerhoff, 2002]. It has been also reported that MMP-13 plays a critical role in ECM turnover and cell–cell interactions, as well as tumor metastasis [Chu et al., 2007].

Connective tissue growth factor (CTGF, also known as CCN2) belongs to the CCN family [Bork, 1998]. This family consists of six members, CTGF, NOVH, CYR61, WISP1, WISP2, and WISP3 [Perbal, 2004] that all possess an N-terminal signal peptide identifying them as secreted proteins. CCN proteins probably carry out their biological activity through binding and activating of the cell surface integrins [Perbal, 2004]. Focal adhesion kinase (FAK), a potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated signaling [Crouch et al., 1996; Hadden and Henke, 2000]. However, the downstream signaling pathways that mediate integrin-FAK signaling are diverse, and the factors determining which pathway is used remain obscure. It has been reported that the CCN proteins involved the stimulation of cellular proliferation, migration, adhesion, ECM formation, and also the regulation of angiogenesis and tumorigenesis [Lau and Lam, 1999]. Overexpression of CTGF, WISP1, and CYR61 in tumor cells have been linked to tumor size and lymph node metastasis [Xie et al., 2001], suggesting that these CCN proteins are involved in the progression of human cancers.

Previous studies have shown that CTGF modulates cell migration and invasion in cancer cells [Chang et al., 2004a; Chen et al., 2007]. CTGF-mediated migration may involve activation of integrin receptors [Chen et al., 2007]. However, the effect of CTGF on MMPs expression and migration activity in human chondrosarcoma cells is mostly unknown. We hypothesized that CTGF might be capable of regulating chondrosarcoma cell migration and MMP expression. Here we found that CTGF increased the migration and the expression of MMP-13 in human chondrosarcoma cells. In addition, αvβ3 integrin, FAK, MAPK kinase (MEK), extracellular signal-regulated kinase (ERK), and NF-κB signaling pathways may be involved in the increase of MMP-13 expression and cell migration by CTGF. Furthermore, the discovery of the CTGF-mediated αvβ3 integrin/NF-κB-dependent pathway helps us understand the mechanism of human chondrosarcoma metasatais and may help us develop an effective therapy in the future.

MATERIALS AND METHODS

MATERIALS

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for FAK, p-MEK, MEK, p-ERK, ERK, IKKα/β, p-IκBα, α-tubulin, MMP-13, and MMP-13 small interfering RNA (siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MEK inhibitors (PD98059 and U0126) [Choi et al., 2008], NF-κB inhibitor (TPCK), and IκB protease inhibitor (PDTC) [Tang et al., 2007] were purchased from Calbiochem (San Diego, CA). Rabbit polyclonal antibody specific for phosphor-FAK (Tyr^{397}), phosphor-IKKα/β ($Ser^{180/181}$), and phosphor-p65 (Ser^{536}) were purchased from Cell Signaling (Danvers, MA). Rabbit polyclonal antibodies specific for αvβ3 integrin were purchased from Chemicon (Temecula, CA). A selective αvβ3 integrin antagonist cyclic RGD (RGD) peptide and the cyclic RAD (RAD) peptide were purchased from Peptides International (Louisville, KY). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA). The phosphorylation site mutant of FAK(Y397F) was a gift from Dr. J. A. Girault (Institut du Fer à Moulin, Moulin, France). The ERK2 (K52R) dominant-negative mutant was a gift from Dr. M. Cobb (South-Western Medical Center, Dallas, TX). The IKKα(KM) and IKKβ(KM) mutants were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

CELL CULTURE

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) [Yeh et al., 2008]. The JJ012 cells were cultured in DMEM/α-MEM supplemented with 10% Fetal Bovine Serum and maintained at 37°C in a humidified atmosphere of 5% CO_2. The human chondrosarcoma cell line (SW1353) was obtained from the American Type Culture Collection. The cells were cultured in DMEM/α-MEM supplemented with 10% Fetal Bovine Serum and maintained at 37°C in a humidified atmosphere of 5% CO_2.

MIGRATION ASSAY

The migration assay was performed using Transwell (Costar, NY; pore size, 8 μm) in 24-well dishes. Before performing the migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the PD98059, U0126, PDTC, TPCK 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 CTGF was placed in the lower chamber. The plates were incubated for 24 h at 37°C in 5% CO_2, cells were 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 for 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 CTGF treatment (corrected migrating cell number = counted migrating cell number/percentage of viable cells) [Tang et al., 2008].

FLOW CYTOMETRIC ANALYSIS

Human chondrosarcoma cells were plated in 6-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 rinsing in PBS, the cells were incubated with rabbit anti-human antibody against αv or β3 integrin, (1:100) for 1 h at 4°C. Cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:150; Leinco Tec., Inc., St. Louis, MO) for 45 min and analyzed by Flow cytometry using FACSCalibur and CellQuest software (BD Biosciences).

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). The zymographic activities were revealed by staining with 1% Coomassie blue.

mRNA ANALYSIS BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from cancer cells using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reversely transcribed into cDNA using oligo(dT) primer, then amplified for 33 cycles using two oligonucleotide primers:

αv integrin sense ACTGGGAGCACAAGGAGAACC and
anti-sense CCGCTTAGTGATGAGATGGTC;
β3 integrin sense CCTACATGACCGAAAATACCT and
anti-sense AATCCCTCCCCACAAATACTG;
MMP-13 sense TGCTCGCATTCTCCTTCAGGA and
anti-sense ATGCATCCAGGGGTCCTGGC;
GAPDH sense ACCACAGTCCATGCCATCAC and
anti-sense TCCACCACCCTGTTGCTGTA.

Each PCR cycle was carried out for 30 s at 94°C, 30 s at 55°C, and 1 min at 68°C.

PCR products were then separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide [Tang et al., 2008].

QUANTITATIVE REAL-TIME PCR

The quantitative real-time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). Hundred nanograms 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). qPCR assays were carried out in triplicate on an ABI Prism 7900 sequence detection system. The cycling conditions were 10 min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

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 h at room temperature and then probed with rabbit anti-human antibodies against IκBα, p-IκBα, IKKαβ or p-ERK (1:1,000) for 1 h at room temperature. After three washings, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1,000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

TRANSFECTION AND REPORTER GENE ASSAY

Human chondrosarcoma cells were co-transfected with 0.8 μg NF-κB-luciferase plasmid and 0.4 μg β-galactosidase expression vector. JJ102 cells were grown to 80% confluent in 12-well plates and were transfected the following day by Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. After a 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) 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. The value of negative-control (transfected with vector control plasmid) was 53 ± 6. The value of positive-control (transfected with κB-luciferase plasmid) was 4851 ± 312 (Fig. 5H).

