Original Article

IGF-I enhances α5β1 integrin 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. Integrins are the major adhesive molecules in mammalian cells and have been associated with metastasis of cancer cells. Insulin-like growth factor-I (IGF)-I plays an important role in regulating cell growth, proliferation, survival, and metabolism. However, the effects of IGF-I in migration and integrin expression in chondrosarcoma cells are largely unknown. In this study, we found that IGF-I increased the migration and the expression of α5β1 integrin in human chondrosarcoma cells. Pretreatment of cells with IGF-I receptor antibody reduced IGF-I-induced cell migration and integrin expression. Activations of phosphatidylinositol 3-kinase (PI3K), Akt, and nuclear factor-κB (NF-κB) pathways after IGF-I treatment were demonstrated, and IGF-I-induced expression of integrin and migration activity was inhibited by the specific inhibitor and mutant of PI3K, Akt, and NF-κB cascades. Taken together, our results indicated that IGF-I enhances the migration of chondrosarcoma cells by increasing α5β1 integrin expression through the IGF-I receptor/PI3K/Akt/NF-κB signal transduction pathway.

Running title: IGF-I induces the migration of chondrosarcoma

Key words: IGF-I; Migration; Chondrosarcoma; PI3K; IGF-IR

Introduction

Chondrosarcoma is a malignant primary bone tumor with a poor response to currently-used chemotherapy or radiation treatment, making the management of chondrosarcomas a complicated challenge (Terek et al., 1998). Clinically, surgical resection remains the primary mode of therapy for chondrosarcoma. Due to the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis and, therefore, it is important to explore a novel and adequate remedy (Yuan et al., 2005).

The insulin-like growth factor (IGF) plays an important role in regulating cell growth, proliferation, survival, and metabolism. IGF-I, a 7.7-kDa peptide, is both a systemic and local growth factor (Yu and Rohan, 2000). IGF-I assumes an important role in both normal and neoplastic growth (Samani et al., 2007). The IGF axis consists of two ligands (IGF-I and IGF-II), two surface receptors (IGF-IR and IGF-IIR), six binding proteins (IGFBP-1 to IGFBP-6) that regulate the availability to the receptors, and a group of IGFBP proteases that cleave IGFBP and modulate the action of IGFs (Adhami et al., 2006). IGF-I binds to IGF-IR and the tyrosine kinase on the cytoplasmic domain of IGF-IR transduces IGF-I signaling into cells (Adhami et al., 2006; Pollak et al., 2004). Multiple signaling proteins are activated downstream of these receptors, including the extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3′-kinase (PI3K), and Akt. IGF-I signaling has been implicated in a variety of human cancers and promotes tumorigenesis (Pollak et al., 2004).

Decades of scrutiny into the molecular bases of cancer have largely focused on what causes oncogenic transformation and the incipient emergence of tumors (Gupta and Massague, 2006). The invasion of tumor cells is a complex, multistage process. To facilitate 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). Integrins are a family of transmembrane adhesion receptors comprising 19 α and 8 β subunits that interact

noncovalently to form up to 24 different heterodimeric receptors. The combination of different integrin subunits on the cell surface allows cells to recognize and respond to a variety of different ECM proteins including fibronectin, laminin, collagen, and vitronectin (Humphries, 2000). Because integrins are the primary receptors for cellular adhesion to ECM molecules, they act as crucial transducers of bidirectional cell signaling, regulating cell survival, differentiation, proliferation, migration, and tissue remodeling (Stupack, 2007). Activation and elevated expression of integrin-coupled signaling effectors have been implicated in the induction of a wide variety of human cancers, including those of the breast, colon, prostate, and ovaries (White et al., 2004). In addition, integrin has also been implicated in metastasis of lung, breast, bladder, and colon cancers (Heyder et al., 2005; Seales et al., 2005; Takenaka et al., 2000).

Previous studies have shown that IGF-I modulates cell migration and invasion in human cancer cells (Bauer et al., 2006; Graham et al., 2008). However, the effect of IGF-I on integrin expression and migration activity in human chondrosarcoma cells is mostly unknown. In this study, we explored whether IGF-I increased the migration and integrin expression of in human chondrosarcoma cells. In addition, IGF-IR, PI3K, Akt, IKKα/β and NF-κB signaling pathways may be involved in the increase of integrin expression and cell migration by IGF-I.

Materials and Methods

Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p-Akt(sc-7985-R), Akt(sc-55523), p-p85, p85(sc-56939), IKKα/β(sc-7607), p-IκBα(sc-8404), IκBα(sc-81296), p65(sc-71677), and α-tubulin (sc-23948) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody specific for α2(MAB1988), α5(CBL497P), β1(MAB2000), β3(MAB2637), α2β1(MAB1998), and α5β1(MAB1969) integrin were purchased from Chemicon (Temecula, CA).

