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Interleukin-11 Increases Cell Motility and Up-Regulates Intercellular Adhesion Molecule-1 Expression in Human Chondrosarcoma Cells

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ABSTRACT

Interleukin-11 (IL-11) was originally identified as the cytokine that could induce the proliferation of human cells. Recent studies have shown that IL-11 plays a critical role in tumor growth, angiogenesis, and metastasis. Chondrosarcoma is a type of highly malignant tumor with a potent capacity to invade locally and cause distant metastasis. However, the effects of IL-11 on human chondrosarcoma cells are largely unknown. Here, we found that IL-11 increased the migration and expression of intercellular adhesion molecule-1 (ICAM)-1 in human chondrosarcoma cells. We also found that human chondrosarcoma tissues had significant expression of the IL-11 which was higher than that in primary chondrocytes. The phosphatidylinositol 3-kinase (PI3K), Akt, and NF-κB pathways were activated by IL-11 treatment, and the IL-11-induced expression of ICAM-1 and migration activity were inhibited by the specific inhibitors and mutant forms of PI3K, Akt, and NF-κB cascades. Taken together, our results indicate that IL-11 enhanced the migration of the chondrosarcoma cells by increasing ICAM-1 expression through the IL-11Rα receptor, PI3K, Akt, and NF-κB signal transduction pathway. J. Cell. Biochem. 113: 3353–3362, 2012.

KEY WORDS: IL-11; CHONDROSARCOMA; ICAM-1; MIGRATION

Chondrosarcoma is a malignant primary bone tumor associated with a poor response to the current 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. Because of the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis; therefore, novel and adequate therapy strategies are necessary [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, 2010]. 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 [Zimmerman and Blanco, 2008; Lawson and Wolf, 2009]. The extracellular domain of ICAM-1 is essential for the transendothelial migration of leukocytes from the capillary bed into

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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.

Interleukin-11 (IL-11) was originally identified as the cytokine that could induce the proliferation of IL-6-dependent T1165 cells [Paul et al., 1990]. IL-11 had various bio-functions, such as being an inducer of acute-phase protein, a thrombopoietic factor, a competence factor for hematopoietic stem cells, and an inducer of antibody secretion [Girasole et al., 1994]. IL-11 transduces its signal through gp130 (common co-receptor for this family) and the interleukin-11 receptor α (IL-11Rα; IL-11-specific receptor) by forming a hexameric complex involving two molecules each of IL-11, IL-11Rα, and gp130 [Matadeen et al., 2007]. Its signal is mediated in different cell types through activation of Jak1/2 or phosphatidylinositol 3′-kinase (PI3K)/Akt pathway [Dahmen et al., 1998; Fischer and Hilfiker-Kleiner, 2007]. Recent studies have suggested that IL-11 is involved in progression of carcinomas, including gastric and colorectal adenocarcinoma [Yoshizaki et al., 2006; Nakayama et al., 2007].

Previous studies have shown that IL-11 modulates cell migration and invasion in human cancer cells [Nakayama et al., 2007; Suman et al., 2009]. However, the effects of IL-11 on ICAM-1 expression and migration activity in human chondrosarcoma cells are mostly unknown. In this study, the effects of IL-11 on ICAM-1 expression and the migration of human chondrosarcoma cells were explored. In addition, the role of the PI3K, Akt, and NF-κB signaling pathways in the IL-11-mediated increase of ICAM-1 expression and cell migration by IL-11 were investigated.

MATERIALS AND METHODS

MATERIALS

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin (sc-81178), p85 (sc-376112), p-p85(Tyr^{508}) (sc-12929), Akt(sc-55523), p-Akt(Ser^{473}) (sc-7985-R), p-IKK(Thr^{23}) (sc-21660), IKK(sc-7607), p-p65(Ser^{536}) (sc-33020), p65(sc-71676) and the siRNAs against ICAM-1(sc-29354), IL-11Rα(sc-35647), gp130(sc-29333), 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) and control shRNA (sc-108060) and IL-11 shRNA (sc-39636-SH) plasmids were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TPCK and PDTC were purchased from Calbiochem (San Diego, CA). The recombinant human IL-11 was purchased from PeproTech (Rocky Hill, NJ). Mouse monoclonal antibody specific for ICAM-1 and IL-11 were purchased from R&D Systems (Minneapolis, MN). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA). The p85α and Akt (Akt K179A) dominant-negative mutants were gifts from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The pSV-β-galactosidase vector and the luciferase assay kit were purchased from Promega (Madison, MA). All that other chemicals were purchased 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). The human chondrosarcoma cell line (SW1353) was obtained from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium/α-minimum essential medium supplemented with 20 mM HEPES, 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2.

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 China Medical University Hospital. Tissue specimens were ground and then sonicated in a lysis buffer. The protein level was analyzed using Western blot analysis.

MIGRATION ASSAY

The migration assay was performed by using Transwell chambers (pore size, 8 μm; Costar, NY) in 24-well dishes. Before the migration assay, the cells were pretreated for 30 min with different concentrations of inhibitors, including the Ly294002, Akt inhibitor, 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 IL-11 was placed in the lower chamber. The plates were incubated for 24 h at 37°C in 5% CO_2, and then the cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. The 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 [Chen et al., 2011].

WOUND-HEALING MIGRATION ASSAY

For the wound-healing migration assay, cells were seeded on 12-well plates at a density of 1×10^5 cells/well in culture medium. At 24 h after seeding, the confluent monolayer of culture was scratched with a fine pipette tip. Images of migratory cells from the boundary were observed and acquired at 0 and 24 h with a digital camera and a light microscope (Olympus, Japan). Number of migratory cells was counted from the resulting four phase images for each point and then averaged for each experimental condition. The data presented are generated from three separate assays.

QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from chondrosarcoma 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 [Hsieh et al., 2003; Wang et al., 2003]. The quantitative real-time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). Two microliter cDNA templates were added per 25-μl reaction with sequence-specific primers and Taqman® probes. The sequences for all target gene primers and probes were purchased commercially (β-actin was used as internal control; Applied Biosystems). The qPCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions involved 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 the target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T). Relative gene expression was determined by the $\Delta\Delta C_t$ method, where C_t = threshold cycle.

WESTERN BLOT ANALYSIS

Cellular lysates were prepared as described previously [Huang et al., 2003; Tseng et al., 2003]. 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 Akt, p-Akt, p85, or p-p85 (1:1,000) for 1 h at room temperature. After three washes with PBS, 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 then washed with PBS for three times and visualized by enhanced chemiluminescence using a Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

IMMUNOFLUOROCYTOCHEMISTRY

Cells were cultured in 12-mm coverslips. After treatment with IL-11, cells were fixed with 4% paraformaldehyde at room temperature. Thirty minutes later, 4% non-fat milk in PBS containing 0.5% Triton X-100 was added to the cells. Cells were incubated with rabbit anti-p65 (1:100) for 1 h at room temperature. Cells were then washed again and labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:500; Leinco Technology Inc., St Louis, MO) for 1 h. Finally, cells were washed, mounted, and detected using a Zeiss fluorescence microscope.

SIRNA AND MUTANT TRANSFECTION

Cells were transfected with siRNAs (100 nM) or dominant-negative mutants (0.5 μg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation analysis was performed as described previously [Huang and Chen, 2005]. DNA immunoprecipitated by anti-p65 Ab 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 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].

ESTABLISHMENT OF STABLY TRANSFECTED CELLS

The IL-11 shRNA or control shRNA plasmids were transfected into the cancer cells using the Lipofectamine 2000 transfection reagent. Twenty-four hours after the transfection, stable transfectants were selected in puromycin (Life Technologies) at a concentration of 10 μg/ml. Thereafter, the selection medium was replaced every 3 days. After 2 weeks of selection in puromycin, the clones of the resistant cells were isolated.

STATISTICS

The values given are means ± SEM. Statistical analyses between two samples were 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 significant.

RESULTS

IL-11-DIRECTED CHONDROSARCOMA CELLS MIGRATION VIA THE IL-11Rα RECEPTOR

IL-11 has been reported to stimulate directional migration and invasion of human cancer cells [Nakayama et al., 2007; Suman et al., 2009]. However, little is known about the expression of the IL-11 in human chondrosarcoma cells. We examined human chondrosarcoma patients for expression of the IL-11 using Western blotting and qPCR. Expression of the protein and the mRNA levels of IL-11 in chondrosarcoma patients were significantly higher than in primary chondrocytes (Fig. 1A,B). The qualification data of IL-11 mRNA expression were shown in Figure 1B. We next examined the migratory activity of human chondrosarcoma cells by using the Transwell assay. IL-11-directed human chondrosarcoma cell (JJ012 and SW1353 cells) migration (Fig. 1C). On the other hand, IL-11 also increased wound-healing activity in human chondrosarcoma cells (Fig. 1D). Thus, expression of IL-11 was associated with an invasive and/or metastatic phenotype of chondrosarcoma cells. Previous studies have shown that IL-11 affects cell migration through binding to cell surface IL-11Rα receptor [Yoshizaki et al., 2006; Nakayama et al., 2007]. Transfection of cells with IL-11Rα siRNA reduced IL-11 increased cell migration (Fig. 1E). It has been reported that IL-11 transduces its signal through gp130 and IL-11Rα receptor by forming a hexameric complex [Matadeen et al., 2007]. On the other hand, transfection of cells with gp130 siRNA also blocked IL-11-mediated cell migration (Fig. 1E). These data suggest that IL-11-induced cancer migration may occur via IL-11Rα receptor.

IL-11-DIRECTED CHONDROSARCOMA CELL MIGRATION INVOLVES ICAM-1 UP-REGULATION

ICAM-1 has been reported in cell motility of human chondrosarcoma cells [Fong et al., 2012]. We therefore, hypothesized that ICAM-1 may be involved in IL-11-directed migration of human chondrosarcoma. Treatment of cells with IL-11 increased ICAM-1 mRNA expression in a dose-dependent manner (Fig. 2A). In addition, IL-11 also increased ICAM-1 protein expression time- and dose-dependently (Fig. 2B). Pretreatment of cells with ICAM-1 mAb