STATISTICS

The values given are means ± SEM. Statistical analysis between two samples was performed using Student's *t*-test. Statistical comparisons of more than two groups were performed using one-way analysis of variance (ANOVA) with Bonferroni's post hoc test. In all cases, $P < 0.05$ was considered as significant.

RESULTS

CTGF-DIRECTED CHONDROSARCOMA CELL MIGRATION THROUGH αVβ3 INTEGRIN

CTGF has been reported stimulates directional migration and invasion of human cancer cells [Chang et al., 2004a; Chen et al., 2007]. The CTGF for chondrosarcoma cell migration was examined using the Transwell assay with correction of CTGF-induced proliferation effects on human chondrosarcoma cells. Treatment

of CTGF (5–50 ng/ml) with chondrosarcoma cells (JJ012) directed cell migration (Fig. 1A). Previous study has shown CTGF affects cell migration through αvβ3 integrin signaling [Chen et al., 2007]. We therefore hypothesized that αvβ3 integrin signaling pathway may be involved in CTGF-directed chondrosarcoma cell migration. Pretreatment of cells for 30 min with anti-αvβ3 mAb (3, 10 μg/ml) markedly inhibited the CTGF-induced cancer migration (Fig. 1B). The cyclic RGD peptide (RGD) has been reported to bind αvβ3 at high affinity and block its function effectively at low concentrations [Pfaff et al., 1994]. Pretreatment of cells for 30 min with cyclic RGD but not cyclic RAD markedly inhibited the CTGF-induced cell migration (Fig. 1B). Moreover, CTGF also up-regulated cell surface and mRNA expression of αv and β3 integrin by using Flow cytometry analysis, RT-PCR, and qPCR, respectively (Fig. 1C–E), suggesting that the amplification loop strengthens the CTGF-αvβ3 signaling pathway. In addition, treatment of cells with CTGF also increased the migration and cell surface αv and β3 integrin expression in the other chondrosarcoma cell line (SW1353) (Fig. 1F,G). Pretreatment of SW1353 cells with αvβ3 mAb or RGD antagonized the CTGF-induced cell migration (Fig. 1F). These data suggest that CTGF-induced cancer migration may occur via activation of αvβ3 integrin receptor.

INVOLVEMENT OF MMP-13 IN THE CTGF-DIRECTED CHONDROSARCOMA CELL MIGRATION

Previous studies have shown a significant expression of MMP-1, -2, -9, and -13 in human cancer cells [Egeblad and Werb, 2002; Ohbayashi, 2002]. We therefore, hypothesized that any of these MMPs may be involved in CTGF-directed chondrosarcoma migration. Treatment of cells with CTGF induced the expression of MMP-13 but not other MMPs by using qPCR (Fig. 2A). CTGF further increased protein expression of MMP-13 in JJ012 cells in a time-dependent manner (Fig. 2B; upper panel). MMP-13 expression was also increased in the supernatant, and its enzyme activity was up-regulated at 6 h and peaked at 24 h (Fig. 2B; lower panel). When the JJ012 cells were transfected with MMP-13 or control siRNA for 24 h, the RT-PCR and Western blot analysis showed that the expression of RNA or protein levels of MMP-13 was suppressed by transfection with MMP-13 siRNA (Fig. 2C; left panel). Transfection of cells with MMP-13 siRNA reduced the CTGF-increased cell migration (Fig. 2C; right panel). On the other hand, MMP-13 transcription and gelatinase activity was abolished by αvβ3 mAb and RGD peptide (Fig. 2D,E), confirming the involvement of CTGF in MMP-13 regulation.

FAK, MEK, AND ERK SIGNALING PATHWAYS ARE INVOLVED IN THE CTGF-MEDIATED CELL MIGRATION OF CHONDROSARCOMA CELLS

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 Figure 3A, FAK phosphorylation increased in a time-dependent manner in response to CTGF stimulation, reaching the maximum between 10 and 25 min. Transfection of cells with FAK(Y397F) mutant reduced the CTGF-mediated cell migration (Fig. 3B). Transfection of cells with FAK(Y397F) mutant also reduced the CTGF-induced MMP-13 expression and activity (Fig. 3C,D). On the other hand, transfection of cells with FAK(Y397F) mutant antagonized CTGF-mediated FAK phosphorylation (Fig. 3E). MEK/ERK signaling pathway can be activated by a variety of growth factors, such as insulin, nerve growth factors, and CTGF [Chen et al., 2007; Rangaswami and Kundu, 2007]. We then examined whether CTGF stimulation also enhances the activation of the MEK/ERK pathway. CTGF-induced migration of JJ012 cells were greatly reduced with treatment of MEK inhibitors PD98059 and U0126 (Fig. 4A). The MEK inhibitors PD98095 and U0126 also inhibited the CTGF-increased MMP-13 expression and activity (Fig. 4B,C). Pretreatment of cells with PD98059 or U0126 reduced CTGF-increased MEK phosphorylation (Fig. 4D). In addition, treatment of cells with CTGF increased phosphorylation of ERK (Fig. 4E). Transfection of cells with ERK2 mutant reduced the CTGF-mediated cell migration and MMP-13 activity (Fig. 4F–H). ERK2 mutant also reduced CTGF-increased ERK phosphorylation (Fig. 4I). Furthermore, CTGF-induced ERK phosphorylation was markedly inhibited if cells were pretreated for 30 min with αvβ3 mAb and RGD or transfection for 24 h with FAK(Y397F) mutant (Fig. 4J). Taken together, these results indicate that the αvβ3 integrin/FAK/MEK and ERK pathway is involved in CTGF-induced migration activity and MMP-13 up-regulation in human chondrosarcoma cells.

INVOLVEMENT OF NF-κB IN CTGF-INDUCED CELL MIGRATION AND MMP-13 EXPRESSION

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human cancer cells [Tang et al., 2008; Yeh et al., 2008]. To examine whether NF-κB activation is involved in CTGF-induced cancer migration, an NF-κB inhibitor, PDTC, was used. Figure 5A shows that JJ012 cells pretreated with PDTC (10 μM) inhibited CTGF-induced chondrosarcoma cell migration. Furthermore, JJ012 cells pretreated with TPCK (3 μM), an IκB protease inhibitor, also reduced CTGF-induced cancer cell migration (Fig. 5A). Treatment of cells with PDTC and TPCK reduced the CTGF-mediated mRNA expression and enzyme activity of MMP-13 (Fig. 5B,C). We further examined the upstream molecules involved in CTGF-induced NF-κB activation. Stimulation of cells with CTGF induced IKKα/β phosphorylation in a time-dependent manner (Fig. 5D). Pretreatment of cells with PDTC and TPCK reduced CTGF-induced IKK phosphorylation (Fig. 5E). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the CTGF-induced cancer cell migration and MMP-13 activity (Fig. 5F,G). These data suggest that IKKα/β activation is involved in CTGF-induced migration activity of human chondrosarcoma cells. Treatment of chondrosarcoma cells with CTGF also caused IκBα phosphorylation in a time-dependent manner (Fig. 5D). Previous studies showed that p65 Ser^{536} phosphorylation increases NF-κB transactivation [Madrid et al., 2001; Viatour et al., 2005], and the antibody specific against phosphorylated p65 Ser^{536} was used to examine p65 phosphorylation. Treatment of JJ012 cells with CTGF for various time intervals resulted in p65 Ser^{536} phosphorylation, which began at 10 min and was sustained for 60 min (Fig. 5D). To directly determine NF-κB activation after CTGF treatment, JJ012 cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Figure 5H, CTGF (5–50 ng/ml) treatment of

