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WISP-1 increases MMP-2 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. WISP-1 is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of matricellular proteins. However, the effect of WISP-1 on migration activity in human chondrosarcoma cells is mostly unknown. Here we found that WISP-1 increased the migration and expression of matrix metalloproteinase (MMP)-2 in human chondrosarcoma cells (JJ012 cells). We also found that human chondrosarcoma tissues had significant expression of the WISP-1 which was higher than that in normal cartilage. α5β1 monoclonal antibody and MAPK kinase (MEK) inhibitors (PD98059 and U0126) inhibited the WISP-1-induced increase of the migration and MMP-2 up-regulation of chondrosarcoma cells. WISP-1 stimulation increased the phosphorylation of focal adhesion kinase (FAK), MEK and extracellular signal-regulated kinase (ERK). In addition, NF-κB inhibitors also suppressed the cell migration and MMP-2 expression enhanced by WISP-1. Moreover, WISP-1 increased NF-κB luciferase activity and binding of p65 to the NF-κB element on the MMP-2 promoter. Taken together, our results indicated that WISP-1 enhances the migration of chondrosarcoma cells by increasing MMP-2 expression through the α5β1 integrin receptor, FAK, MEK, ERK, p65 and NF-κB signal transduction pathway.

1. Introduction

Chondrosarcoma is the second most common malignancy of bone and it has a poor response to chemotherapy or radiation treatment currently used, making the management of chondrosarcomas a complicated challenge [1]. Clinically, surgical resection remains the primary mode of therapy for chondrosarcoma. In the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis and therefore, it is important to explore novel and adequate remedies [2]. Since chondrosarcoma is a type of highly malignant tumor with a potent capacity to invade locally and metastasize distantly [2], 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 [3]. 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 [4]. Matrix metalloproteinases (MMPs) have important roles in these processes because their proteolytic activities assist in degradation of ECM and basement membrane [5–7]. MMPs, cytokines, growth factors and chemokines have been shown to regulate tumor cell invasion through autocrine or paracrine pathways [4]. Previous studies demonstrated the expression of MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 in human chondrosarcoma cells [8,9].

WISP-1 is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of matricellular proteins, with developmental functions [10,11]. Recent studies have shown that the CCN protein family members also play important roles in tumorigenesis, including cancer cell proliferation, survival, adhesion, and

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invasion [12,13]. CCN proteins are mostly secreted and extracellular matrix associated and have been proposed to connect signaling pathways and facilitate cross talks between epithelium and stroma [10]. WISP-1 is strongly expressed in the fibrovascular stroma of breast tumors developing in Wnt-1 transgenic mice [14]. Forced overexpression of WISP-1 in normal rat kidney fibroblasts was sufficient to induce their transformation [15]. Moreover, increasing evidence has suggested that WISP-1 proteins are involved in tumorigenesis, and variation of expression of these molecules has been observed in several types of cancers [16,17].

Previous studies have shown that CCN1 (Cyr61) and CCN2 (CTGF) modulates cell migration and invasion in human chondrosarcoma cells [8,18]. However, the effect of WISP-1 on migration activity in human chondrosarcoma cells is mostly unknown. Here we show that WISP-1 increase migration and upregulate MMP-2 expression in human chondrosarcoma cells. In addition, α5β1 integrin receptor, focal adhesion kinase (FAK), MAPK kinase (MEK), ERK and NF-κB signaling pathways were involved.

2. Materials and methods

2.1. Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p-MEK, MEK, p-ERK, ERK, IKKα/β, p-IκBα, p65, α-tubulin, FAK siRNA, MMP-2 siRNA, control siRNA, control shRNA plasmid and WISP-1 shRNA plasmid were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody specific for α5β1 integrin was purchased from Chemicon (Temecula, CA, USA). U0126, PD98059, TPCK and PDTC were purchased from Calbiochem (San Diego, CA, USA). 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, USA). The recombinant human WISP-1 was purchased from PeproTech (Rocky Hill, NJ, USA). The phosphorylation site mutant of FAK(Y397F) was a gift from Dr. J.A. Girault (Institut du Fer a'Moulin, Moulin, France). The MEK1 dominant-negative mutant was a gift from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The ERK2 dominant-negative mutant was a gift from Dr. M. Cobb (South-Western Medical Center, Dallas, TX). The IKKα(KM) and IKKβ(KM) mutants were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA, USA). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The human chondrosarcoma cell line (JJ012) was kindly provided from the laboratory of Dr. Sean P Scully (University of Miami School of Medicine, Miami, FL, USA). The human chondrosarcoma cell lines (SW1353 and HS18.90) were obtained from the American Type Culture Collection. 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.

2.3. Patients and specimen preparation

Upon approval by the local ethics committee, specimens of tumor tissue or normal 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 the China Medical University Hospital. Tissue specimens were ground and then sonicated in a lysis buffer. The protein level was analyzed by using Western blot analysis.

2.4. Migration assay

The migration assay was performed by using Transwell (Costar, NY, USA; pore size, 8-μm) in 24-well dishes. Before migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the U0126, PD98059, PDTC, TPCK or vehicle control (0.1% DMSO). 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 WISP-1 was placed in the lower chamber. The plates were incubated for 24 h at 37 °C in 5% CO_2, then cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times. The number of invading cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of WISP-1 treatment (corrected invading cell number = counted invading cell number/percentage of viable cells) [19].

2.5. 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 h 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, Lincolin, NE, USA).

2.6. Western blot analysis

The cellular lysates were prepared as described previously [20]. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against IκBα, p-IκB or IKKα/β (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA).

2.7. Zymography analysis

The supernatants of JJ012 cells were mixed with sample buffer without reducing agent or heating. The sample was loaded into a gelatin (1 mg/ml) containing SDS–polyacrylamide gel and underwent electrophoresis with constant voltage. Afterwards, the gel was washed with 2.5% Triton X-100 to remove SDS, rinsed with 50 mM Tris–HCl, pH 7.5, and then incubated overnight at room temperature with the developing buffer (50 mM Tris–HCl, pH 7.5, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% thimerosal, 1% Triton X-100).