Ly294002, Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-((R)-2-O-methyl-3-O-octadecylcarbonate)), TPCK, and PDTC were purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal antibody specific for phosphor-IKKα/β ($Ser^{180/181}$)(No.2681) and phosphor-p65 (Ser^{536})(No.3036) were purchased from Cell Signaling (Danvers, MA, USA). The recombinant human IGF-I was purchased from PeproTech (Rocky Hill, NJ, USA). Mouse monoclonal antibody specific for IGF-IR was purchased from R&D Systems (Minneapolis, MN, USA). The p85α [(Δp85; deletion of 35 amino acids from residues 479-513 of p85);(Transfection of cells with p85 mutant reduced p85 and Akt phosphorylation; Supplementary Figure S1)] and Akt [(Akt K179A); (Transfection of cells with Akt mutant reduced Akt phosphorylation and kinase activity; Supplementary Figure S1)] mutants were gifts from Dr. W. M. Fu (National Taiwan University, Taipei, Taiwan). The IKKα (KM) and IKKβ (KM) mutants were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA, USA). 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).

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, USA). The human chondrosarcoma cell line (SW1353), prostate cancer cell line (PC3), lung cancer cell line (A549), and breast cancer cell line (MDA-231) 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.

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 IGF-IR mAb, Ly294002, Akt inhibitor, PDTC, and TPCK [These inhibitors did not affect the basal migration activity of chondrosarcoma (Supplementary Figure S2)] 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 IGF-I 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 IGF-I treatment (corrected invading cell number = counted invading cell number/percentage of viable cells) (Fong et al., 2008).

Flow cytometric analysis

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

Western blot analysis

The cellular lysates were prepared as described previously (Tang et al., 2008). Proteins were resolved on SDS-PAGE and transferred to Immobilon

polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 hr at room temperature and then probed with rabbit anti-human antibodies against p-p85, p-Akt, IκBα, p-IκB, or IKKα/β (1:1000) for 1 hr at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 hr at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA).

Quantitative real-time PCR

Total RNA was extracted from chondrosarcomas using a TRIzol kit (MDBio Inc., Taipei, Taiwan). Two μg of total RNA was reverse transcribed into cDNA using oligo(dT) primer. The quantitative real-time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). Two μl of cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems, CA). qPCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 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).

siRNA transfection

The siRNA against IGF-IR (sc-29358) and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transfected with siRNAs (100 nM) or dominant-negative mutant (1 μg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions (Tang et al.).

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 hr transfection, the cells were then incubated with the indicated agents. After a further 24 hr incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI, USA) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector.

Statistics

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

Results

IGF-I-directed chondrosarcoma cells migration involves α5β1 integrin up-regulation

IGF-I has been suggested to stimulate directional migration and invasion of human cancer cells (Bauer et al., 2006; Graham et al., 2008). IGF-I-triggered migration in chondrosarcoma cells was examined using the Transwell assay with correction of

IGF-I-induced proliferation effects on human chondrosarcoma cells (Fong et al., 2008). IGF-I directed human chondrosarcoma cell (JJ012 cells) migration (Fig. 1A). In addition, IGF-I also dose-dependently directed other human chondrosarcoma cell migration (SW1353 cells) (Fig. 1A). We also found that IGF-I increased migration in other cancer cell type [PC-3 (prostate cancer), A549 (lung cancer), and MDA-231 (breast cancer)] (Fig. 1B). Therefore, IGF-I-induced cell migration is a common phenomenon in human cancer cells. Previous studies have shown significant expression of integrin in human chondrosarcoma cells (Chen et al., 2009; Lai et al., 2009). We therefore, hypothesized that integrin may be involved in IGF-I-directed chondrosarcoma cell migration. Flow cytometry analysis showed that IGF-I induced the cell surface expression of α5 and β1 but not α2, β3, and α2β1 integrin (Fig. 1C). To confirm this finding, expression of mRNA for the integrins in response to IGF-I was analyzed by qPCR. Treatment of JJ012 cells with IGF-I induced the mRNA expression of α5 and β1 but not α2 and β3 integrin (Fig. 1D). Pretreatment of cells for 30 min with anti-α5β1 monoclonal antibody (mAb) (10 μg/ml) markedly inhibited the IGF-I-induced cell migration (Fig. 1E). The migration activity contains cell adhesion and cell motility. Therefore, the reducing cell adhesion after anti-α5β1 monoclonal antibody treatment may involves α5β1-reduced cell migration in chondrosarcoma. These data suggest that IGF-I-induced cancer migration may occur via activation of α5β1 integrin receptor.