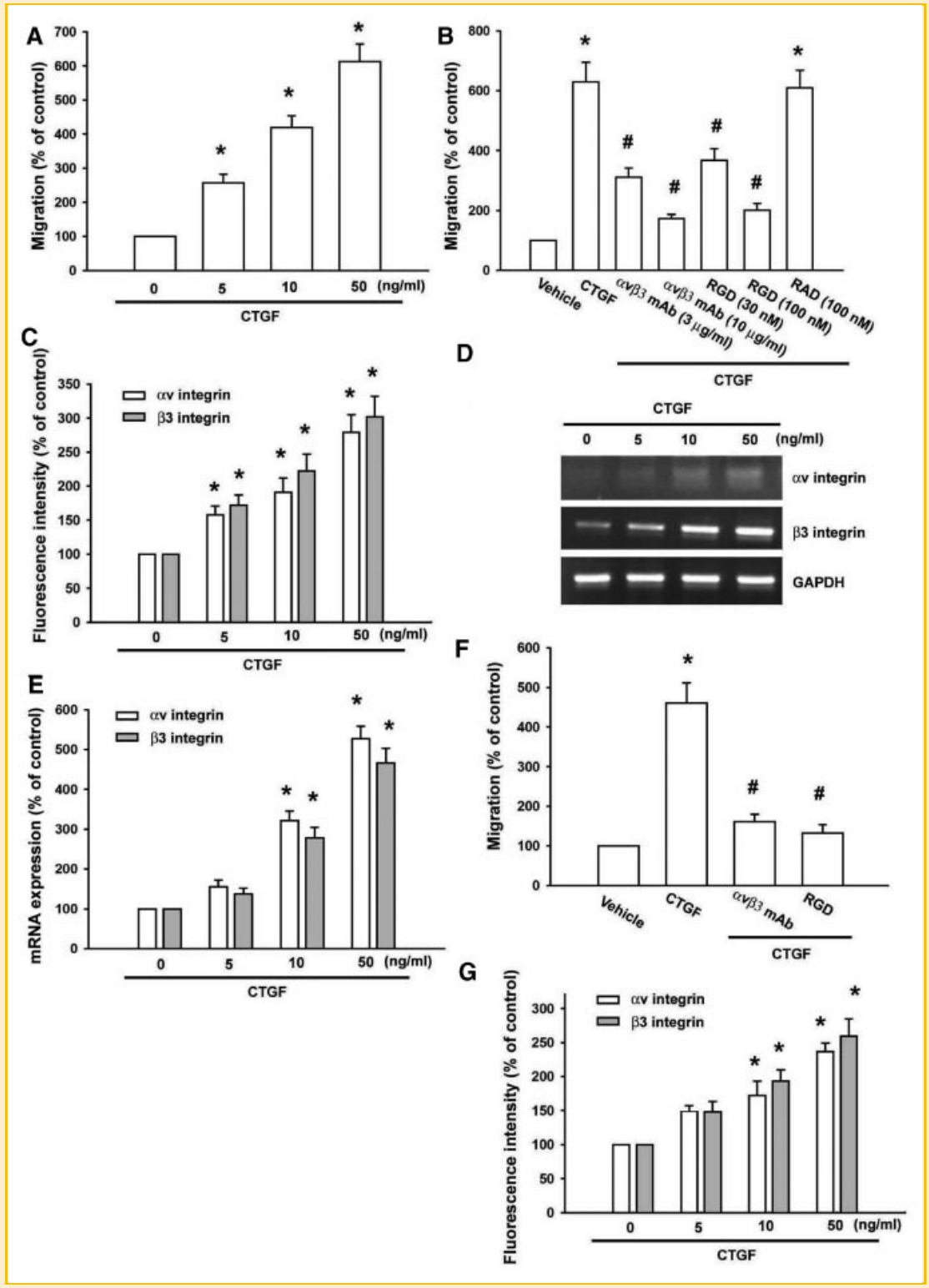

Fig. 1. CTGF induced the migration activity of human chondrosarcoma cells JJ012 cells were incubated with various concentrations of CTGF, and in vitro migration activities measured with the Transwell after 24 h showed that all supported the JJ012 cell migrations in a dose-dependent way (A). JJ012 cells were pretreated with αvβ3 mAb (3, 10 μg/ml), cyclic RGD (30, 100 nM), cyclic RAD (100 nM) for 30 min followed by stimulation with CTGF (50 ng/ml). The in vitro migration activity measured after 24 h showed that αvβ3 mAb and cyclic RGD but not cyclic RAD could inhibit the activities (B). JJ012 cells were incubated with CTGF (5–50 ng/ml) for 24 h, and the cell surface expression of αv and β3 integrin were determined using Flow cytometer (C). JJ012 cells were incubated with CTGF (5–50 ng/ml) for 24 h, and the mRNA expression of αv and β3 integrin were determined using RT-PCR and qPCR (D,E). SW1353 cells were pretreated with αvβ3 mAb (10 μg/ml) or cyclic RGD (100 nM) for 30 min followed by stimulation with CTGF (50 ng/ml). The in vitro migration activity measured after 24 h showed that αvβ3 mAb and cyclic RGD could inhibit the activities (F). SW1353 cells were incubated with CTGF (5–50 ng/ml) for 24 h, and the cell surface expression of αv and β3 integrin were determined using Flow cytometry (G). Results are expressed as the mean ± SE $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with CTGF-treated group.