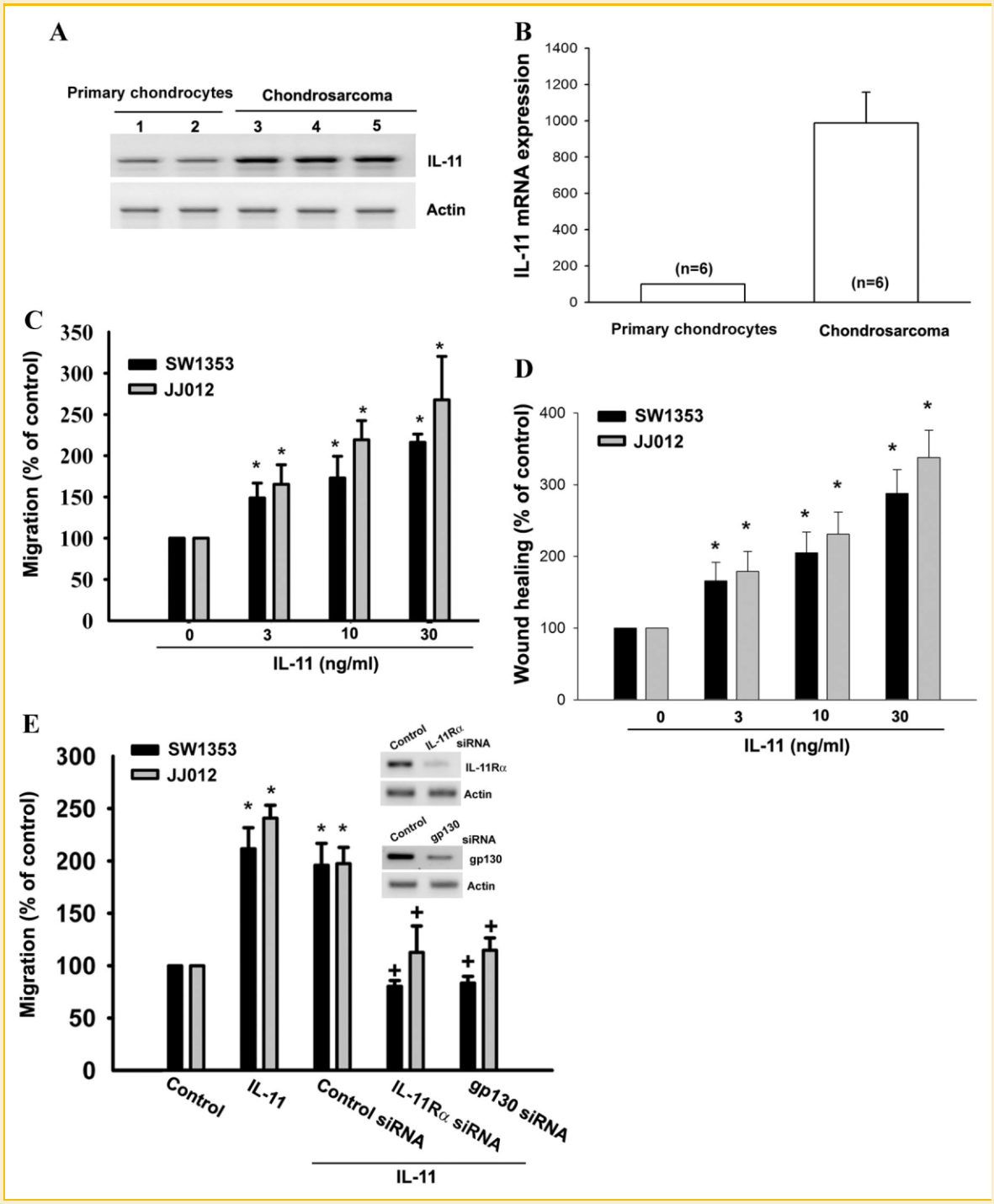

Fig. 1. IL-11-directed migration of human chondrosarcoma cells through IL-11Rα receptor. A: Total proteins were extracted from chondrosarcoma patients and primary chondrocytes, and subjected to Western blot analysis for IL-11. B: Total RNA were extracted from chondrosarcoma patients and primary chondrocytes, and subjected to qPCR analysis for IL-11. C: Cells were incubated with IL-11 for 24 h, and in vitro migration was measured by Transwell after 24 h. D: Cells were treated with IL-11 for 24 h, the wound-scratching assay was performed. E: Cells were transfected with IL-11Rα, gp130 or control siRNA for 24 h followed by stimulation with IL-11. The in vitro migration was examined by Transwell. Results are expressed as the mean ± SEM. $^{*}P < 0.05$ compared with control; $+P < 0.05$ compared with IL-11-treated group.

markedly reduced IL-11-enhanced cell migration (Fig. 2C). Furthermore, transfection of cells with ICAM-1 siRNA also inhibited the IL-11-induced cell migration (Fig. 2C). These data suggest that IL-11-induced cancer migration was enabled by ICAM-1 up-regulation.

PI3K AND AKT SIGNALING PATHWAYS ARE INVOLVED IN IL-11-MEDIATED ICAM-1 UP-REGULATION AND CELL MIGRATION OF CHONDROSARCOMA CELLS

The PI3K/Akt signaling pathway can be activated by a variety of factors including insulin and different growth factors [Horowitz

