Macrophage migration inhibitory factor increases cell motility and up-regulates αvβ3 integrin in human chondrosarcoma cells

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ABSTRACT

The macrophage migration-inhibitory factor (MIF) is a pro-inflammatory cytokine first known for its effect on macrophage migration and activation. Recent studies have shown that MIP 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 MIF on human chondrosarcoma cells are largely unknown. In the present study, MIF was found to increase the migration and the expression of αvβ3 integrin in human chondrosarcoma cells. The phosphatidylinositol 3-kinase (PI3K), Akt, and NF-κB pathways were activated by MIF treatment, and the MIF-induced expression of integrin and migration activity were inhibited by the specific inhibitors and mutant forms of PI3K, Akt, and NF-κB cascades. In addition, migration-prone sublines demonstrated that increased cell migration ability was correlated with increased expression of MIF and αvβ3 integrin. Taken together, our results indicate that MIF enhanced the migration of the chondrosarcoma cells by increasing αvβ3 integrin expression through the PI3K/Akt/NF-κB signal transduction pathway.

Running title: MIF induces cancer migration

Key Words: MIF; Chondrosarcoma; Integrin; Migration

INTRODUCTION

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].

The macrophage migration-inhibitory factor (MIF) is a pro-inflammatory cytokine first known for its effect on macrophage migration and activation [Bloom and Bennett, 1966]. MIF is now known to have an important role in directing inflammatory response, and elevated levels of MIF have been found in many cases of pathological inflammation and in healing wounds [Calandra and Roger, 2003]. Recent studies have shown that MIF is over-expressed in a variety of human tumors, including those of the colon, prostate, breast, lung, and liver, as well as in melanoma and glioblastoma multiforme [Ren et al., 2003; Takahashi et al., 1998]. In some of these tumors, MIF has been implicated in oncogene-induced malignant transformation and in promoting tumor cell invasion, proliferation, angiogenesis, and metastasis [Ogawa et al., 2000; Takahashi et al., 1998]. Moreover, MIF expression has been shown to closely correlate with tumor aggressiveness and metastatic potential, and not surprisingly. MIF has been shown to be a poor prognostic factor in patients with lung adenocarcinoma, breast, and hepatocellular carcinomas [Takahashi et al., 1998; Xu et al., 2008].

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, and suppress anoikis and reorganize their cytoskeletons [Woodhouse et al., 1997]. Integrins are a

family of transmembrane adhesion receptors composed of 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 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 MIF modulates cell migration and invasion in human cancer cells [Ogawa et al., 2000; Takahashi et al., 1998]. However, the effects of MIF on integrin expression and migration activity in human chondrosarcoma cells are mostly unknown. In this study, the effects of MIF on integrin expression and the migration of human chondrosarcoma cells were explored. In addition, the role of the phosphatidylinositol 3′-kinase (PI3K), Akt, and NF-κB signaling pathways in the MIF-mediated increase of integrin expression and cell migration by MIF were investigated.

MATERIALS AND METHODS

Materials:

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, p85, p-p85, Akt, p-Akt, p65, p-p65, and control shRNA and MIF shRNA plasmids were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody specific for αvβ3 integrin was purchased from Chemicon (Temecula, CA). TPCK and PDTC were purchased from Calbiochem (San Diego, CA). The recombinant human MIF was purchased from PeproTech (Rocky Hill, NJ). 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 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 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 MIF 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 3 times [Chen et al., 2011].

Flow cytometric analysis

The human chondrosarcoma cells were plated in 6-well dishes. The cells were then washed with PBS and detached with trypsin at 37°C. The cells were fixed for 10 min in PBS containing 1% paraformaldehyde. After rinsing in PBS, the cells were incubated with a mouse anti-human antibody against αvβ3 integrin (1:100) for 1 h at 4°C. The cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:100; Leinco Tec. Inc., St. Louis, MO) for 45 min and analyzed by flow cytometry using FACS Calibur and CellQuest software (BD Biosciences).

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]. The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). 2 μl 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, CA). The qPCR assays were carried out in triplicate on an 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).