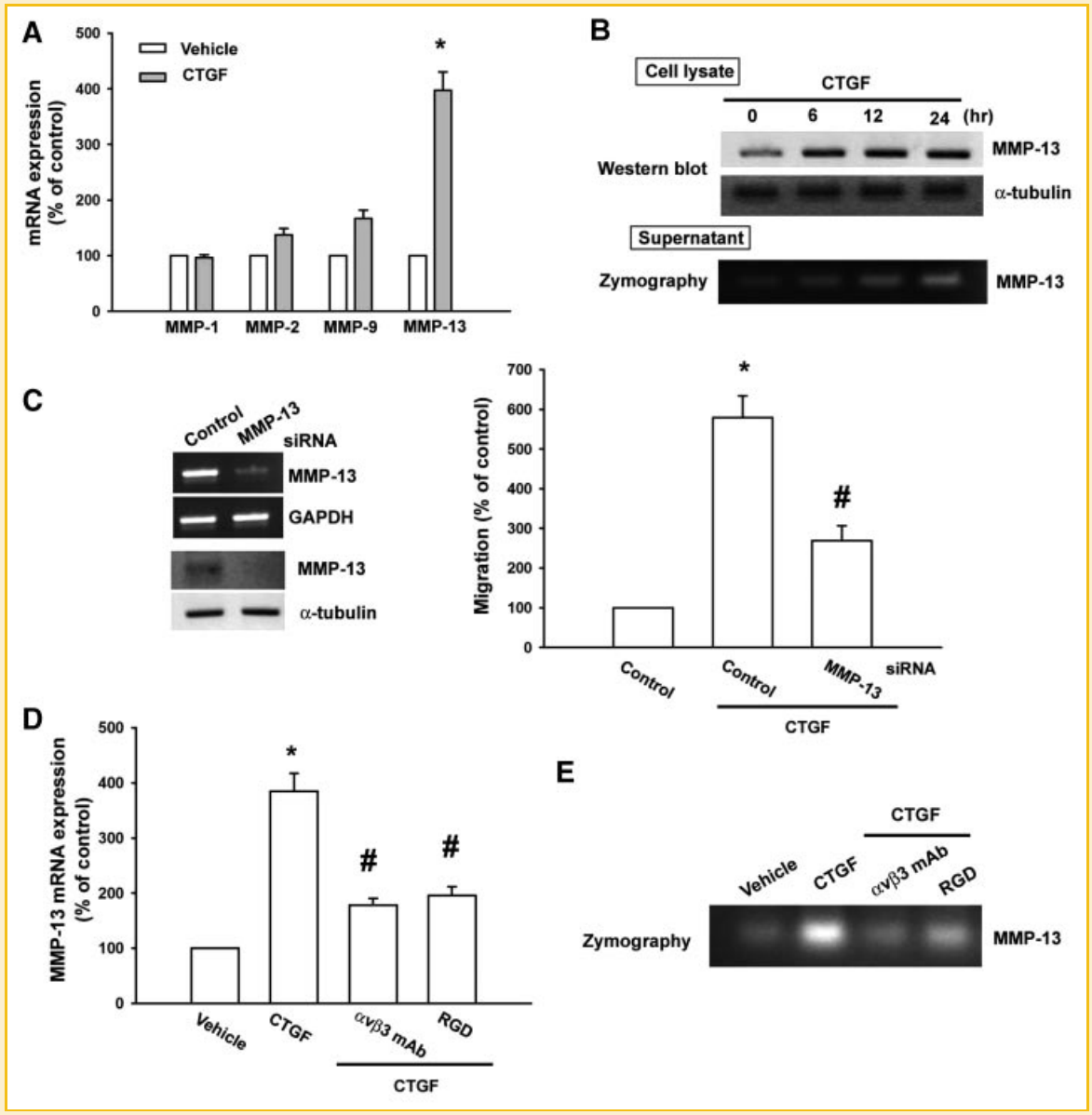

Fig. 2. CTGF-directed migration activity of human chondrosarcoma cells involves up-regulation of MMP-13. JJ012 cells were incubated with CTGF (50 ng/ml) for 24 h or for indicated time intervals. Cell lysates were then collected and the mRNA level of MMP-1, -2, -9, and -13 was determined using qPCR (A). Cells were incubated with CTGF (50 ng/ml) for indicated time intervals. The cultured medium and cell lysates were then collected. Both the protein level of MMP-13 in cell lysates determined by Western blot analysis and the enzyme activity of MMP-13 in supernatant determined using zymography were increased in a time-dependent manner (B). Cells were transfected with MMP-13 or control siRNA for 24 h, and the mRNA and protein levels of MMP-13 were examined using RT-PCR and Western blot analysis (C; left panel). Cells were transfected with MMP-13 or control siRNA for 24 h, and in vitro migration was measured with the Transwell after 24 h (C; right panel). Cells were pretreated with αvβ3 antibody (10 μg/ml) and RGD (100 nM) for 30 min followed by stimulation with CTGF (50 ng/ml) for 24 h, and the mRNA level and enzyme activity of MMP-13 was determined by using qPCR (D) and zymography (E). Results are expressed as the mean ± SE $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with CTGF-treated group.

JJ012 cells for 24 h caused a concentration-dependent increase in κB-luciferase activity.

αVβ3 INTEGRIN, FAK, ERK SIGNAL TRANSDUCTION-MEDIATED CTGF-INDUCED IKKα/β PHOSPHORYLATION, P65 PHOSPHORYLATION, AND κB LUCIFERASE ACTIVITY

To further investigate whether CTGF-induced IKKα/β phosphorylation, p65 Ser^{536} phosphorylation, and NF-κB activation occur through the αvβ3 integrin, FAK, ERK pathway, JJ012 cells were pretreated for 30 min with RGD and PD98059 or transfected for 24 h with FAK(Y397F) mutant, which inhibited the CTGF-induced increase in IKKαβ, IκBα, and p65 phosphorylation as shown in Figure 6A. In addition, the CTGF-induced increase in κB-luciferase activity was also inhibited by treatment with αvβ3 mAb, RGD, PD98059, U0126, PDTC, and TPCK (Fig. 6B). Co-transfection of cells with FAK(Y397F), ERK2, IKKα, or IKKβ mutant also reduced the CTGF-increased κB luciferase activity (Fig. 6C). Taken together, these data suggest that the activation of αvβ3 integrin, FAK, ERK