Involvement of IGF-I receptor in IGF-I-mediated migration of chondrosarcoma

Interaction of IGF-I with its specific receptor IGF-IR on the surface of cancer cells has been reported to induce cancer invasion (Bauer et al., 2006; Girnita et al., 2006). Therefore, we next examined whether IGF-IR was involved in IGF-I-mediated cell migration in human chondrosarcoma cells. Pretreatment of cells with IGF-IR Ab reduced IGF-I-induced cell migration and α5β1 integrin expression (Fig. 2A&B). Transfection of cells with IGF-IR siRNA reduced IGF-IR expression and IGF-I-increased Akt and ERK phosphorylation (Supplementary Figure S3). In addition,

transfection of cells with IGF-IR siRNA effectively inhibited the migration activity and α5β1 integrin expression of chondrosarcoma cells (Fig. 2C&D). These data suggest that IGF-I/IGF-IR interactions play a key role in the migration of chondrosarcoma.

PI3K and Akt signaling pathways are involved in IGF-I-mediated α5β1 integirn up-regulation and cell migration of chondrosarcoma cells

PI3K/Akt can be activated by a variety of growth factors, such as insulin, nerve growth factors, and IGF-I (Bibollet-Bahena and Almazan, 2009; Horowitz et al., 2004; Huang et al., 2009). We examined whether IGF-I stimulation also enhanced PI3K activation. Stimulation of JJ012 cells led to a significant increase in phosphorylation of p85 (Fig. 3A). IGF-I-induced migration and integrin expression of chondrosarcoma cells were greatly reduced by treatment with PI3K inhibitors Ly294002 and wortmannin (Fig. 3B&C). In addition, transfection of cells with p85 mutant also inhibited IGF-I-induced migration and α5β1 integrin expression of chondrosarcoma cells (Fig. 3D&E). Ser473 residue phosphorylation of Akt by a PI3K-dependent signaling pathway causes enzymatic activation (Qiao et al., 2008). To examine the crucial role of PI3K/Akt in cancer migration and integrin up-regulation, we next determined Akt Ser473 phosphorylation in response to IGF-I treatment. As shown in Fig. 4A, treatment of JJ012 cells with IGF-I resulted in time-dependent phosphorylation of Akt Ser473. Pretreatment of cells with Akt inhibitor antagonized IGF-I-induced migration and integrin expression of chondrosarcoma cells (Fig. 4B&C). In addition, the Akt mutant also reduced IGF-I-mediated cell migration and integin up-regulation (Fig. 4B&C). On the other hand, Akt inhibitor or mutant reduced basal and IGF-I-increased Akt phosphorylation (Supplementary Figure S4). Based on these results, it appears that IGF-I/IGF-IR axis acts through the PI3K and Akt-dependent signaling pathway to enhance α5β1 integrin expression and cell migration in human chondrosarcoma cells.

NF-κB signaling pathway is involved in IGF-I-induced integrin up-regulation and

migration activity

As mentioned above, NF-κB activation is necessary for the migration and invasion of human cancer cells (Fong et al., 2008; Yeh et al., 2008). To examine whether NF-κB activation is involved in IGF-I-induced cancer migration, an NF-κB inhibitor, PDTC, was used. Fig. 5B shows that cells pretreated with PDTC inhibited IGF-I-induced chondrosarcoma cell migration. Furthermore, cells pretreated with TPCK, an IκB protease inhibitor, also inhibited IGF-I-induced cancer cell migration (Fig. 5B). In addition, treatment of cells with PDTC or TPCK also antagonized IGF-I-induced expression of α5β1 integrin (Fig. 5C). These results indicated that NF-κB activation is important for IGF-I-induced cancer cell migration and the expression α5β1 integrin.

We further examined the upstream molecules involved in IGF-I-induced NF-κB activation. Stimulation of JJ012 cells with IGF-Ị induced IKKα/β phosphorylation in a time-dependent manner (Fig. 5A). Furthermore, transfection with IKKα or IKKβ mutants markedly inhibited the IGF-I-induced cancer cells migration (Fig. 5D). These data suggest that IKKα/β activation is involved in IGF-I-induced migration of human chondrosarcoma. Treatment of IGF-I in chondrosarcoma cells also caused IκBα phosphorylation in a time-dependent manner (Fig. 5A). In addition, treatment of JJ012 cells with IGF-I for various time intervals resulted in p65 phosphorylation (Fig. 5A). To directly determine NF-κB activation after IGF-I treatment, JJ012 cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Fig 6A, IGF-I treatment of JJ012 cells for 24 hr increased κB-luciferase activity. In addition, IGF-IR Ab, Ly294002, wortmannin and Akt inhibitor antagonized the IGF-I-induced κB-luciferase activity (Fig. 6A). Co-transfection of cells with p85, Akt, IKKα or IKKβ mutant and IGF-IR siRNA also reduced IGF-I-increased κB-luciferase activity (Fig. 6B). Taken together, these data suggest that activation of IGF-IR/PI3K/Akt is required for IGF-I-induced NF-κB activation in chondrosarcoma cells.