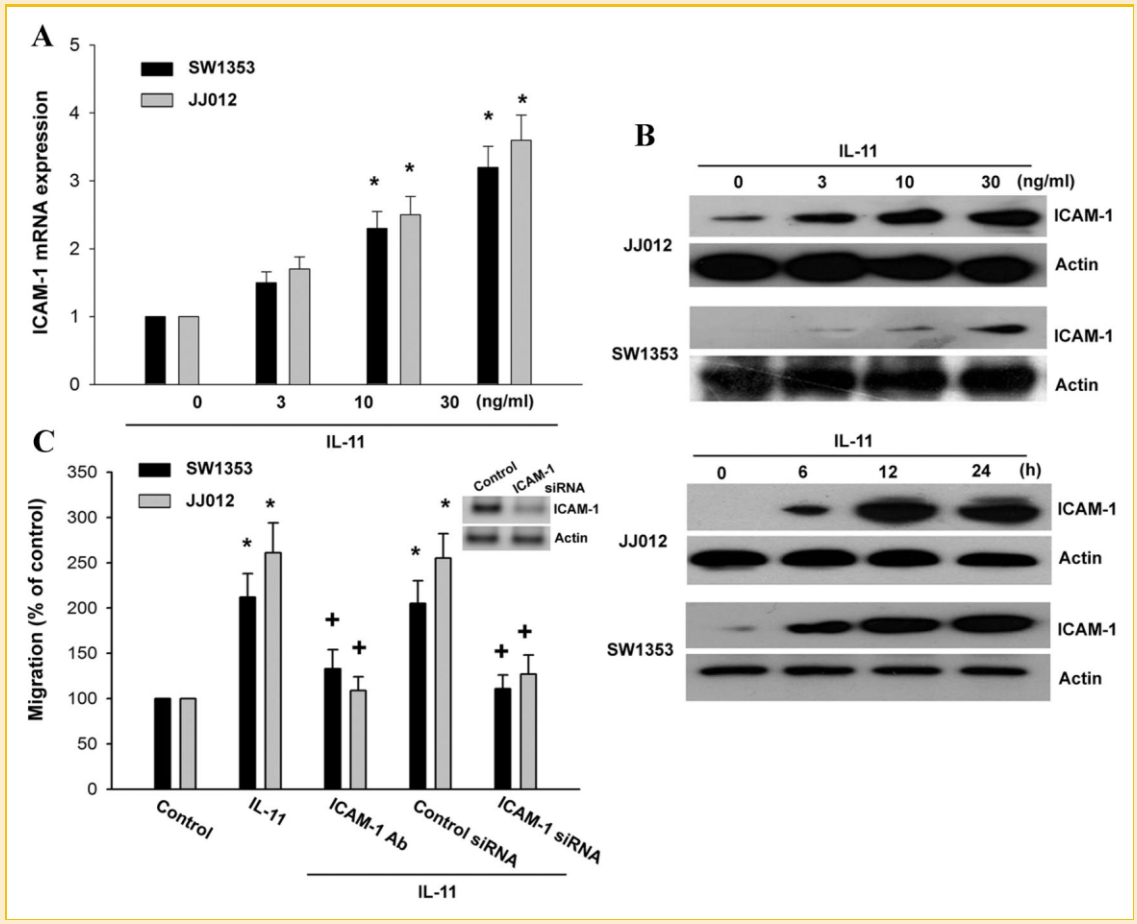

Fig. 2. IL-11 increased cell migration in chondrosarcoma cells involves up-regulation of ICAM-1. A: Cells were incubated with IL-11 (30 ng/ml) for indicated time intervals, the mRNA expression of ICAM-1 were examined by qPCR. B: Cells were incubated with IL-11 (1–30 ng/ml) for 24 h or IL-11 (30 ng/ml) for indicated time intervals the protein expression of ICAM-1 were examined by Western blotting ($n = 5$). C: Cells were pretreated for 30 min with ICAM-1 Ab (3 μg/ml) or transfected with ICAM-1 siRNA for 24 h followed by stimulation with IL-11. The in vitro migration was examined by Transwell. Results are expressed as the mean ± SEM. $^{*}P < 0.05$ compared with control; $+P < 0.05$ compared with IL-11-treated group.

et al., 2004; Bibollet-Bahena and Almazan, 2009; Chuang et al., 2009]. We examined whether IL-11 stimulation also enhanced PI3K activation. Stimulation of JJ012 cells with IL-11 led to a significant increase in phosphorylation of p85 (Fig. 3A). IL-11-induced migration and ICAM-1 expression of chondrosarcoma cells were greatly reduced by treatment with PI3K inhibitors Ly294002 and wortmannin (Fig. 3B–D). In addition, transfection of cells with a p85 mutant also inhibited IL-11-induced migration and ICAM-1 expression of chondrosarcoma cells (Fig. 3B–D). Akt phosphorylation at Ser473 by a PI3K-dependent signaling pathway causes enzymatic activation [Qiao et al., 2008]. To examine the crucial role of PI3K/Akt signaling in cancer migration and ICAM-1 up-regulation, we determined Akt Ser473 phosphorylation in response to IL-11 treatment. As shown in Figure 4A, treatment of JJ012 cells with IL-11 resulted in time-dependent phosphorylation of Akt Ser473. Pretreatment of cells with Akt inhibitor antagonized IL-11-induced migration and ICAM-1 expression of chondrosarcoma cells (Fig. 4B–D). In addition, the Akt mutant also reduced IL-11-mediated cell migration and ICAM-1 up-regulation (Fig. 4B–D). Based on these results, it appears that IL-11 acts through the PI3K and Akt-dependent signaling pathway to enhance ICAM-1 expression and cell migration in human chondrosarcoma cells.

INVOLVEMENT OF NF-κB IN IL-11-INDUCED CELL MIGRATION AND ICAM-1 EXPRESSION

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human chondrosarcoma cells [Su et al., 2009]. To examine whether NF-κB activation is involved in the signal transduction pathway leading to migration and ICAM-1 expression caused by IL-11, the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was used. Figure 5A–C shows that PDTC inhibited the enhancement of migration and ICAM-1 expression induced by IL-11. Furthermore, pretreatment of cells with TPCK (a non-specific protease inhibitor which can inhibit NF-κB [Ha et al., 2009]) also antagonized the potentiating action of IL-11 (Fig. 5A–C). These results indicate that NF-κB activation is important for IL-11-induced cancer cell migration and ICAM-1 expression.