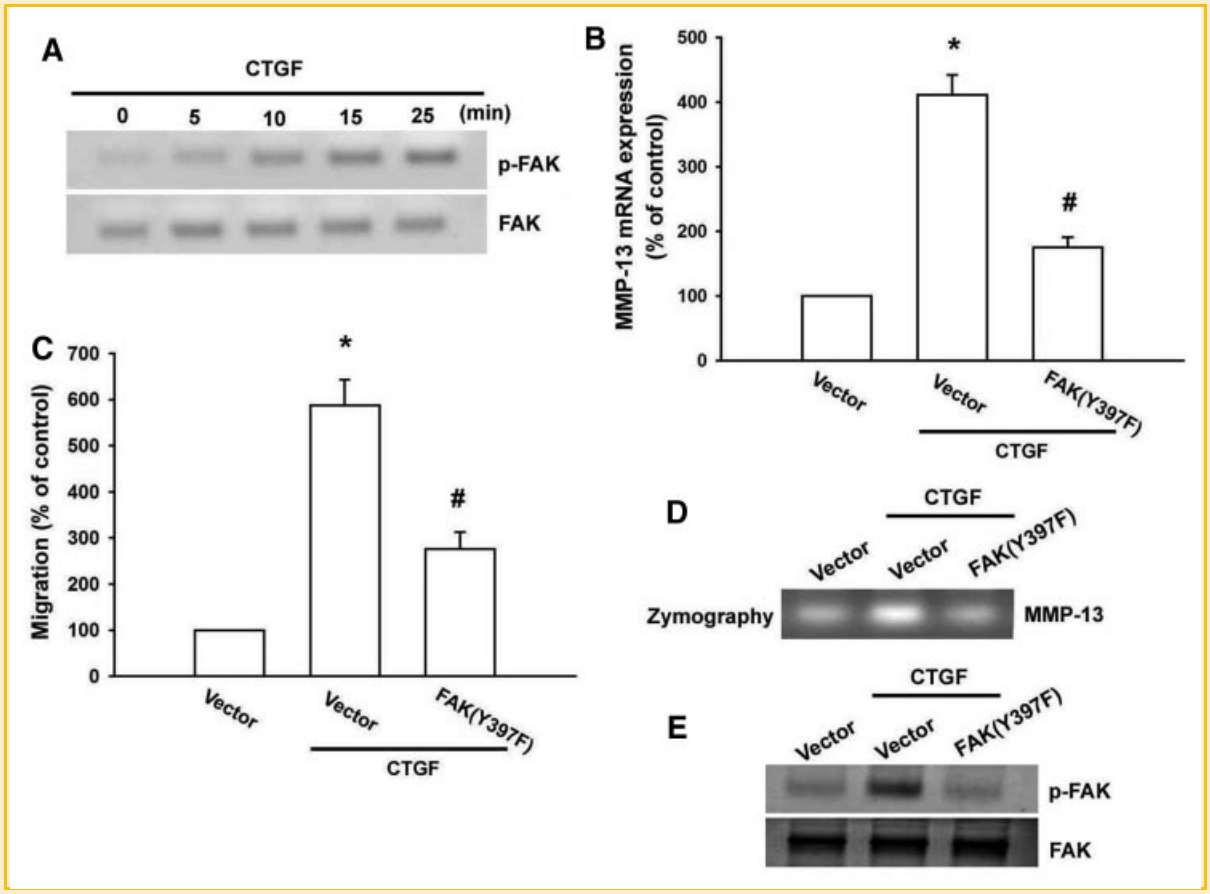

Fig. 3. Involvement of FAK signaling pathway in response to CTGF in chondrosarcoma cells. A: JJ012 cells were incubated with CTGF (50 ng/ml) for indicated time intervals, and p-FAK expression was determined by Western blot analysis. Note that CTGF activated the FAK pathway in JJ012 cells. B: Cells were transfected with mutant of FAK for 24 h followed by stimulation with CTGF (50 ng/ml), and in vitro migration was measured with the Transwell after 24 h. JJ012 cells were transfected with mutant of FAK for 24 h followed by stimulation with CTGF (50 ng/ml), and the mRNA level and enzyme activity of MMP-13 was determined by using qPCR (C) and zymography (D). Cells were transfected with mutant of FAK for 24 h followed by stimulation with CTGF (50 ng/ml) for 25 min, and the FAK phosphorylation was examined by Western blot analysis (E). Results are expressed as the mean ± SE $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with CTGF-treated group.

pathway is required for CTGF-induced IKKα/β phosphorylation, p65 Ser^{536} phosphorylation, and NF-κB activation in chondrosarcoma cells.

DISCUSSION

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which are 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. But due to the relatively indolent growth rates of many low- and moderate-grade chondrosarcomas, approximately 15% of patients dying from metastatic disease do so more than 5 years after initial diagnosis [Springfield, 1998]. Therefore, it is important to development an effective adjuvant therapy for preventing chondrosarcoma metastasis. We hypothesized that CTGF and its αvβ3 integrin receptor would help direct the metastasis of chondrosarcoma cells. We found that CTGF increased the migration of chondrosarcoma cells. One of the mechanisms underlying CTGF-directed migration was transcriptional up-regulation of MMP-13 and activation of αvβ3 integrin, FAK, MEK, ERK, 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, -9, and -13 have been found to correlate with malignant grade and metastasis [Egeblad and Werb, 2002; Ohbayashi, 2002]. It has been reported that MMP plays an important role in CTGF-induced metastasis in human cancer cells [Maeta et al., 2007]. In this study, we found that CTGF induced MMP-13 expression and secretion in human chondrosarcoma cells without significantly changing the expression of MMP-1, -2, and -9 mRNAs. In addition, the inhibition of CTGF-enhanced MMP-13 protein expression with siRNA significantly suppressed CTGF-induced migration. Therefore, MMP-13 may be the CTGF-responsive mediator, and it causes the degradation of ECM that 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 [Hynes, 2002;