2.8. Quantitative real time PCR

Total RNA was extracted from chondrosarcomas by using a TRIzol kit (MDBio Inc., Taipei, Taiwan). Two microgram of total

RNA was reverse transcribed into cDNA by using oligo(dT) primer. The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA, USA). One hundred nanogram of total cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems, Foster City, CA, USA). qPCR assays were carried out in triplicate on an StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

2.9. Transfection and reporter gene assay

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

2.10. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously [27]. 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′-CCCCTGTTCAAGATGGAGTC-3′ and 5′-CCCAGGTTGCTTCCTTACCT-3′ were utilized to amplify across the human MMP-2 promoter region (−673 to −517).

2.11. Establish of stably transfected cells

WISP-1 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, Grand Island, NY) 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.

2.12. Statistics

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

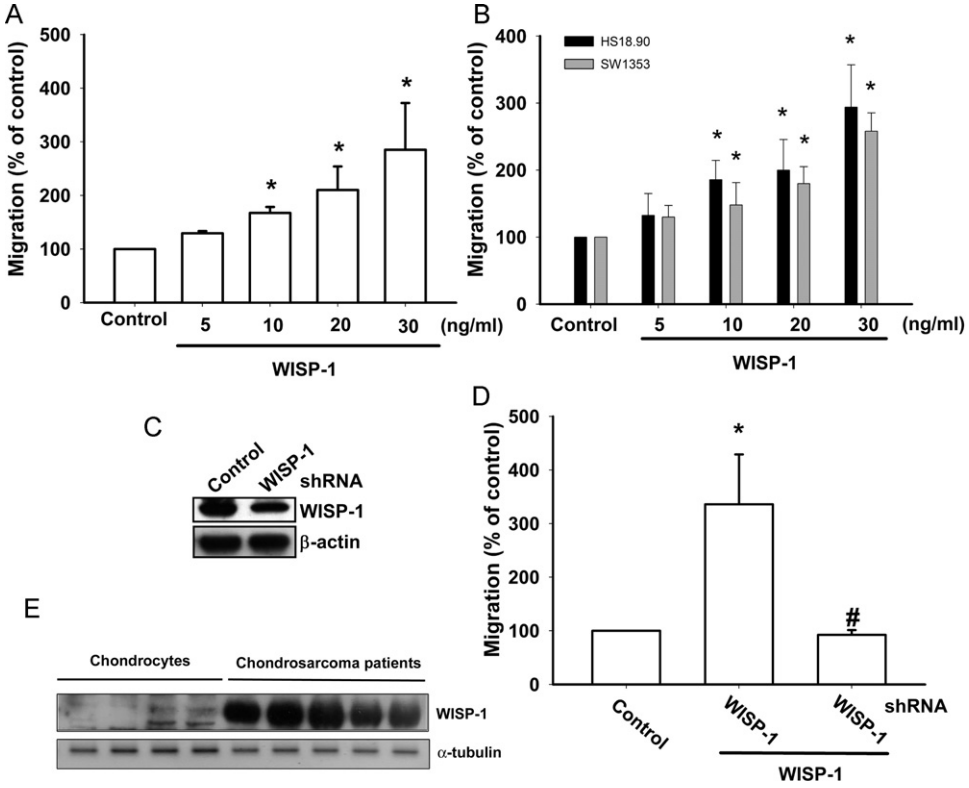

Fig. 1. WISP-1 induced the migration activity of human chondrosarcoma cells JJ012 (A), SH18.90 or SW1353 (B) cells were incubated with WISP-1 for 24 h, and *in vitro* migration was measured with the Transwell after 24 h. (C) JJ012 cells were transfected with WISP-1 shRNA for 24 h, the WISP-1 expression was examined by western blot. (D) JJ012 cells were transfected with WISP-1 shRNA for 24 h followed by stimulation with WISP-1, and *in vitro* migration was measured after 24 h. (E) Total proteins were extracted from chondrosarcoma patients and normal cartilage, and subjected to Western blot analysis for WISP-1. Results are expressed as the mean ± S.E. (*) $p < 0.05$ compared with control.

3. Result

3.1. WISP-1-directed chondrosarcoma cells migration through α5β1 integrin

WISP-1-triggered migration in chondrosarcoma cells was examined by using the Transwell assay with correction of WISP-1-induced proliferation effects on human chondrosarcoma cells [19]. WISP-1 directed human chondrosarcoma cell (JJ012 cell) migration (Fig. 1A). In addition, WISP-1 also dose-dependently directed other human chondrosarcoma cell migration (SW1353 and HS18.90 cells) (Fig. 1B). Transfection of cells with WISP-1 shRNA reduced WISP-1 expression (Fig. 1C). Besides, transfection of cells with WISP-1 shRNA also inhibited WISP-1-mediated migration activity (Fig. 1D). We also examined human chondrosarcoma tissues for the expression of the WISP-1 using Western blot analysis. Expression of protein levels of WISP-1 in human chondrosarcoma tissues were significantly higher than those in normal cartilage (Fig. 1E). Thus, expression of WISP-1 was associated with a metastatic phenotype of chondrosarcoma cells. Previous study has shown CCN affects cells migration through binding to cell surface integrin receptors [18,21]. We therefore, hypothesized that integrins signaling pathway may be involved in WISP-1-directed chondrosarcoma cells migration. Stimulation of cells with WISP-1 induced α5 and β1 but not αv, α2, β3 and β5 integrin expression (Fig. 2A). Flow cytometry analysis also showed