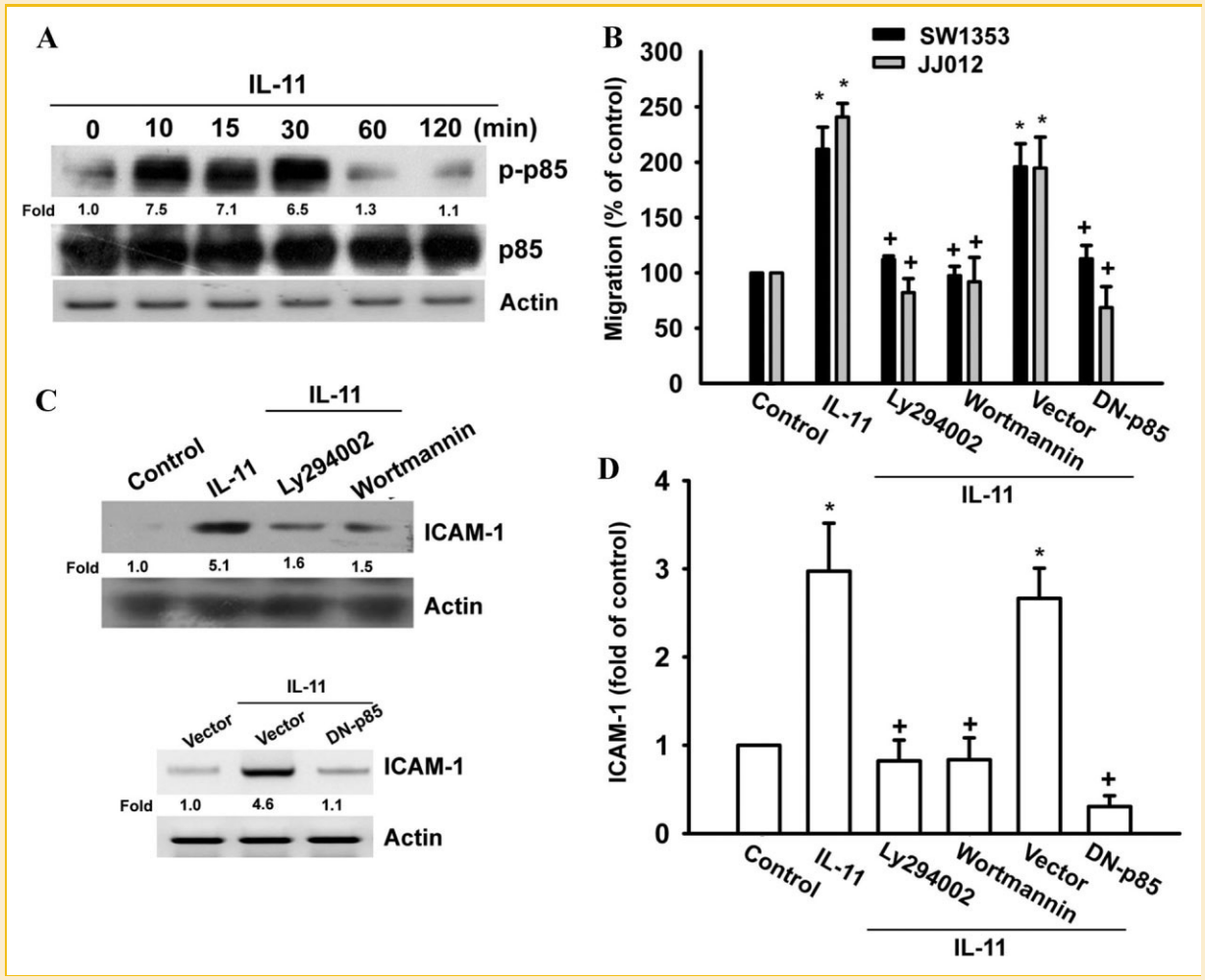

Fig. 3. PI3K is involved in IL-11-induced migration and ICAM-1 up-regulation in human chondrosarcoma cells. A: JJ012 cells were incubated with IL-11 (30 ng/ml) for indicated time intervals, and p-p85 was examined by Western blotting ($n = 5$). B: Cells were pretreated for 30 min with Ly294002 (10 μM) and wortmannin (1 μM) or transfected with dominant negative (DN) mutant of p85 for 24 h followed by stimulation with IL-11. The in vitro migration was examined by Transwell. C,D: JJ012 cells were pretreated for 30 min with Ly294002 and wortmannin or transfected with p85 mutant for 24 h followed by stimulation with IL-11. The ICAM-1 expression was examined by Western blotting and qPCR. Results are expressed as the mean ± SEM. $^{*}P < 0.05$ compared with control; $+P < 0.05$ compared with IL-11-treated group.

We further examined the upstream molecules involved in IL-11-induced NF-κB activation. Stimulation of cells with IL-11 induced IKKα/β phosphorylation in a time-dependent manner (Fig. 5D). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the IL-11-induced cell migration and ICAM-1 expression (Fig. 5A,C). These data suggest that IKKα/β activation is involved in IL-11-induced the migration activity of human chondrosarcoma cells. Based on previous studies showing that p65 Ser^{536} phosphorylation increases NF-κB transactivation [Madrid et al., 2001], an antibody against phosphorylated p65 Ser^{536} was used to examine p65 phosphorylation. Treatment of chondrosarcoma cells with IL-11 for various time intervals resulted in p65 Ser^{536} phosphorylation (Fig. 5D). Pretreatment of cells with Ly294002 and Akt inhibitor or transfection of cells with p85 and Akt mutant reduced IL-11-induced IKK and p65 phosphorylation (Fig. 5E). We next investigated whether p65 binds to the NF-κB element on the ICAM-1 promoter after IL-11 stimulation. The in vivo recruitment of p65 to the ICAM-1 promoter was assessed by the chromatin immunoprecipitation assay. In vivo binding of p65 to the NF-κB element of the ICAM-1 promoter occurred after IL-11 stimulation (Fig. 5F). Binding of p65 to the NF-κB element by IL-11 was attenuated by Ly294002, wortmannin, and Akt inhibitor (Fig. 5E). Pretreatment of cells with Ly294002, wortmannin, and Akt inhibitor also reduced IL-11-induced accumulation of p65 into the nucleus (Fig. 5G). Taken together, these data suggest that activation of IL-Rα receptor, PI3K, and Akt are required for IL-11-induced NF-κB activation in human chondrosarcoma cells.