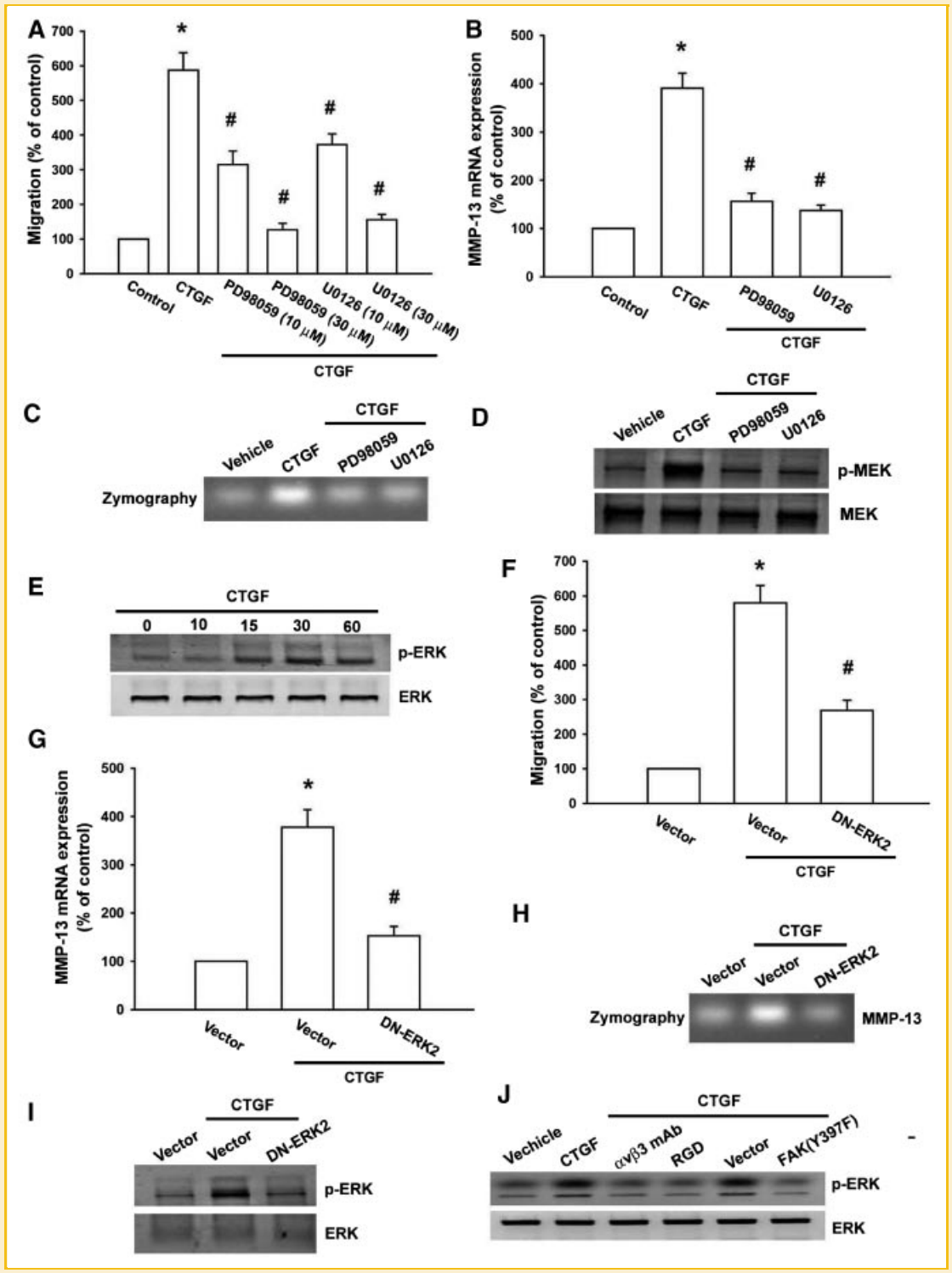

Fig. 4. MEK/ERK pathway is involved in CTGF-mediated migration in human chondrosarcoma cells. A: JJ012 cells were pretreated with PD98059 (10, 30 μg/ml) and U0126 (10, 30 μM) for 30 min followed by stimulation with CTGF (50 ng/ml) for 24 h, and in vitro migration was measured with the Transwell after 24 h. JJ012 cells were pretreated with PD98059 (30 μg/ml) and U0126 (30 μM) for 30 min followed by stimulation with CTGF (50 ng/ml) for 24 h, and the mRNA level and enzyme activity of MMP-13 was determined by using qPCR (B) and zymography (C). JJ012 cells were pretreated with PD98059 and U0126 for 30 min followed by stimulation with CTGF for 60 min, and the MEK phosphorylation was examined by Western blot analysis (D). JJ012 cells were incubated with CTGF (50 ng/ml) for indicated time intervals, and p-ERK expression was determined by Western blot analysis. Note that CTGF activated the ERK pathway in JJ012 cells (E). Cells were transfected with mutant of ERK2 for 24 h followed by stimulation with CTGF (50 ng/ml), and in vitro migration was measured with the Transwell after 24 h (F). JJ012 cells were transfected with mutant of ERK2 for 24 h followed by stimulation with CTGF (50 ng/ml), and the mRNA and enzyme activity of MMP-13 was determined by using qPCR (G) and zymography (H). JJ012 cells were transfected with ERK2 mutant or vector for 24 h followed by stimulation with CTGF for 60 min, the ERK phosphorylation was examined by Western blot analysis (I). Cells were pretreated for 30 min with αvβ3 antibody (10 μg/ml) and RGD (100 nM) or transfected with DN-mutant of FAK(Y397F) for 24 h followed by stimulation with CTGF (50 ng/ml), and p-ERK expression was determined by Western blot analysis 60 min following CTGF stimulation (J). Results are expressed as the mean ± SE $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with CTGF-treated group.