Discussion

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, which have seen dramatic increases in long-term survival with the advent of systemic chemotherapy, chondrosarcoma continues to have a poor prognosis due to 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 who die 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 IGF-I would help to direct the metastasis of chondrosarcoma cells. We found that IGF-I increased the migration of chondrosarcoma cells. One of the mechanisms underlying IGF-I-directed migration was up-regulation of $\alpha 5\beta 1$ integrin and activation of IGF-IR, PI3K, Akt, and NF-κB pathways.

The IGF-I receptor belongs to the cytokine receptor superfamily (Bruchim et al., 2009). Human cancer cells express IGF-IR (Gualberto and Karp, 2009); in addition, IGF-IR is involved in IGF-I-mediated cell motility (LeRoith and Roberts, 2003). The role of IGF-IR in the motility of human chondrosarcoma cells is, however, largely unknown. We found that the IGF-IR Ab and siRNA reduced IGF-I-increased cell migration and integrin expression. Therefore, IGF-IR is very important in IGF-I-mediated motility. Collectively, the interpretation of our data would appear to encourage us to conclude that IGF-I/IGF-IR plays a novel role in regulating chondrosarcoma migration in experimental setting, and it would also appear to be feasible as a biological marker to predict the relative likelihood/extent of peritoneal metastasis following chondrosarcoma.

A variety of growth factors stimulate the expression of integrin via signal-transduction pathways that converge to activate NF-κB complex of transcription factors (Tang and Lu, 2009). The PI3K/Akt pathway is a major cascade mediating activation of the NF-κB signaling pathway in human cancer cells (Pan et al., 1999). Phosphorylation of the p85 subunit is required for activation of the p110

catalytic subunit of PI3K (Qureshi et al., 2007). We found IGF-I-enhanced the p85 subunit phosphorylation in human chondrosarcoma cells. Pretreatment of cells with PI3K inhibitors Ly294002 and wortmannin antagonized an increase in migration and integrin expression by IGF-I stimulation. This was further confirmed by the result that the dominant-negative mutant of p85 inhibited the enhancement of migration by IGF-I. Moreover, we also found that IGF-I activated Akt Ser473 phosphorylation, while Akt inhibitor inhibited IGF-I-mediated cell migration. Since many Akt inhibitors are not fully specific. Therefore, we used Akt mutant to confirm the results from Akt inhibitor. We found that transfection of cells with Akt mutant reduced IGF-I-enhanced migration and integrin expression. Our data indicates that PI3K/Akt could play an important role in the expression of α5β1 integrin and migration of human chondrosarcoma cells. It has been reported that NF-κB element is important in α5β1 integrin transcription activity (Tang and Lu, 2009). In this study, NF-κB inhibitors reduced the IGF-I-mediated cell migration and integrin activation in chondrosarcoma cells. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that IGF-I-induced an increase in NF-κB activity. In this study, we found that IGF-I-induced NF-κB activity was inhibited by IGF-IR Ab, Ly294002, wortmannin and Akt inhibitor or p85, Akt, IKKα, and IKKβ mutant. These results indicate that IGF-IR, PI3K, and Akt signaling pathways are involved in IGF-I-mediated NF-κB transactivation.

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 IGF-I increases the activity of α5β1 integrin via the IGF-IR, PI3K, Akt, IKKα/β, and NF-κB-dependent pathway and enhances migration of human chondrosarcoma cells (Fig. 6C). Furthermore, the discovery of IGF-I/IGF-IR-mediated signaling pathway helps us understand the mechanism of human chondrosarcoma metastasis and may lead us to develop effective therapy in the future.

Acknowledgments

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Figure legends

Fig. 1 IGF-I-directed migration of human chondrosarcoma cells involves up-regulation of α5β1 integrin

(A) Cells were incubated with IGF-I for 24 hr, and *in vitro* migration was measured by Transwell after 24 hr. (B) Cells were incubated with IGF-I for 24 hr, and *in vitro* migration was measured by Transwell after 24 hr. (C) JJ012 cells were incubated with IGF-I (30 ng/ml) for 24 hr, and the cell surface expression of α2, α5, β1, β3, α2β1, or αvβ3 integrin was determined using flow cytometry. (D) JJ012 cells were incubated with IGF-I (30 ng/ml) for 24 hr, and the mRNA levels of α2, α5, β1, or β3 integrin was determined using qPCR. (E) Cells were pretreated with α5β1 monoclonal antibody (10 μg/ml) for 30 min followed by stimulation with IGF-I (30 ng/ml). The *in vitro* migration activity measured after 24 hr. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with IGF-I-treated group.