DECREASE CELL MOTILITY IN IL-11-SHRNA OVER-EXPRESSION CLONE

To further confirm that IL-11-mediated cell migration and ICAM-1 expression in human chondrosarcoma cells, an IL-11-shRNA expression cell lines was established. The IL-11 expression level in stable transfectants was assessed by Western blotting, which

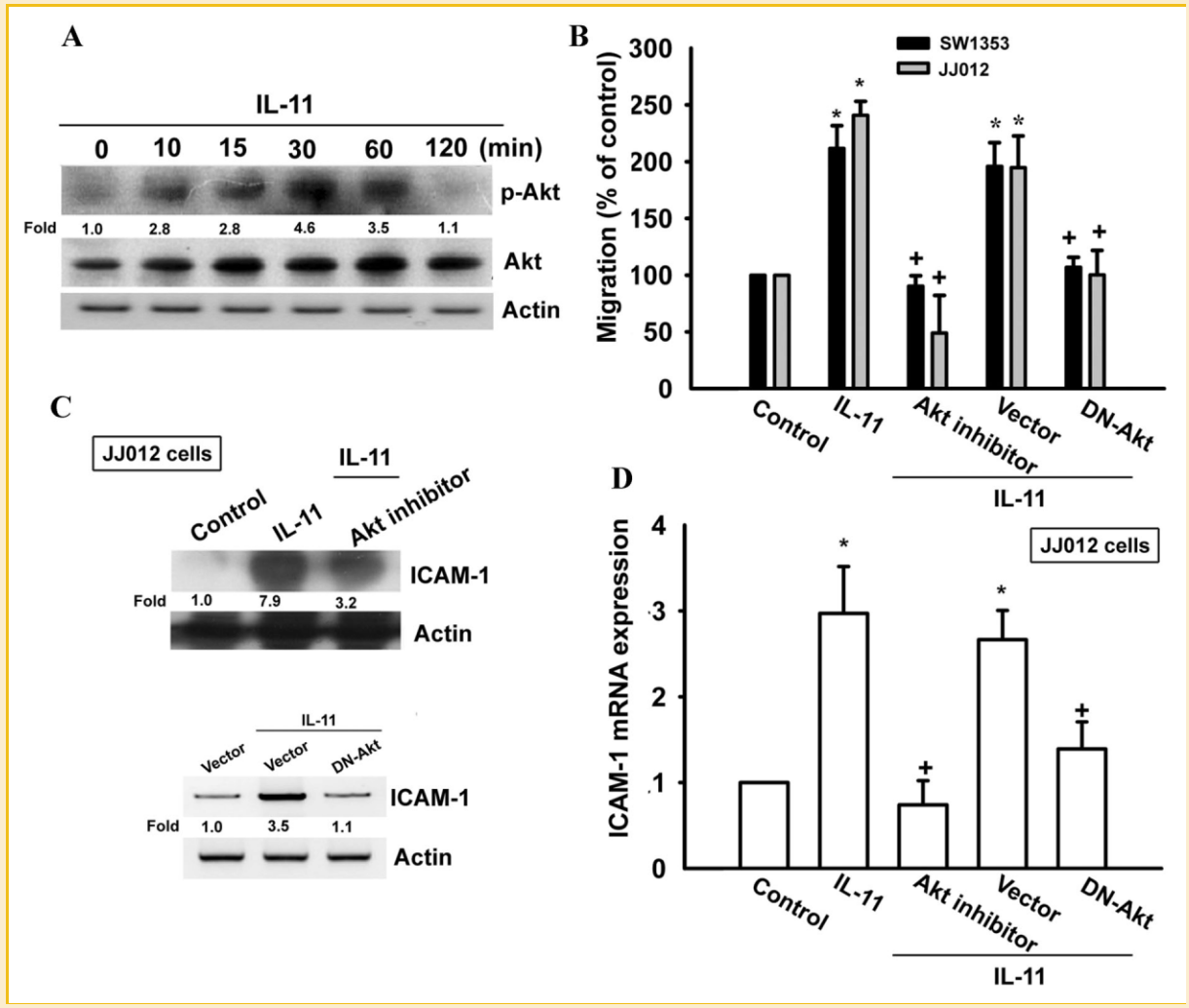

Fig. 4. Akt is involved in IL-11-induced migration and ICAM-1 up-regulation in human chondrosarcoma cells. A: JJ012 cells were incubated with IL-11 (30 ng/ml) for indicated time intervals, and p-Akt was examined by Western blotting ($n = 4$). B: Cells were pretreated for 30 min with Akt inhibitor (10 μM) or transfected with dominant negative (DN) mutant of Akt for 24 h followed by stimulation with IL-11. The in vitro migration was examined by Transwell. B–D: Cells were pretreated for 30 min with Akt inhibitor or transfected with Akt mutant of Akt for 24 h followed by stimulation with IL-11. The ICAM-1 expression was examined by Western blotting and qPCR. Results are expressed as the mean ± SEM. $^{*}P < 0.05$ compared with control; $+P < 0.05$ compared with IL-11-treated group.

showed a dramatic reduction of IL-11 expression in JJ012/IL-11-shRNA cells (Fig. 6A). Based on the reported activity of IL-11 as a mitogen in human cancer cells [Yoshizaki et al., 2006; Nakayama et al., 2007], the cellular growth rate of control cells and transfectants was investigated by MTT assay 1–6 days after cell seeding. No appreciable difference in cell proliferation was evident between these cells (data not shown), suggesting that IL-11 did not have a mitogenic effect on the human chondrosarcoma cells. The analysis of the migratory ability of these transfectants using a Transwell migration assay revealed that the knockdown of the IL-11 expression inhibited the migratory ability by approximately 60% in JJ012 cells (Fig. 6A). In addition, knockdown IL-11 also reduced ICAM-1 expression in JJ012 cells (Fig. 6B). Therefore, human chondrosarcoma cells with higher ability to migrate expressed more IL-11 and ICAM-1.