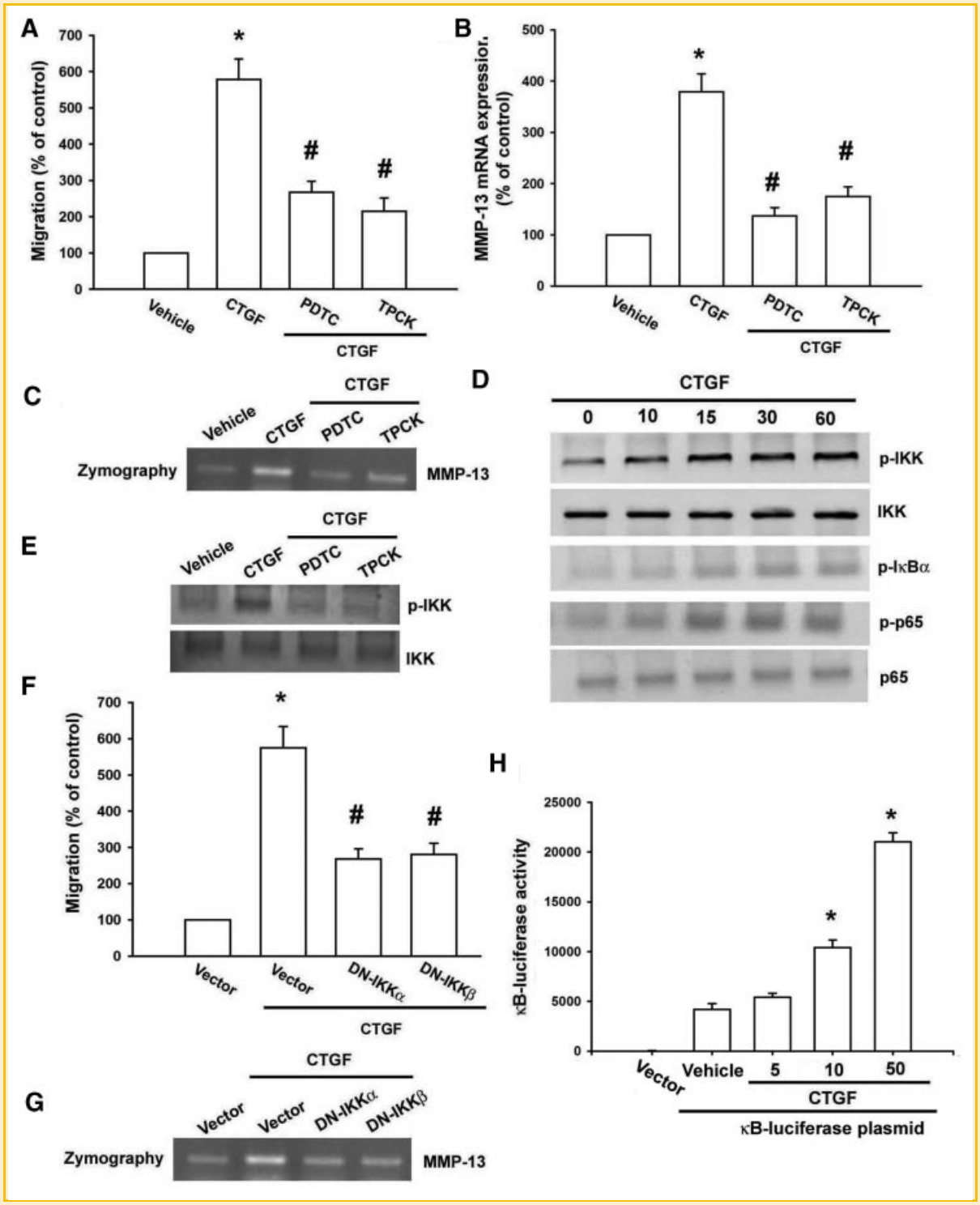

Fig. 5. CTGF induces cell migration and MMP-13 up-regulation through NF-κB. A: Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with CTGF (50 ng/ml), and in vitro migration was measured with the Transwell after 24 h. B: Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with CTGF (50 ng/ml) for 24 h, and the mRNA level and enzyme activity of MMP-13 was determined by using qPCR (B) and zymography (C). Cells were incubated with CTGF (50 ng/ml) for indicated time intervals, and p-IKKα/β p-IκBα and p-p65 expression was determined by Western blot analysis (D). Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with CTGF (50 ng/ml) for 60 min, and the IKK phosphorylation was examined by Western blot (E). Cells were transfected with mutant of IKKα and IKKβ for 24 h followed by stimulation with CTGF (50 ng/ml), and in vitro migration was measured with the Transwell after 24 h (F). JJ012 cells were transfected with mutant of IKKα and IKKβ for 24 h followed by stimulation with CTGF (50 ng/ml), and the enzyme activity of MMP-13 was determined by using and zymography (G). Cells were transfected with κB promoter plasmid for 24 h, and were then incubated with CTGF (5–50 ng/ml) for 24 h. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity (H). Results are expressed as the mean ± SE $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with CTGF-treated group.

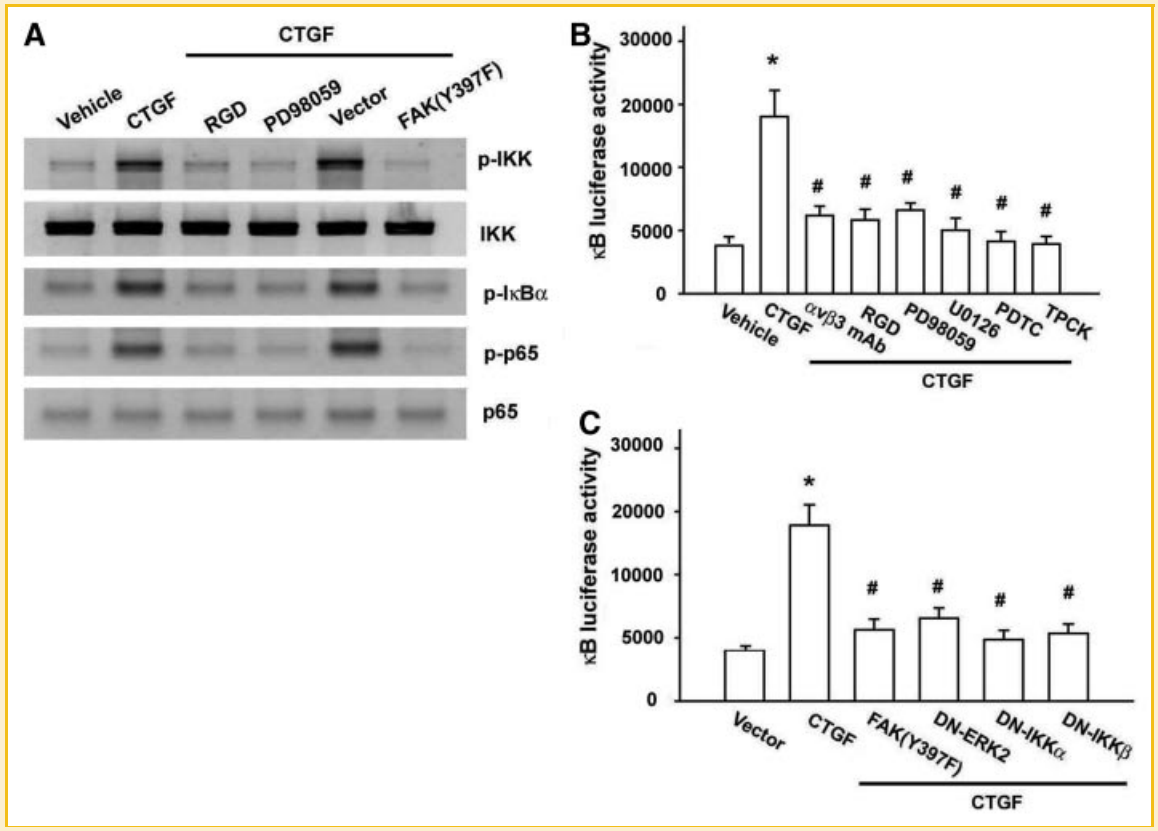

Fig. 6. RGD, ERK, and NF-κB inhibitors antagonized the CTGF-induced IKKα/β phoshorylation, p65 Ser^{536} phosphorylation, and κB luciferase activity in chondrosarcoma cells. A: JJ012 cells were pretreated with RGD and PD98059 for 30 min or transfected with FAK(397F) mutant for 24 h before treatment with CTGF (50 ng/ml) for another 60 min, after which IKKα/β, IκBα phosphorylation, p65 Ser^{536} phosphorylation were determined by immunoblotting with antibodies specific for phospho-IKKα/β phospho-IκBα and phospho-p65, respectively. B,C: JJ012 cells transiently transfected with κB-luciferase plasmid for 24 h were either co-transfected with FAK, ERK2, IKKα, and IKKβ mutant or pretreated with αvβ3 mAb, RGD, PD98059, U0126, PDTC, and TPCK for 30 min, before incubation with CTGF (50 ng/ml) for 24 h. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity. Results are expressed as the mean ± SE. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with CTGF-treated group.