Fig. 2 Involvement of IGF-IR in IGF-I-directed migration of human chondrosarcoma.

(A) Cells were pretreated with IGF-IR antibody (10 μg/ml) for 30 min followed by stimulation with IGF-I (30 ng/ml). The *in vitro* migration (A) and integrin expression (B) was examined by Transwell and flow cytometry analysis. Cells were transfected with IGF-IR or control siRNA for 24 hr followed by stimulation with IGF-I. The *in vitro* migration (C) and integrin expression (D) was examined by Transwell and flow cytometry analysis. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with IGF-I-treated group.

Fig. 3 PI3K is involved in IGF-I-induced migration and integrin up-regulation in human chondrosarcoma cells.

(A) JJ012 cells were incubated with IGF-I (30 ng/ml) for indicated time

intervals, and p-p85 was examined by Western blot analysis (n=5). (B-E) 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 hr followed by stimulation with IGF-I (30 ng/ml). The *in vitro* migration and integrin expression was examined by Transwell and flow cytometry analysis. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with IGF-I-treated group.

Fig. 4 Akt is involved in IGF-I-induced migration and integrin up-regulation in human chondrosarcoma cells.

(A) JJ012 cells were incubated with IGF-I (30 ng/ml) for indicated time intervals, and p-Akt was examined by Western blot analysis (n=5). (B&C) Cells were pretreated for 30 min with Akt inhibitor (10 μM) or transfected with dominant negative (DN) mutant of Akt for 24 hr followed by stimulation with IGF-I (30 ng/ml). The *in vitro* migration and integrin expression was examined by Transwell and flow cytometry analysis. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with IGF-I-treated group.

Fig. 5 IGF-I induces cell migration and integrin up-regulation through NF-κB.

(A) JJ012 cells were incubated with IGF-I for indicated time intervals, and IKK, IκBα, and p65 phophorylation was examined by Western blot analysis (n=5). Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with IGF-I (30 ng/ml). The *in vitro* migration (B) and integrin expression (C) was examined by Transwell and flow cytometry analysis. Cells were transfected with dominant negative (DN) mutant of IKKα or IKKβ for 24 hr followed by stimulation with IGF-I (30 ng/ml). The *in vitro* migration and integrin expression was examined by Transwell and flow cytometry analysis. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #,

$p < 0.05$ compared with IGF-I-treated group.

Fig. 6 IGF-IR/PI3K/Akt pathway is involved in IGF-I-mediated NF-κB activation and integrin expression.

JJ012 cells were pretreated with IGF-IR Ab (10 μg/ml), Ly294002 (10 μM), wortmannin (1 μM) and Akt inhibitor (10 μM) for 30 min (A) or transfected with mutant of p85, Akt, IKKα and IKKβ (B) before exposure to IGF-I (30 ng/ml). NF-κB luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with IGF-I-treated group. (C) Schematic presentation of the signaling pathways involved in IGF-I-induced migration and α5β1 integrin expression of chondrosarcoma cells. IGF-I activates IGF-IR, PI3K, and Akt pathways, which in turn induces NF-κB activation, which lead to α5β1 integrin expression and increases the migration of human chondrosarcoma cells.