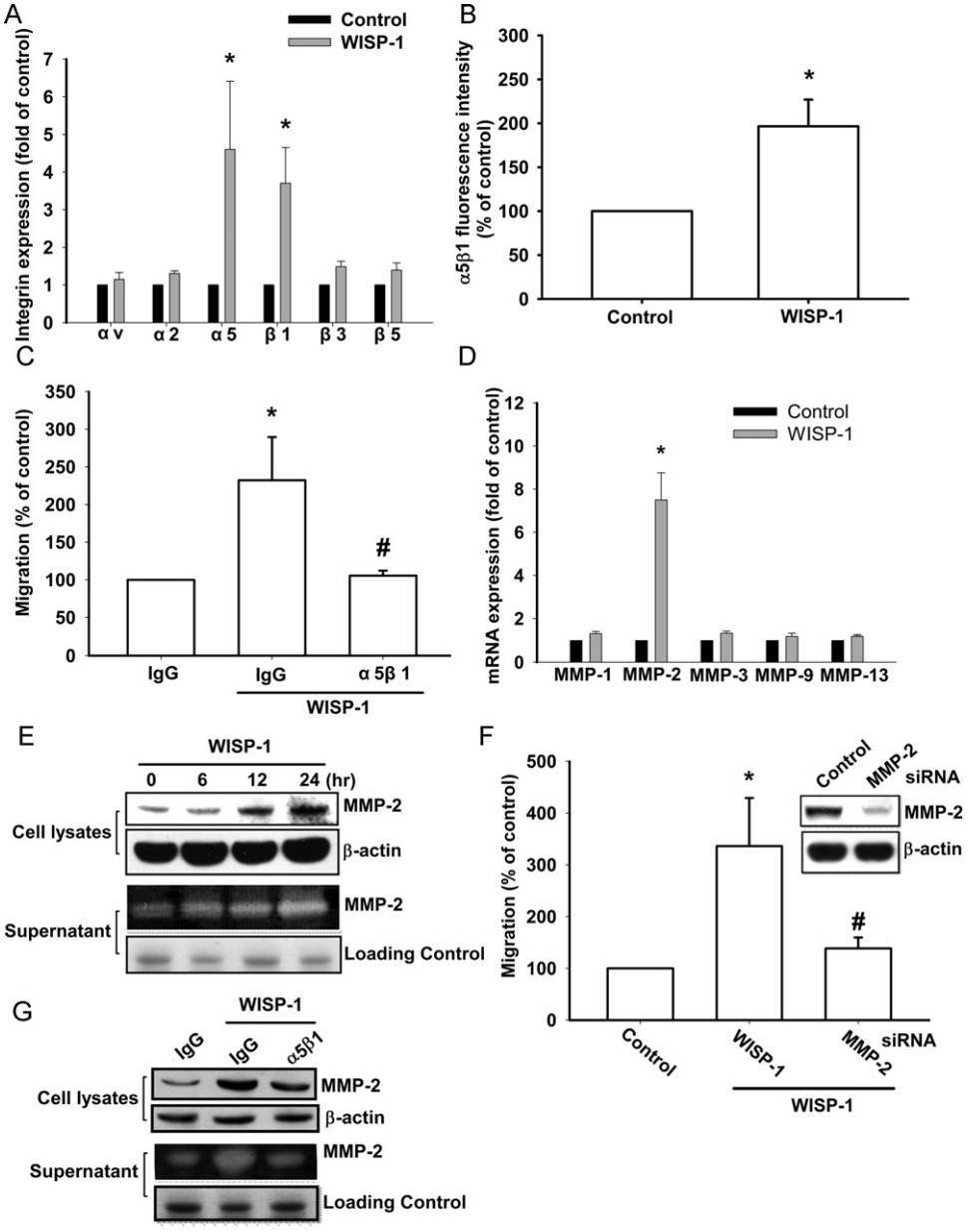

Fig. 2. WISP-1-directed migration activity of human chondrosarcoma cells involves upregulation of MMP-2. (A) JJ012 cells were incubated with WISP-1, and mRNA expression of αv, α2, α5, β1, β3 and β5 integrin was determined using qPCR. (B) JJ012 cells were incubated with WISP-1, and the cell surface expression of α5β1 integrin was determined using flow cytometry. (C) JJ012 cells were pretreated with α5β1 monoclonal antibody for 30 min followed by stimulation with WISP-1. The *in vitro* migration activity measured after 24 h. (D) JJ012 cells were incubated with WISP-1 for 24 h, the mRNA level of MMP-1, -2, -3, -9 and -13 was determined using qPCR. (E) JJ012 cells were incubated with WISP-1 for indicated time intervals. The cultured medium and cell lysates were then collected. The protein level of MMP-2 in cell lysates and enzyme activity of MMP-2 in supernatant were examined by Western blot and zymography. (F) JJ012 cells were transfected with MMP-2 siRNA for 24 h followed by stimulation with WISP-1, and *in vitro* migration was measured after 24 h. (G) J012 cells were pretreated with α5β1 monoclonal antibody for 30 min followed by stimulation with WISP-1 for 24 h. The protein level of MMP-2 in cell lysates and enzyme activity of MMP-2 in supernatant were examined by Western blot and zymography. Results are expressed as the mean ± S.E. (*) $p < 0.05$ compared with control; (#) $p < 0.05$ compared with WISP-1-treated group.

that WISP-1-induced the cell surface expression of α5β1 integrin (Fig. 2B). Pretreatment of cells for 30 min with anti-α5β1 monoclonal antibody (mAb) (10 μg/ml) markedly inhibited the WISP-1-induced cell migration (Fig. 2C). These data suggest that WISP-1-induced cancer migration may occur via α5β1 integrin receptor.

3.2. Involvement of MMP-2 in the WISP-1-directed cell migration of chondrosarcoma

Previous studies have shown a significant expression of MMP-1, -2, -3, -9 and -13 in human chondrosarcoma cells [8,9]. We therefore, hypothesized that any of these MMPs may be involved in WISP-1-directed chondrosarcoma migration. Treatment of cells with WISP-1 induced the expression of MMP-2 but not other MMPs by using qPCR (Fig. 2D). Furthermore, WISP-1 further increased protein expression of MMP-2 in JJ012 cells (Fig. 2E). MMP-2 expression was also increased in the supernatant, and its enzyme activity was up-regulated (Fig. 2E). JJ012 cells were transfected with MMP-2 or control siRNA for 24 h, and the Western blot analysis showed that the expression of protein levels of MMP-2 was suppressed by transfection with MMP-2 siRNA (Fig. 2F). Transfection of cells with MMP-2 siRNA reduced the WISP-1-increased cells migration (Fig. 2F). On the other hand, MMP-2 protein expression and enzyme activity were abolished by α5β1 integrin mAb (Fig. 2G), confirming the involvement of WISP-1 in MMP-2 regulation.