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 (1:1000) for 1 h at room temperature. After 3 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 a Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Transfection and reporter gene assay

The human chondrosarcoma cells were co-transfected with 0.8 μg luciferase plasmid and 0.4 μg β-galactosidase expression vector. The cells were grown to 80% confluence in 12 well plates and transfected the following day by Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. After 24 h transfection, the cells were incubated with the indicated agents. After a further 24 h incubation, the media were removed, and the cells were washed once with cold PBS. To prepare the lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) 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 that of the transfection efficiency monitored by the co-transfected β-galactosidase expression vector [Lu et al., 2010].

Establishment of migration-prone sublines

Subpopulations of the JJ012 cells were selected according to their differential migration ability determined using the cell culture insert system described previously. After 24 h migration, cells that penetrated through pores and migrated to the underside of the filters were trypsinized and harvested for a second-round selection. The original cells that did not pass through membrane pores were designated as JJ012-S0. After 10 rounds of selection, the migration-prone subline was designated as JJ012-S10 [Tang et al., 2011].

Establishment of stably transfected cells

The MIF 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 ± 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.

RESULTS

MIF-directed chondrosarcoma cell migration involves αvβ3 integrin up-regulation

The effect of MIF on the stimulation of directional migration and invasion of human cancer cells has been suggested in prior studies [Ogawa et al., 2000; Takahashi et al., 1998]. MIF-triggered migration in chondrosarcoma cells was examined using

the Transwell assay. MIF promoted the migration of the human chondrosarcoma cells (JJ012 cells; Fig 1A) and increased the invasive ability of the JJ012 cells through the Matrigel basement membrane matrix (Fig. 1B). However, MIF did not affect the proliferation of the chondrosarcoma cell lines (JJ012 and SW1353 cells) by the MTT assay (data not shown)[Fang et al., 2003]. Previous study has shown a significant expression of integrin in human chondrosarcoma cells [Lai et al., 2009]. We therefore hypothesized that integrin may be involved in MIF-directed chondrosarcoma cell migration. The qPCR analysis showed that MIF induced αv and β3 but not α2, α5, β1, and β5 integrin expression (Fig. 1C). To confirm this finding, expression of cell surface integrin in response to MIF was analyzed by flow cytometry. Treatment of JJ012 cells with MIF induced the cell surface expression of αvβ3 integrin (Fig. 1D). Pretreatment of cells for 30 min with anti-αvβ3 monoclonal antibody (mAb), but not α2β1 and α5β1 mAbs markedly inhibited the MIF-induced cell migration (Fig. 1E). The cyclic RGD peptide (cyclo-RGDfV) has been reported to bind αvβ3 at high affinity and block its function effectively at low concentrations [Brooks et al., 1996]. Treatment of cells with cyclic RGD, but not cyclic RAD, inhibited the MIF-induced migration of chondrosarcoma cells (Fig. 1F). These data suggest that MIF-induced tumor cell migration may occur via activation of αvβ3 integrin receptor.

PI3K and Akt signaling pathways are involved in MIF-mediated αvβ3 integrin 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 [Bibollet-Bahena and Almazan, 2009; Horowitz et al., 2004; Huang et al., 2009]. We examined whether MIF stimulation also enhanced PI3K activation. Stimulation of JJ012 cells with MIF led to a significant increase in phosphorylation of p85 (Fig. 2A). MIF-induced migration and integrin expression of chondrosarcoma cells were greatly reduced by treatment with PI3K inhibitors Ly294002 and wortmannin (Fig. 2B-E). In addition, transfection of cells with a p85 mutant also inhibited MIF-induced migration and αvβ3 integrin

expression of chondrosarcoma cells (Fig. 2C-E). 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 integrin up-regulation, we determined Akt Ser473 phosphorylation in response to MIF treatment. As shown in Fig. 3A, treatment of JJ012 cells with MIF resulted in time-dependent phosphorylation of Akt Ser473. Pretreatment of cells with Akt inhibitor antagonized MIF-induced migration and integrin expression of chondrosarcoma cells (Fig. 3B-D). In addition, the Akt mutant also reduced MIF-mediated cell migration and integin up-regulation (Fig. 3B-D). Furthermore, transfection of cells with p85 mutant reduced MIF-induced Akt phosphorylation (Fig. 3E). Based on these results, it appears that MIF acts through the PI3K and Akt-dependent signaling pathway to enhance αvβ3 integrin expression and cell migration in human chondrosarcoma cells.