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Figure 1

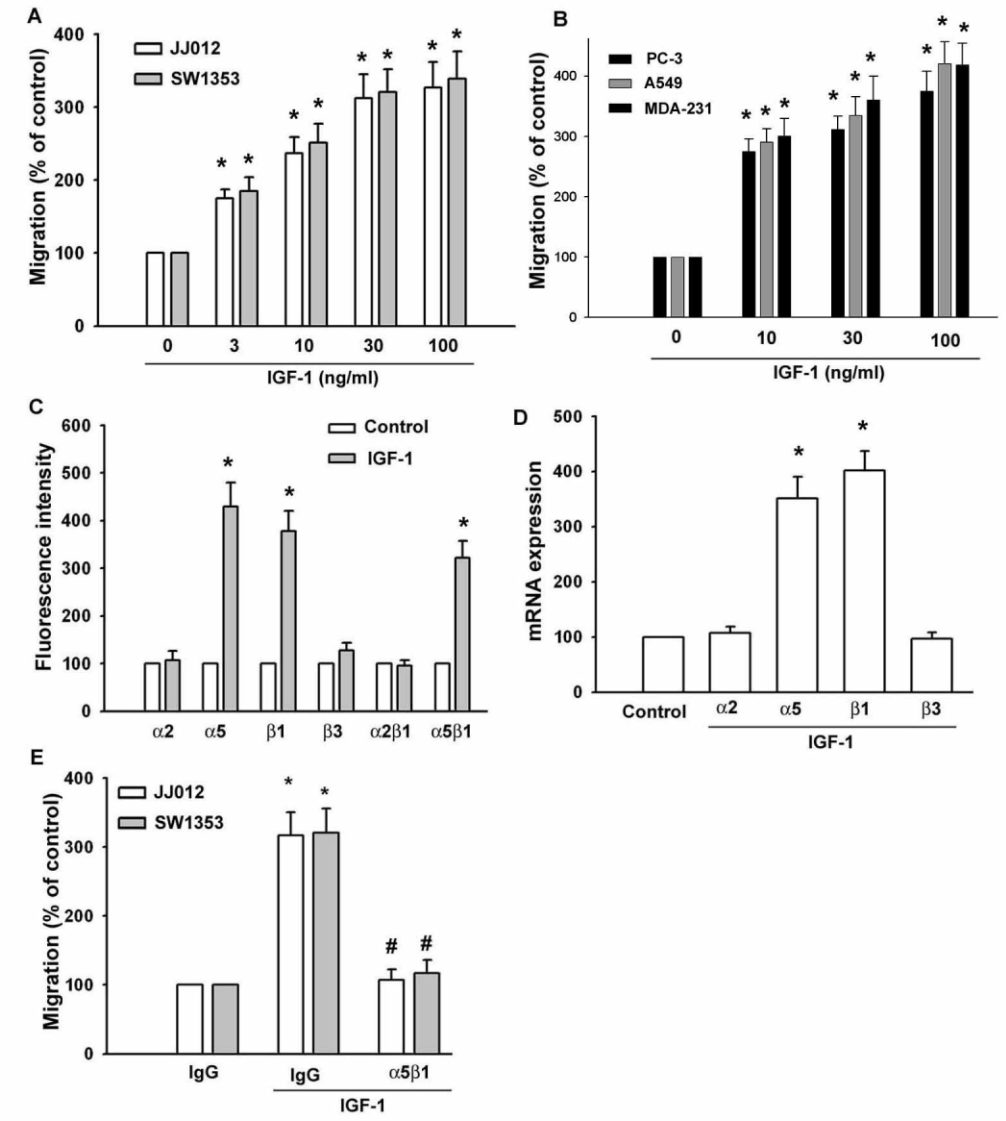
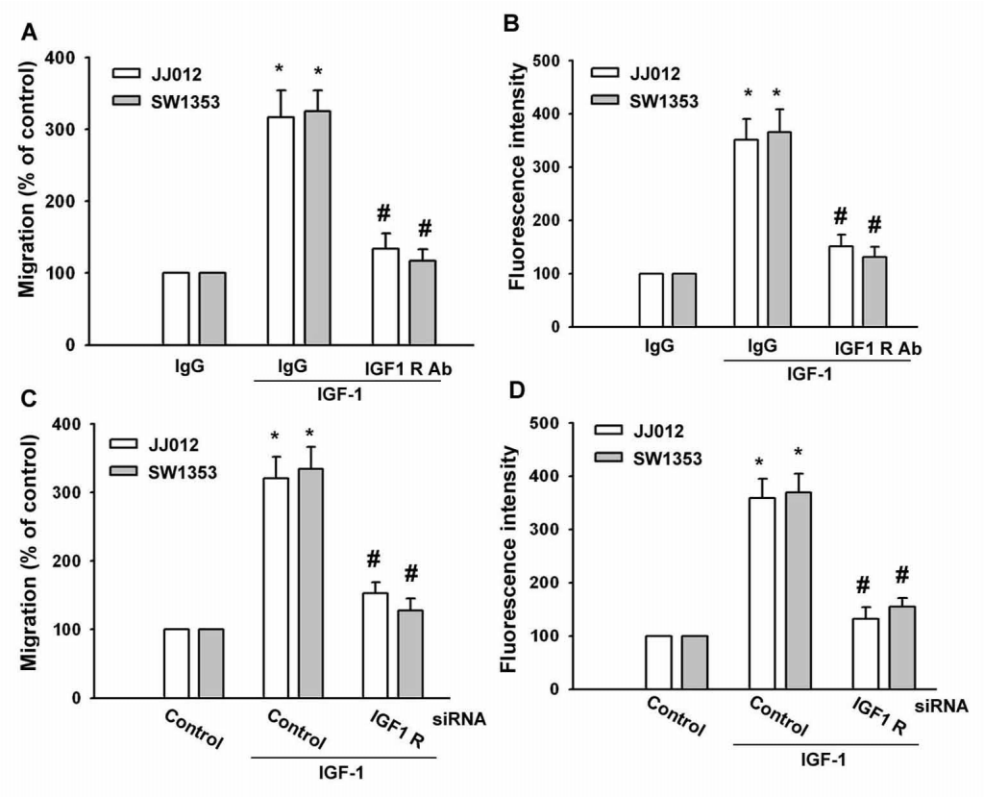