3.3. FAK, MEK and ERK signaling pathways are involved in the WISP-1-mediated cells migration of chondrosarcoma cells

FAK has been shown to be capable of regulating integrin-mediated signaling [22]. 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 WISP-1 stimulation. Transfection of cells with FAK(Y397F) mutant reduced the WISP-1-mediated cells migration and MMP-2 expression (Fig. 3B and C). On the other hand, transfection of cells with FAK siRNA also reduced the WISP-1-induced migration and MMP-2 expression (Fig. 3B and C). MEK/ERK signaling pathway can be activated by a variety of growth factors, such as insulin and growth factors [23]. We then examined whether WISP-1 stimulation also enhances the activation of the MEK/ERK pathway. Stimulation of cells with WISP-1 induced MEK and ERK phosphorylation (Fig. 4A). WISP-1-induced the 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 WISP-1-increased MMP-2 expression and activity (Fig. 4D and E). Transfection of cells with MEK1 or ERK2 mutant reduced the WISP-1-mediated cell migration and MMP-2 expression (Fig. 4C and D). Furthermore, WISP-1-induced MEK and ERK phosphorylation were markedly inhibited if cells were pretreated for 30 min with α5β1 mAb or transfection for 24 h with FAK(Y397F) mutant (Fig. 4F). Taken together, these results indicate that the α5β1 integrin/FAK/MEK and ERK pathway is involved in WISP-1-induced migration activity and MMP-2 up-regulation in human chondrosarcoma cells.

3.4. Involvement of NF-κB in WISP-1-induced cells migration and MMP-2 expression

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human chondrosarcoma cells [24]. To examine whether NF-κB activation is involved in the signal transduction pathway caused by WISP-1 that leads to cell migration and MMP-2 expression, the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was used. Fig. 5A–C shows that PDTC inhibited the enhancement of cell migration and MMP-2 expression induced by WISP-1. Furthermore, pretreatment of chondrosarcoma cells with an IκB protease inhibitor [L-1-tosylamido-2-phenylenylethyl chloromethyl ketone (TPCK)] antagonized the potentiating action of cell migration and MMP-2 expression (Fig. 6A–C). To directly determine NF-κB activation after WISP-1 treatment, chondrosarcoma cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 5D, WISP-1 treatment of chondrosarcoma cells for 24 h caused increase in κB-luciferase activity dose-dependently. These results indicated that NF-κB activation is important for WISP-1-induced

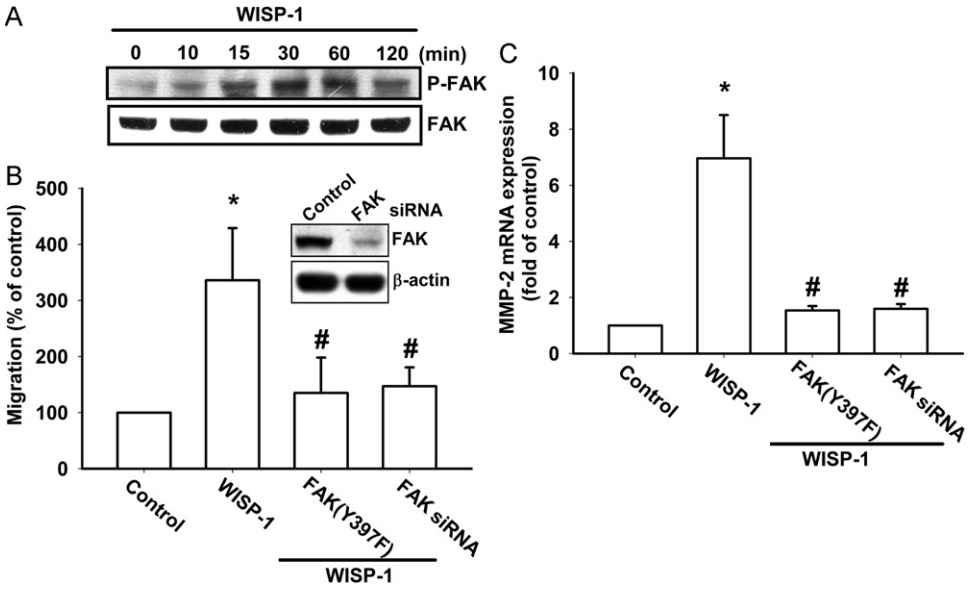

Fig. 3. Involvement of FAK signaling pathway in response to WISP-1 in chondrosarcoma cells. (A) JJ012 cells were incubated with WISP-1 for indicated time intervals, and p-FAK expression was determined by Western blot analysis. (B; upper panel) JJ012 cells were transfected with FAK siRNA for 24 h, the FAK expression was examined by western blot. (B) JJ012 cells were transfected with mutant or siRNA of FAK for 24 h followed by stimulation with WISP-1, and *in vitro* migration was measured with the Transwell after 24 h. (C) JJ012 cells were transfected with mutant or siRNA of FAK for 24 h followed by stimulation with WISP-1, and mRNA level of MMP-2 was measured after 24 h. Results are expressed as the mean ± S.E. (*) $p < 0.05$ compared with control; (#) $p < 0.05$ compared with WISP-1-treated group.