DISCUSSION

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which cause dramatic increases in long-term survival with the advent of systemic chemotherapy, chondrosarcoma continues to have a poor prognosis due to absence of an effective adjuvant therapy [Fong et al., 2007]. The metastatic potential for conventional chondrosarcomas correlates well with the histologic grade of the tumor. But due to the relatively indolent growth rates of many low- and moderate-grade chondrosarcomas, ~15% of patients dying from metastatic disease do so >5 years after initial diagnosis [Fong et al., 2007]. Therefore, it is important to develop effective adjuvant therapy for preventing chondrosarcoma metastasis. We hypothesized that IL-11 would help to direct the metastasis of chondrosarcoma cells. We found that IL-11 increased

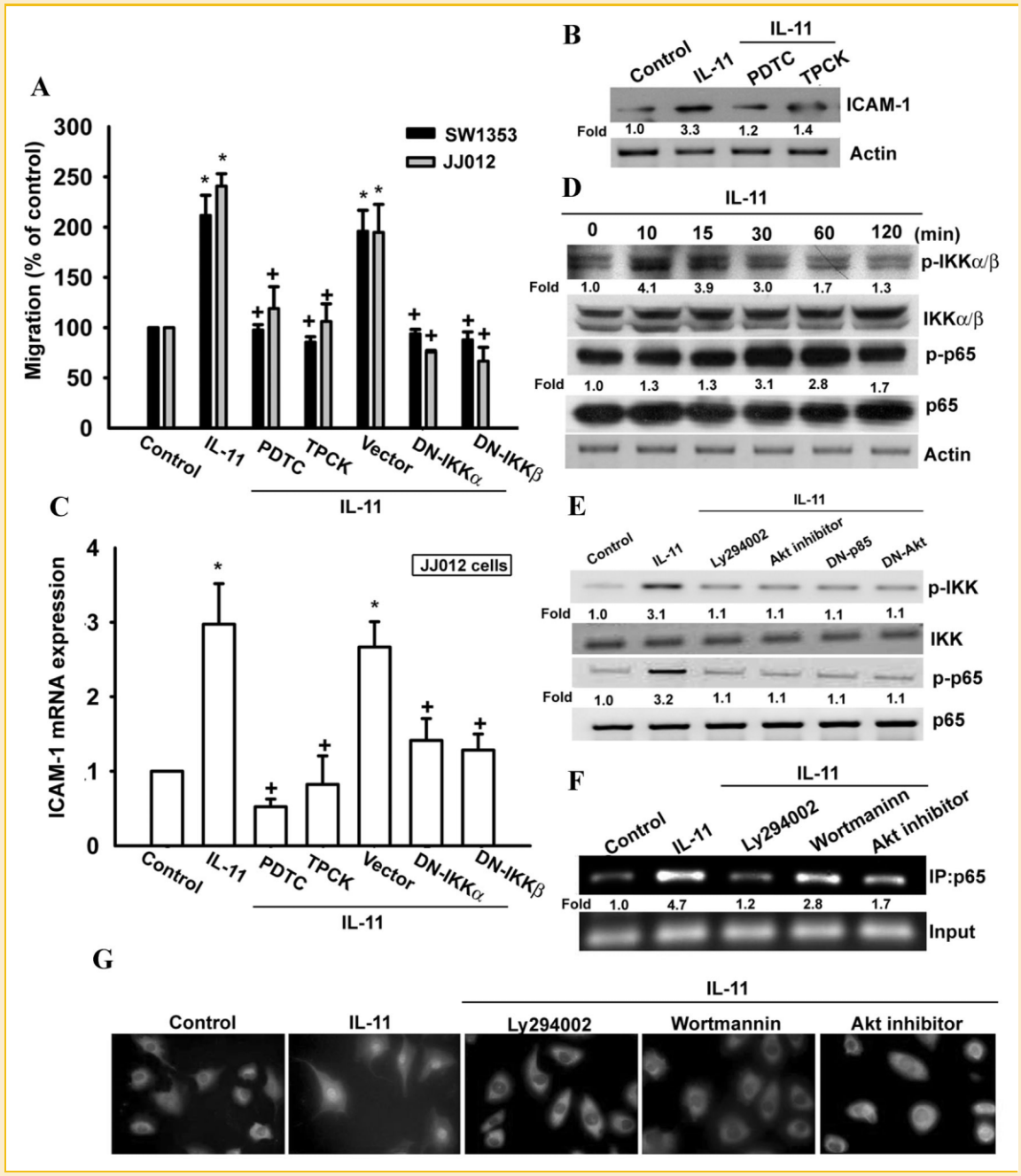

Fig. 5. IL-11 induces cell migration and ICAM-1 up-regulation through NF-κB. A: Cells were pretreated for 30 min with PDTC (10 μM) and TPCK (3 μM) or transfected with dominant negative (DN) mutant of IKKα or IKKβ for 24 h followed by stimulation with IL-11. The in vitro migration was examined by Transwell. B,C: JJ012 cells were pretreated for 30 min with PDTC and TPCK or transfected with IKKα or IKKβ mutant for 24 h followed by stimulation with IL-11. The ICAM-1 expression was examined by Western blotting and qPCR. D: JJ012 cells were incubated with IL-11 (30 ng/ml) for indicated time intervals, and p-IKK and p-p65 was examined by Western blotting ($n = 5$). E: JJ012 cells were pretreated for 30 min with Ly294002 and Akt inhibitor or transfected with p85 and Akt mutant for 24 h followed by stimulation with IL-11, and p-IKK and p-p65 expression was examined by Western blotting ($n = 4$). F: JJ012 cells were pretreated for 30 min with Ly294002, wortmannin, and Akt inhibitor followed by stimulation with IL-11 for 120 min, and the chromatin immunoprecipitation assay was then performed. Chromatin was immunoprecipitated with anti-p65. One percentage of the precipitated chromatin was assayed to verify equal loading (input). G: JJ012 cells were pretreated for 30 min with Ly294002, wortmannin, and Akt inhibitor followed by stimulation with IL-11 for 120 min, and p65 immunofluorescence staining was examined. Results are expressed as the mean ± SEM. $^{*}P < 0.05$ compared with control; $+P < 0.05$ compared with IL-11-treated group.