Figure 2



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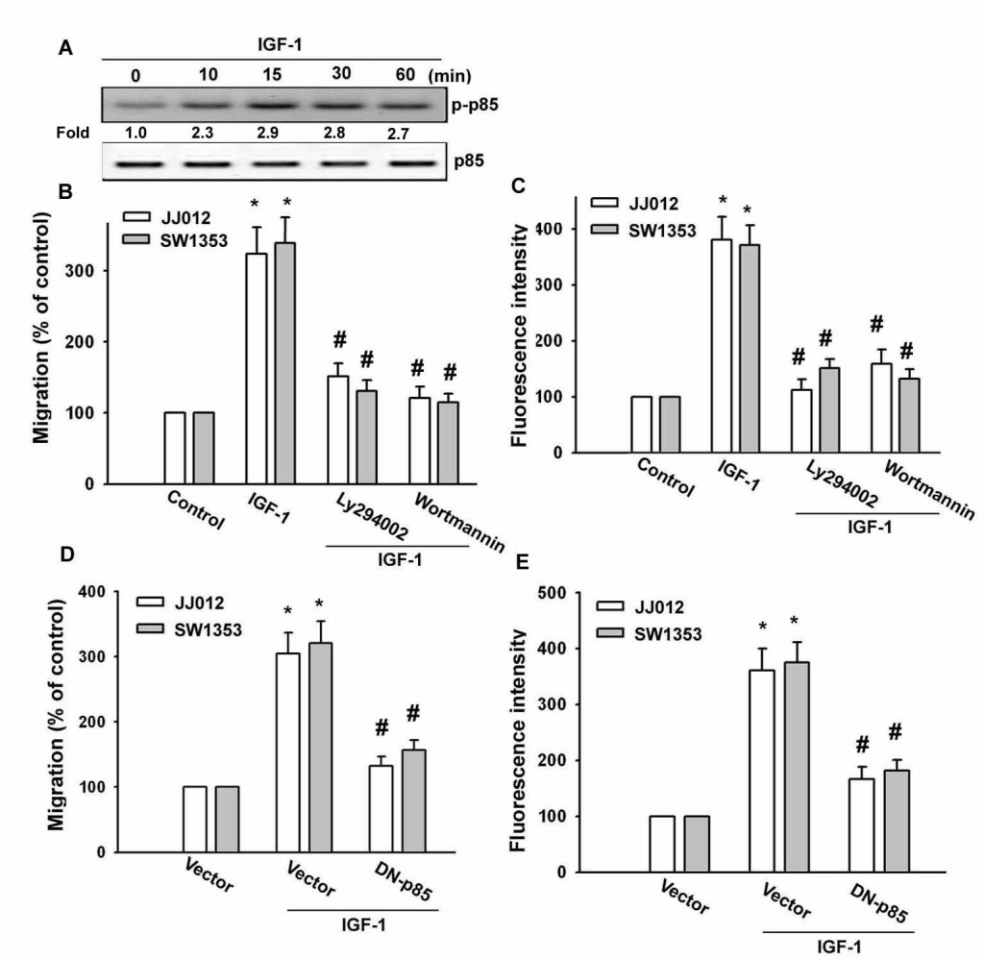