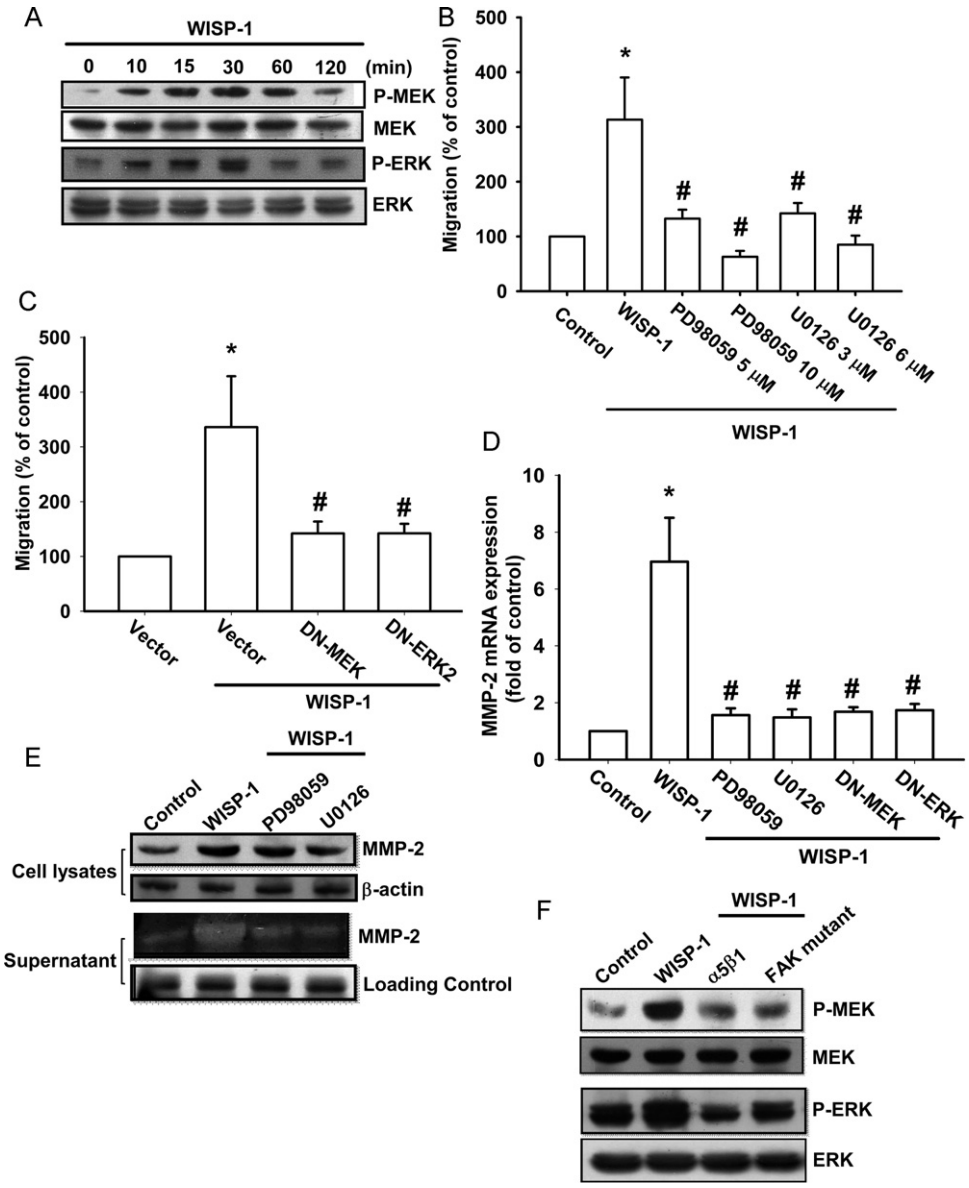

Fig. 4. MEK/ERK pathway is involved in WISP-1-mediated migration in human chondrosarcoma cells. (A) JJ012 cells were incubated with WISP-1 for indicated time intervals, and p-MEK and p-ERK expression was determined by Western blot analysis. (B) JJ012 cells were pretreated with PD98059 and U0126 for 30 min followed by stimulation with WISP-1 for 24 h, and *in vitro* migration was measured with the Transwell after 24 h. (C) JJ012 cells were transfected with mutant of MEK or ERK2 for 24 h followed by stimulation with WISP-1, and *in vitro* migration was measured with the Transwell after 24 h. (D) JJ012 cells were pretreated for 30 min with U0126 and PD98059 or transfected with dominant negative (DN) mutant of MEK1 for 24 h followed by stimulation with WISP-1, and the mRNA of MMP-2 was measured by using qPCR. (E) JJ012 cells were pretreated for 30 min with U0126 and PD98059 followed by stimulation with WISP-1 for 24 h. The protein level of MMP-2 in cell lysates and enzyme activity of MMP-2 in supernatant were examined by Western blot and zymography. (F) JJ012 cells were pretreated for 30 min with α5β1 mAb or transfected with FAK mutant for 24 h followed by stimulation with WISP-1 for 30 min, and the p-MEK and p-ERK was examined by Western blot. Results are expressed as the mean ± S.E. (*) $p < 0.05$ compared with control; (#) $p < 0.05$ compared with WISP-1-treated group.

cancer cell migration and the expression of MMP-2. We further examined the upstream molecules involved in WISP-1-induced NF-κB activation. Stimulation of cells with WISP-1 induced IKKα/β phosphorylation in a time-dependent manner (Fig. 6A). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the WISP-1-induced cell migration and MMP-2 expression (Fig. 6B and C). These data suggest that IKKα/β activation is involved in WISP-1-induced the migration activity of human chondrosarcoma cells. Transfection of cells with ERK2 mutant reduced WISP-1-induced IKK phosphorylation (Fig. 6D). Therefore, ERK2 is upstream molecule in WISP-1-mediated IKK activation. Treatment with chondrosarcoma cells with WISP-1 also caused IκBα phosphorylation in a time-dependent manner (Fig. 6A). Previous studies showed that p65 Ser^{536} phosphorylation increases NF-κB transactivation [25], and the antibody specific against phosphorylated p65 Ser^{536} was used to examine p65 phosphorylation. Treatment of chondrosarcoma cells with WISP-1 for various time intervals resulted in p65 Ser^{536} phosphorylation (Fig. 6A). We next investigated whether p65 binds to the NF-κB element on the MMP-2 promoter after WISP-1 stimulation. The in vivo recruitment of p65 to the MMP-2 promoter (−673 to −517) was assessed by the chromatin immunoprecipitation assay [26]. In vivo binding of p65 to the NF-κB element of the MMP-2 promoter occurred after WISP-1 stimulation (Fig. 6F). Binding of p65 to the NF-κB element and p65 phosphorylation by WISP-1 was attenuated by PD98059 and U0126 (Fig. 6E and F). In addition, the WISP-1-induced increase in κB-luciferase activity was also inhibited by treatment with PD98059, U0126, PDTC and TPCK (Fig. 6G). Co-transfection with