Involvement of NF-κB in MIF-induced cell migration and integrin 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 integrin expression caused by MIF, the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) was used. PDTC has been reported to inhibit NF-κB activation in intact cells [Schreck et al., 1992]. Figure 4A-C show that PDTC inhibited the enhancement of migration and αvβ3 integrin expression induced by MIF. Furthermore, pretreatment of cells with an IκB protease inhibitor TPCK also antagonized the potentiating action of MIF (Fig. 4A-C). These results indicate that NF-κB activation is important for MIF-induced cancer cell migration and integrin expression.

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 MIF for various time intervals resulted in p65 Ser^{536} phosphorylation (Fig. 4D). Pretreatment of cells with Ly294002, wortmannin, or Akt inhibitor reduced MIF-induced p65 phosphorylation (Fig. 4E). To directly assess NF-κB activation after MIF treatment, chondrosarcoma cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 4F, MIF treatment of chondrosarcoma cells for 24 h caused increase in κB-luciferase activity. In addition, the MIF-induced increase in κB-luciferase activity was also inhibited by treatment with Ly294002, wortmannin, or Akt inhibitor (Fig. 4F). Cotransfection of cells with p85 or Akt mutant blocked MIF-enhanced κB-luciferase activity (Fig. 4G). Taken together, these data suggest that activation of PI3K and Akt are required for MIF-induced NF-κB activation in human chondrosarcoma cells.

Increase of MIF and αvβ3 integrin expression in migration-prone cells

To confirm effects of MIF on cell migration and αvβ3 integrin expression in human chondrosarcoma cells, JJ012 sublines with higher cell mobility were selected as described in Materials and Methods. Migration-prone subline JJ012(S10) had higher cell motility compared with original JJ012(S0) (Fig. 5A). Moreover, the JJ012(S10) subline showed markedly increased protein expression of MIF, αv, and β3 integrin (Fig. 5B). Migration-prone sublines were also established from other chondrosarcoma cell lines (SW1353 cells). The migration-prone subline SW1353(S10) had higher cell motility and MIF, αv, and β3 integrin expression compared with the original SW1353(S0). However, there were no significant differences in proliferation ability between the parental and migration-prone sublines (data not shown).

To further confirm that MIF mediated cell migration and αvβ3 integrin expression in human chondrosarcoma cells, a MIF-shRNA expression cell lines was established. The MIF expression level in stable transfectants was assessed by Western blotting, which showed a dramatic reduction of MIF expression in JJ012/MIF-shRNA cells (Fig. 6A). Based on the reported activity of MIF as a mitogen in human cells [Gesser et al., 2011], 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 MIF 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 MIF expression inhibited the migratory ability by approximately 70% in JJ012 cells (Fig. 6A). In addition, knockdown MIF also reduced αv and β3 integrin expression in JJ012 cells (Fig. 6B). Therefore, human chondrosarcoma cells with higher ability to migrate expressed more MIF and αvβ3 integrin.

DISCUSSION

MIF activates target genes involved in differentiation, survival, apoptosis and proliferation. It also manipulates the process of tumorigenesis and tumor progression [Meyer-Siegler et al., 2002; Takahashi et al., 1998]. MIF is implicated in the pathogenesis of several tumors, including prostate, breast, and colon cancer, as well as in melanoma and glioblastoma growth [Meyer-Siegler et al., 2002; Rendon et al., 2007; Shimizu et al., 1999]. MIF is thought to affect tumor progression by (a) stimulating cancer proliferation, (b) inhibiting apoptosis, (c) enhancing vascularization, or (d) inhibiting the lysis of tumor cells by natural killer cells [Rendon et al., 2007; Repp et al., 2000]. However, the effect of MIF on migration activity in chondrosarcoma is mostly unknown. In the present study, we treated human chondrosarcoma cells with MIF to examine its effect on tumor cell migration. We found that exogenous MIF increased migration of chondrosarcoma cells, whereas the overexpression of MIF shRNA inhibited the migratory ability by approximately 70% in JJ012 cells. Moreover, the generation of migration-prone sublines JJ012(S10) demonstrated that cells with increasing migration ability had higher MIF and αvβ3 integrin expression levels. Our data provided the evidence that the expression of MIF is associated with a metastatic phenotype of chondrosarcoma cells.