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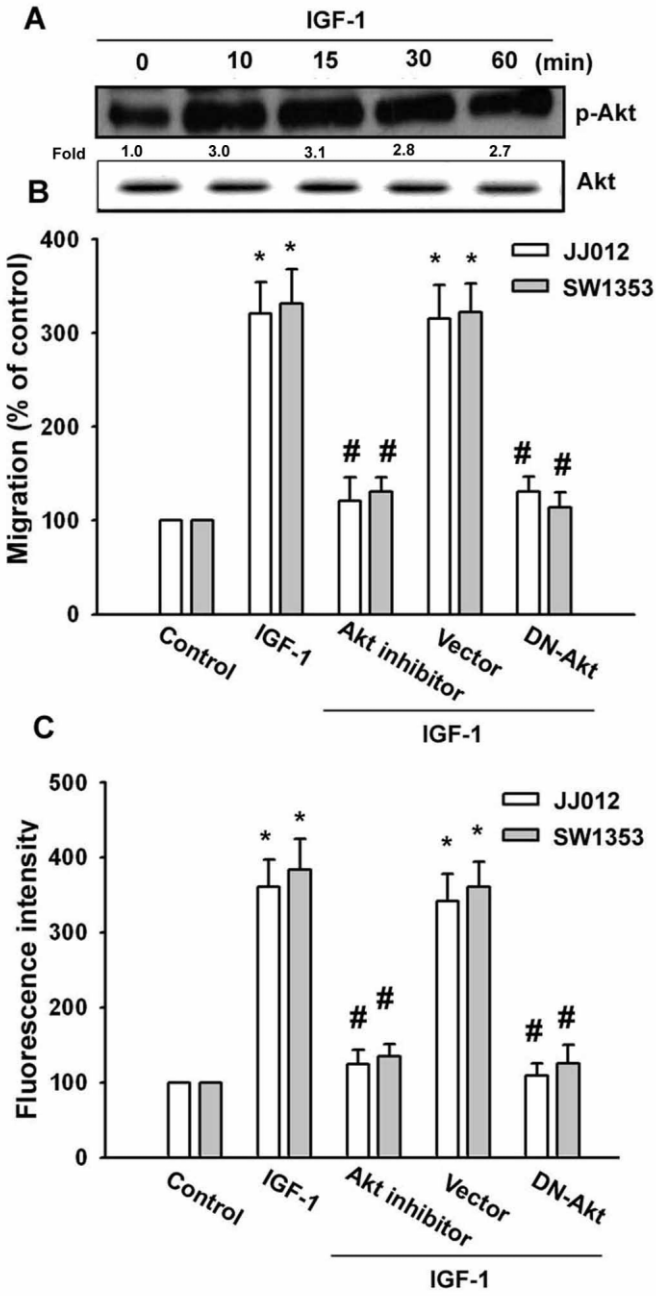

Figure 5

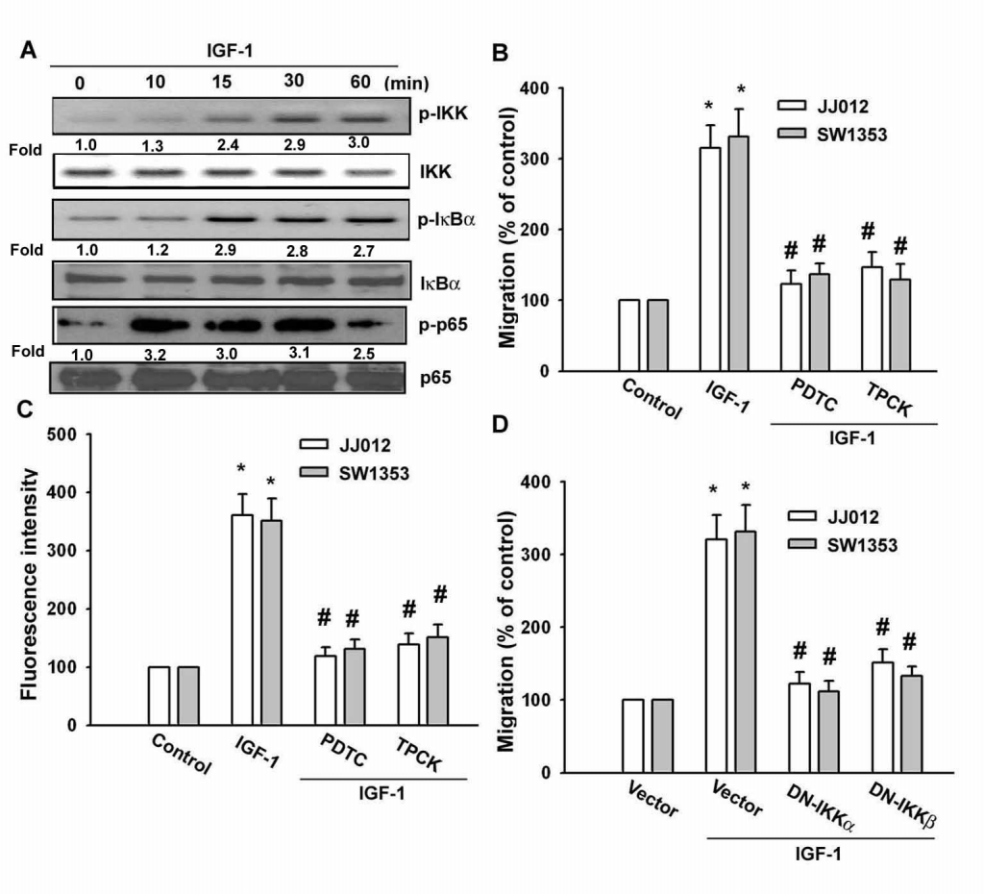

Figure 6