the migration of chondrosarcoma cells. Although JJ012 and SW1353 displayed different levels of basal and IL-11-induced ICAM-1 expression, however, we did not find any significant differences on migration activity after response to IL-11. Therefore, the maximum increase in migration activity after IL-11 stimulation of chondrosarcoma is ~2- to 3-fold. One of the mechanisms underlying IL-11 directed migration was transcriptional up-regulation of ICAM-1 and activation of IL-11Rα receptor, PI3K,

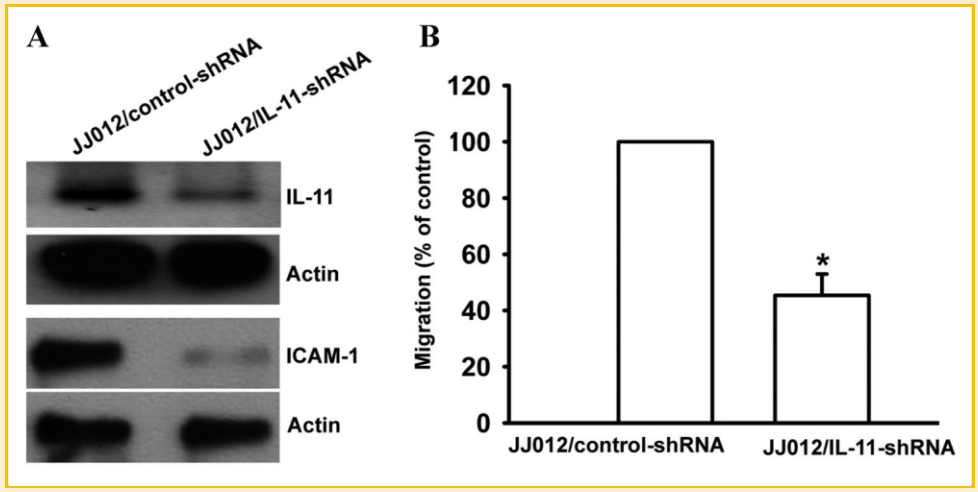

Fig. 6. Knockdown of IL-11 inhibited the migratory ability in chondrosarcoma cells. A: The protein levels of IL-11 and ICAM-1 in JJ012/control-shRNA and JJ012/IL-11-shRNA cells was examined by Western blot analysis. B: The in vitro migration activity of JJ012/control-shRNA and JJ012/IL-11-shRNA cells was measured with the Transwell. Results are expressed as the mean ± SE.

Akt, and NF-κB pathways. Using Western blotting and qPCR analysis, we found that the expression of IL-11 in human chondrosarcoma tissues was significantly higher than that in primary chondrocytes. Moreover, over-expression of IL-11 shRNA inhibited the migratory ability by approximately 60% in JJ012 cells. Therefore, the expression of IL-11 is associated with a metastatic phenotype of chondrosarcoma cells.

A variety of growth factors stimulate cancer metastasis via signal-transduction pathways that converge to activate NF-κB complex of transcription factors [Sliva, 2004]. The PI3K/Akt pathway is a major cascade mediating activation of the NF-κB signaling pathway in human cancer cells [Sliva, 2004]. Phosphorylation of the p85 subunit is required for activation of the p110 catalytic subunit of PI3K [Qureshi et al., 2007]. We found IL-11-enhanced the p85 subunit phosphorylation in human chondrosarcoma cells. Pretreatment of cells with PI3K inhibitors Ly294002 or wortmannin antagonized an increase in migration and ICAM-1 expression by IL-11 stimulation. This was further confirmed by the result that the dominant-negative mutant of p85 inhibited the enhancement of migration by IL-11. Moreover, we also found that IL-11 activated Akt Ser473 phosphorylation, whereas the Akt inhibitor and Akt mutant inhibited IL-11-mediated cell migration and ICAM-1 expression. Our data indicate that PI3K/Akt signaling could play an important role in the expression of ICAM-1 and migration of human chondrosarcoma cells.

NF-κB has been shown to control the induced transcription of ICAM-1 in human cancer cells [Inoue et al., 2011]. The results of this study show that NF-κB activation contributes to IL-11-induced ICAM-1 expression and migration in human chondrosarcoma cells, and that the inhibitors of the NF-κB-dependent signaling pathway, including PDTC or TPCK inhibited IL-11-induced ICAM-1 expression and cancer migration. In an inactivated state, NF-κB is normally held in the cytoplasm by the inhibitor protein IκB. Upon stimulation, such as by TNF-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 [Hatada et al., 2000]. In the present study, we found that treatment of JJ012 cells with IL-11 resulted in increases in IKKα/β phosphorylation. IKK mutants also reduced IL-11 mediated cell migration and ICAM-1 expression. Therefore, IKKα/β are involved in IL-11-induced cell motility in chondrosarcoma cells. Furthermore, IL-11 increased the binding of p65 to the NF-κB element within the ICAM-1 promoter, as shown by a chromatin immunoprecipitation assay. Binding of p65 to the NF-κB element was attenuated by Ly294002, wortmannin, and Akt inhibitor. On the other hand, Ly294002, wortmannin, and Akt inhibitor reduced IL-11-mediated p65 translocation into nucleus. Our data indicated that PI3K, Akt, and NF-κB pathways might play important role in the expression of ICAM-1 and cell migration of human chondrosarcoma cells.

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 IL-11 increases the activity of ICAM-1 via the IL-11Rα receptor, PI3K, Akt, and NF-κB-dependent pathway and to enhance migration of human chondrosarcoma cells. Furthermore, the discovery of IL-11-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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