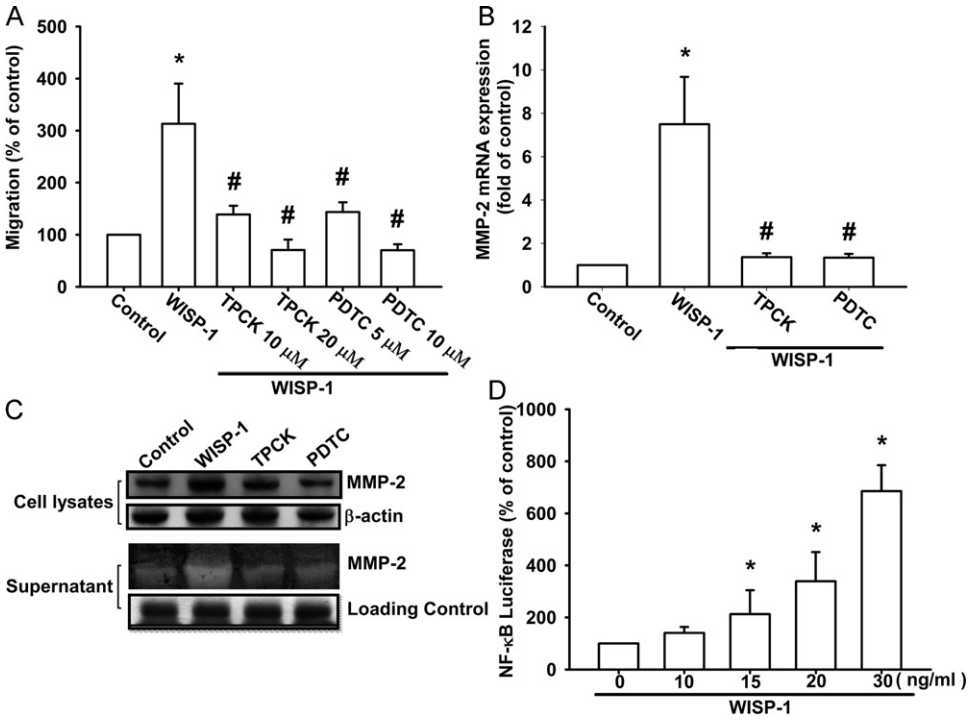

Fig. 5. WISP-1 induces cells migration and MMP-2 up-regulation through NF-κB. (A) JJ012 cells were pretreated for 30 min with PDTC or TPCK followed by stimulation with WISP-1, and *in vitro* migration was measured with the Transwell after 24 h. (B) JJ012 cells were pretreated for 30 min with PDTC or TPCK followed by stimulation with WISP-1, and mRNA level of MMP-2 was measured after 24 h. (C) JJ012 cells were pretreated for 30 min with PDTC or TPCK followed by stimulation with WISP-1. The protein level of MMP-2 in cell lysates and enzyme activity of MMP-2 in supernatant were examined by Western blot and zymography. (D) Cells were treated with WISP-1 for 24 h. The NF-κB luciferase activity was measured, and the results were normalized to the β-galactosidase activity. Results are expressed as the mean ± S.E. (*) $p < 0.05$ compared with control; (#) $p < 0.05$ compared with WISP-1-treated group.

FAK, MEK, ERK, IKKα and IKKβ mutants or FAK siRNA also reduced WISP-1-increased NF-κB luciferase activity (Fig. 6H) Taken together, these data suggest that activation of α5β1 integrin receptor, FAK, MEK and ERK are required for WISP-1-induced NF-κB activation in human chondrosarcoma cells.

3.5. Decrease cell motility in WISP-1-shRNA over-expression clone

To further confirm the WISP-1 mediated cell migration and MMP-2 expression in human chondrosarcoma cells, the WISP-1-shRNA expression cells was established. The WISP-1 expression level in stable transfectants was compared using Western blotting. The expression of WISP-1 was dramatically inhibited by WISP-1-shRNA orientation in JJ012/WISP-1 cells (Fig. 7B). Since, WISP-1 has already been reported to act as a mitogen in osteoblasts [27], we sought 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 (data not shown), suggesting that WISP-1 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 WISP-1 expression inhibited the migratory ability by approximately 60% in JJ012 cells (Fig. 7A). In addition, knockdown WISP-1 also reduced MMP-2 expression in JJ012 cells (Fig. 7B). Therefore, human chondrosarcoma cells with a higher tendency to migrate expressed more WISP-1 and MMP-2.