Miranti and Brugge, 2002]. We demonstrate that CTGF increased phosphorylation of tyrosine 397 of FAK. Furthermore, the FAK(Y397F) mutant antagonized the CTGF-mediated potentiation of migration activity, suggesting that FAK activation is an obligatory event in CTGF-induced migration in these cells. MEK/ERK also plays a critical role in integrin signaling [Kapur et al., 2003]. We found that PD98059 and U0126 (MEK inhibitors) also inhibited CTGF-induced migration. Stimulation of cells with CTGF increased phosphorylation of ERK. The MEK inhibitors and ERK2 mutant also reduced the CTGF-mediated MMP-13 expression and activity. Furthermore, CTGF-induced ERK activation was antagonized by αvβ3 mAb, RGD, and FAK(Y397F) mutant, indicating that the αvβ3 integrin and FAK occur as the upstream molecules involved in CTGF-induced activation of ERK. It has been reported that NF-κB activation is involved in integrin expression and tumor metastasis [Inoue et al., 2007]. In this study, we demonstrate that CTGF-induced migration was inhibited by the NF-κB inhibitors, PDTC and TPCK, indicating that activation of NF-κB might be involved in the induction of migration activity caused by CTGF stimulation. In an inactivated state, NF-κB is normally held in the cytoplasm by the inhibitor protein IκB. Upon stimulation, such as by tumor necrosis factor-α, IκB proteins become phosphorylated by the multisubunit IKK complex, which subsequently targets IκB for ubiquitination, and then are degraded by the 26S proteasome. Finally, the free NF-κB translocates to the nucleus, where it activates the responsive gene [Chang et al., 2004b]. It has been reported that CTGF increased migration of breast cancer cells through αvβ3 integrin, ERK, and S100A4 pathway [Chen et al., 2007]. On the other hand, the IKK pathway has been reported to be involved in CTGF-mediated cancer progression [Chen and Lau, 2009]. In the present study, we found that exposure of JJ012 cells with CTGF resulted in increased IKK, IκBα, and p65 phosphorylation. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that CTGF induced an increase in NF-κB activity. In this study, we found that CTGF-induced NF-κB activity was inhibited by αvβ3 mAb, RGD, PD98059, U0126, PDTC, and TPCK. FAK, ERK2, IKKα, and IKKβ mutants also reduced the CTGF-increased the NF-κB luciferase activity. These results indicate that αvβ3 integrin, FAK, MEK, and ERK may act through IKKα/β to increase p65 phosphorylation and enhance NF-κB transactivation.

In conclusion, we present here a novel mechanism of CTGF-directed migration and MMP-13 up-regulation of chondrosarcoma

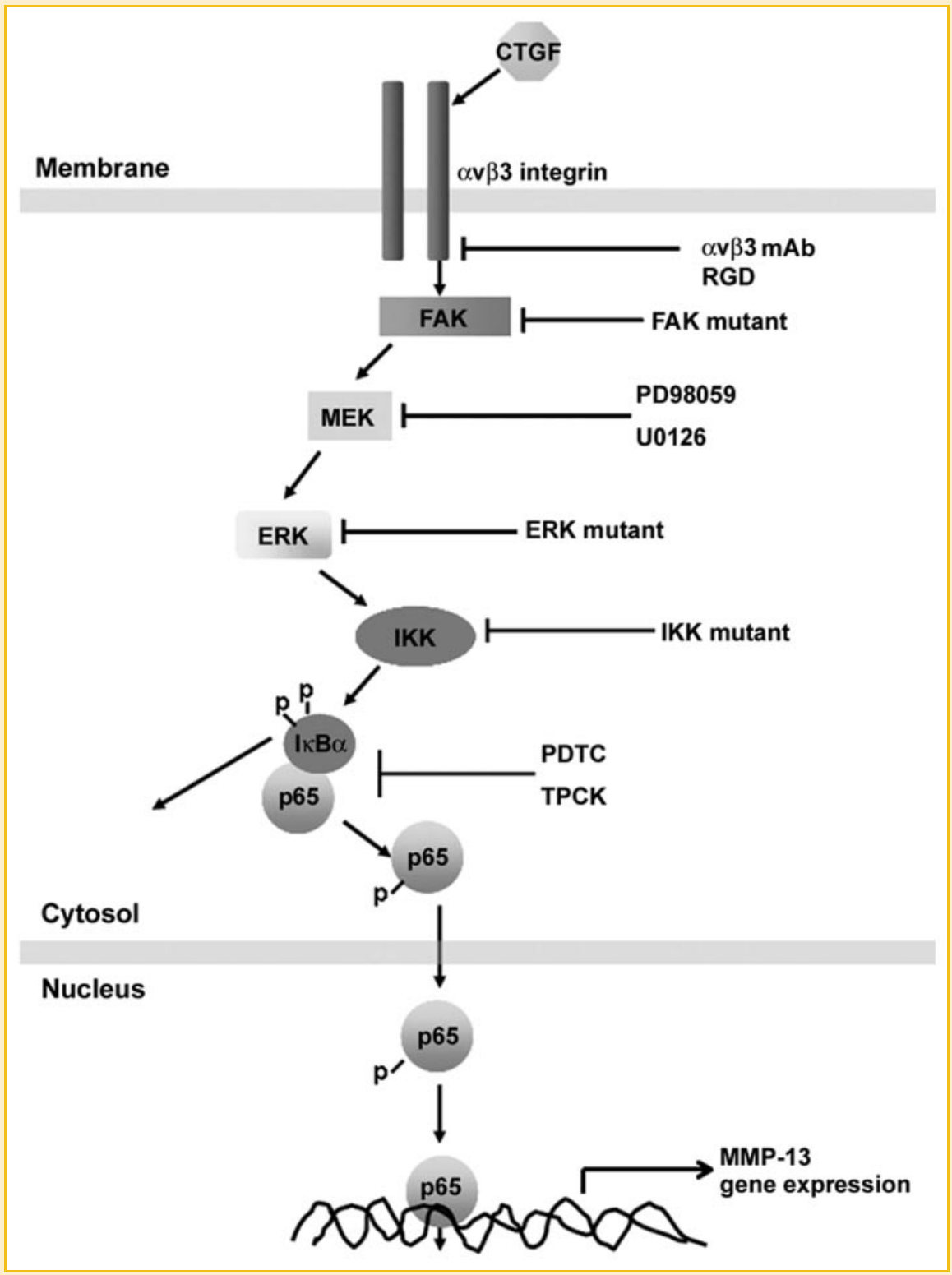

Fig. 7. Schematic presentation of the signaling pathways involved in CTGF-induced MMP-13 expression in human chondrosarcoma cells. CTGF acts through the αvβ3 integrin receptor to activate FAK, MEK and ERK, leading to the activation of p65, resulting in the activation of NF-κB element on the human MMP-13 promoter and the initiation of MMP-13 expression. This MMP-13 induction increases the migration of human chondrosarcoma cells.

cells by activation of αvβ3 integrins, FAK, MEK, ERK, and NF-κB-dependent pathway (Fig. 7).

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