4. Discussion

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which cause dramatic increases in long-term survival with the advent of systemic chemotherapy, chondrosarcoma continues to have a poor prognosis due to absence of an effective adjuvant therapy [28]. 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 [28]. Therefore, it is important to develop effective adjuvant therapy for preventing chondrosarcoma metastasis. We hypothesized that WISP-1 would help to direct the metastasis of chondrosarcoma cells. We found that WISP-1 increased the migration of chondrosarcoma cells. One of the mechanisms underlying WISP-1directed migration was transcriptional up-regulation of MMP-2 and activation of α5β1 integrin, FAK, MEK, ERK and NF-κB pathways. CCN family, comprising six members (Cyr61, CTGF, Nov, WISP-1, WISP-2, WISP-3), is involved in the stimulation of cell proliferation, migration, adhesion, angiogenesis, and tumorigenesis [10,29]. Several studies have shown that Cyr61 and CTGF affects the migratory potential of chondrosarcoma cells [8,18]. Cyr61 can regulate cell proliferation and also regulate the apoptosis process in opposite directions [30]. The Wnt induced secretory proteins WISPs manifest contrasing effects. WISP-1 is believed to stimulate cell motility and promote aggressive behavior whilst WISP-2 can function as a tumor suppressor [31]. However, the effect of WISP-1 on migration activity in chondrosarcoma is mostly unknown. Using Western blot analysis, we found that the expression of protein levels of WISP-1 in chondrosarcoma patients were significantly higher than those in normal cartilage. In addition, exogenous WISP-1 increased migration of chondrosarcoma. Moreover, over-expression of WISP-1 shRNA inhibited the migratory ability by approximately 60% in JJ012 cells. Our data provided the evidence that the expression of WISP-1 is associated with a metastatic phenotype of chondrosarcoma cells.

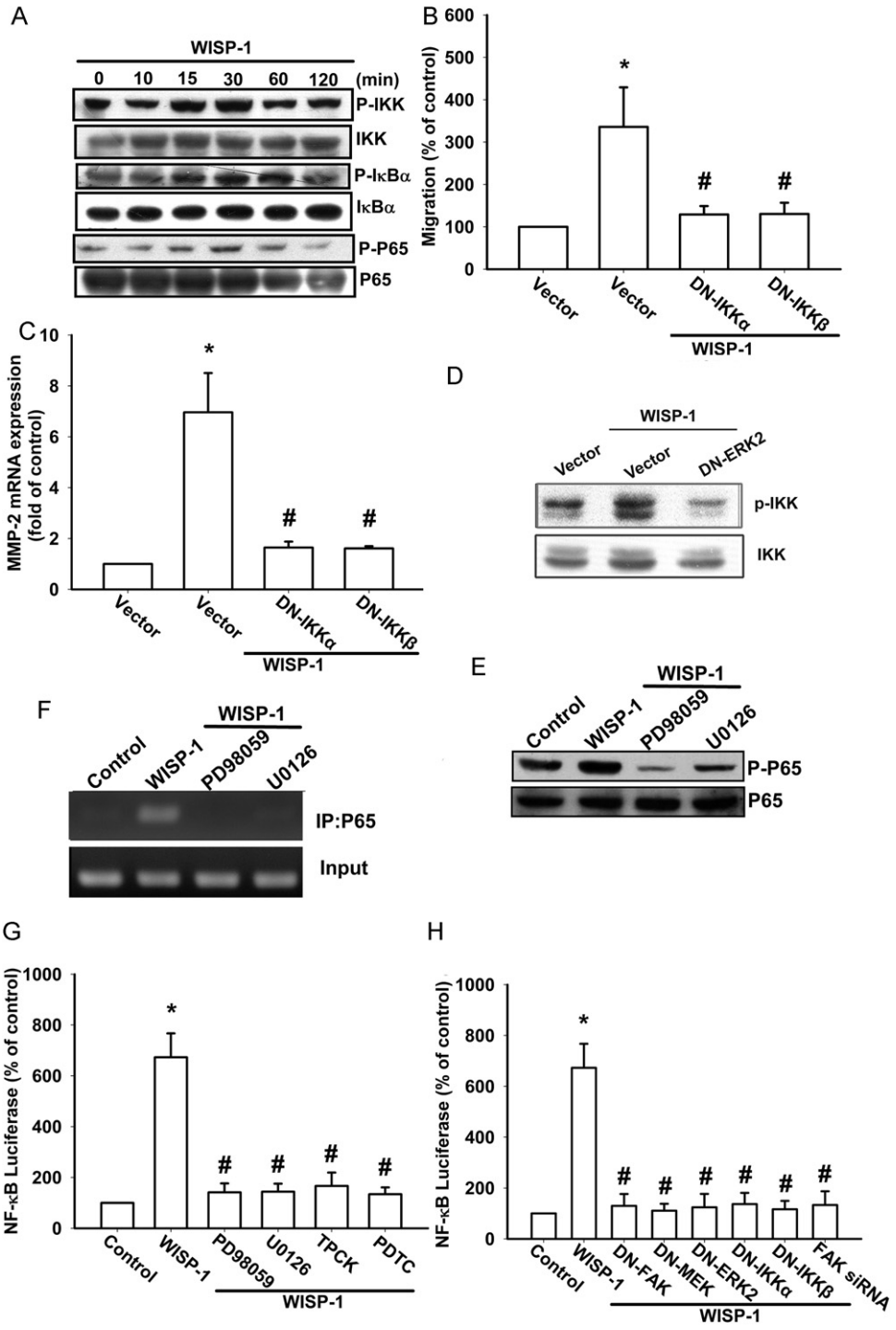

Fig. 6. FAK/MEK/ERK pathway is involved in WISP-1-mediated NF-κB activation and MMP-2 expression. (A) JJ012 cells were incubated with WISP-1 for indicated time intervals, and IKK, IκBα and p65 phophorylation was examined by Western blot analysis. JJ012 cells were transfected with IKKα or IKKβ mutant for 24 h followed by stimulation with WISP-1, and *in vitro* migration and mRNA expression of MMP-2 measured with the Transwell (B) and qPCR (C) after 24 h. (D) JJ012 cells were transfected with ERK2 mutant for 24 h followed by stimulation with WISP-1 for 30 min, and p-IKK expression was examined by Western blot. (E) JJ012 cells were pretreated with PD98059 and U0126 for 30 min. Then they were followed by stimulation with WISP-1 for 30 min, and p65 phosphorylation was examined. (F) JJ012 cells were pretreated with PD98059 and U0126 then stimulated with WISP-1 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). JJ012 cells were pretreated with U0126, PD98059, PDTC and TPCK for 30 min (G) or transfected with mutant of FAK, MEK, ERK, IKKα IKKβ (H) before exposure to WISP-1. 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 WISP-1-treated group.

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 [5,32]. It has been reported that MMP plays important role in CCN-induced migration in human cancer cells [8,33]. In this study, we found that WISP-1 induced MMP-2 expression and secretion in human chondrosarcoma cells without significantly changing the expression of MMP-1, -3, -9 and -13

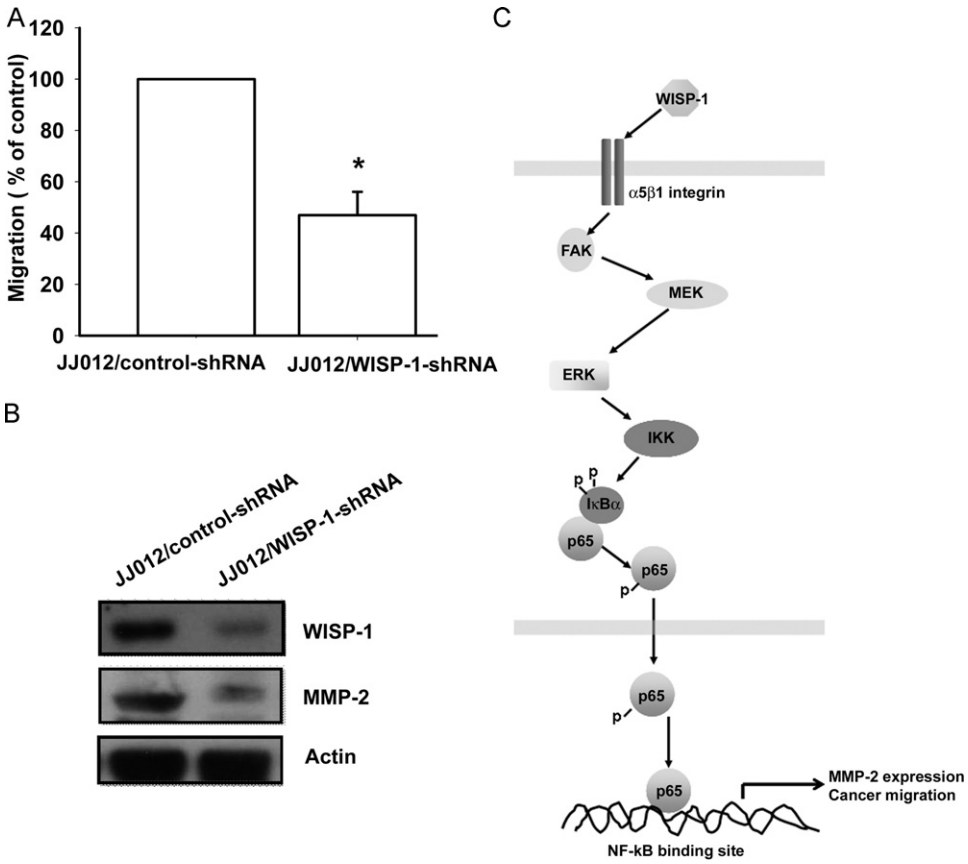

Fig. 7. Knockdown of WISP-1 inhibited the migratory ability in chondrosarcoma cells. (A) The *in vitro* migration activity of JJ012/control-shRNA and JJ012/WISP-1-shRNA cells was measured with the Transwell. (B) The protein levels of WISP-1 and MMP-2 in JJ012/control-shRNA and JJ012/WISP-1-shRNA cells was examined by Western blot analysis. Results are expressed as the mean ± S.E. (*) $p < 0.05$ compared with control. (C) Schematic presentation of the signaling pathways involved in WISP-1-induced migration and MMP-2 expression of chondrosarcoma cells. WISP-1 activates α5β1 integrin, FAK, MEK and ERK pathways, which in turn induces NF-κB activation, which lead to MMP-2 expression and increases the migration of human chondrosarcoma cells.

mRNAs. In addition, the inhibition of WISP-1-enhanced MMP-2 protein expression with siRNA significantly suppressed WISP-1-induced migration. Therefore, MMP-2 may be the WISP-1-responsive mediator, and it causes the degradation of ECM may lead to subsequent cancer migration and metastasis.

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 and differentiation [34]. Previous study has shown that CCNs affects cells migration through binding to cell surface integrin receptors [35]. Here we used integrin antibody to determine the role of integrin and found that α5β1 mAb inhibited WISP-1-induced cell migration, indicating the possible involvement of α5β1 integrin activation in WISP-1-induced migration in chondrosarcoma cells. FAK, a potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated signaling [36]. We demonstrate that WISP-1 increased phosphorylation of tyrosine 397 of FAK. Furthermore, the FAK(Y397F) mutant and FAK siRNA antagonized the WISP-1-mediated potentiation of migration activity, suggesting that FAK activation is an obligatory event in WISP-1-induced migration in these cells. MEK/ERK also plays a critical role in integrin signaling [37]. We found that PD98059 and U0126 (MEK inhibitors) also inhibited WISP-1-induced migration. Stimulation of cells with WISP-1 increased phosphorylation of MEK and ERK. The MEK inhibitors and mutant also reduced the WISP-1-mediated MMP-2 expression and activity. Furthermore, WISP-1-induced MEK and ERK activation was antagonized by α5β1 mAb and FAK(Y397F) mutant, indicating that the α5β1 integrin and FAK occur as the upstream molecules involved in WISP-1-induced activation of MEK/ERK.

The prognosis of patients with chondrosarcoma distant metastasis is generally considered very poor; hence, preventing human chondrosarcoma metastasis is an important issue nowadays. Our study observes that WISP-1 increases the activity of MMP-2 via the α5β1 integrin, FAK, MEK, ERK, IKKα/β and NF-κB-dependent pathway and to enhance migration of human chondrosarcoma cells (Fig. 7C). Furthermore, the discovery of WISP-1-mediated signaling pathway helps us understand the mechanism of human chondrosarcoma metastasis and may lead us to develop effective therapy in the future.

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