MIF engages in high-affinity interactions with the chemokine receptors CXCR2 and CXCR4, and noncognate binding of MIF to CXCR2 and CXCR4 is the molecular basis for MIF-triggered recruitment of monocytes and T cells, respectively, into the atherogenic vessel wall [Bernhagen et al., 2007]. In this study, we found that CXCR2 and CXCR4 mAbs reduced MIF-induced cell migration (Supplementary Fig. S1). In addition, CXCR2 and CXCR4 mAbs also blocked the MIF-induced increase in integrin expression (Supplementary Fig. S1). Therefore, the interaction between MIF and CXCR2/CXCR4 could be important for cancer migration and αvβ3 integrin expression in human chondrosarcoma cells.

Integrins link the ECM 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 [Shattil et al., 2010]. In the present study, we used flow cytometry to demonstrate that MIF increases αvβ3 integrin expression, which plays an important role during tumor metastasis. Furthermore, MIF also increased the mRNA levels of αv and β3 integrins. We also used the anti-αvβ3 integrin antibody to examine the role of αvβ3 integrin and found that it inhibited MIF-induced cancer cell migration, indicating the possible involvement of αvβ3 integrin in MIF-induced migration of the chondrosarcoma cells. This was further confirmed the data showing that the cyclic RGD inhibited the enhancement of migration activity by MIF, providing further evidence of involvement of αvβ3 integrin in MIF-mediated induction of cancer migration.

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 MIF-enhanced the p85 subunit phosphorylation in human chondrosarcoma cells. Pretreatment of cells with PI3K inhibitors Ly294002 or wortmannin antagonized an increase in migration and αvβ3 integrin expression by MIF stimulation. This was further confirmed by the result that the dominant-negative mutant of p85 inhibited the enhancement of migration by MIF. Moreover, we also found that MIF activated Akt Ser473 phosphorylation, whereas the Akt inhibitor and Akt mutant inhibited MIF-mediated cell migration and αvβ3 integrin expression. Our data indicate that PI3K/Akt signaling could play an important role in the expression of integrin and migration of human chondrosarcoma cells.

NF-κB has been shown to control the induced transcription of αvβ3 integrin in human cancer cells [Su et al., 2009]. The results of this study show that NF-κB activation contributes to MIF-induced αvβ3 integrin expression and migration in human chondrosarcoma cells, and that the inhibitors of the NF-κB-dependent

signaling pathway, including PDTC or TPCK inhibited MIF-induced αvβ3 integrin expression and cancer migration. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that MIF-induced an increase in NF-κB activity. p65 is phosphorylated at Ser^{536} by a variety of kinases through various signaling pathways, and this enhances the p65 transactivation potential. The results of this study showed that MIF increased the phosphorylation of p65. On the other hand, Ly294002, wortmannin, and Akt inhibitor reduced MIF-mediated p65 phosphorylation and NF-κB promoter activity. Our data indicated that PI3K, Akt, and NF-κB pathways might play important role in the expression of αvβ3 integrin and cell migration of human chondrosarcoma cells.

Unlike other mesenchymal malignancies, such as osteosarcoma and Ewing's sarcoma, that have seen dramatic increases in long-term survival with the advent of systemic chemotherapy, chondrosarcoma continues to have a poor prognosis due to the absence of an effective adjuvant therapy [Fong et al., 2007]. The metastatic potential of conventional chondrosarcomas correlates well with the histologic grade of the tumor. However, due to the relatively indolent growth rates of many low- and moderate-grade chondrosarcomas, ~15% of patients dying from metastatic disease do so >5 years after initial diagnosis [Fong et al., 2007]. Therefore, it is important to develop an effective adjuvant therapy for prevention of metastasis in chondrosarcoma. MIF expression has been shown to closely correlate with tumor aggressiveness and metastatic potential, and not surprisingly, MIF has been shown to be a poor prognostic factor in patients with lung adenocarcinoma and breast and hepatocellular carcinomas [Takahashi et al., 1998; Xu et al., 2008]. However, we do not have clinical data to show the expression of MIF in chondrosarcoma and healthy patients. Whether MIF acts through autocrine or paracrine pathways to induce cell motility should be examined further. We hypothesized that MIF could promote metastasis of chondrosarcoma cells. We found that MIF increased the migration of these cells and determined that one of the mechanisms underlying MIF-directed migration was the transcriptional upregulation of αvβ3 integrin and activation of the PI3K, Akt, and

NF-κB pathways.

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FIGURE LEGENDS

Fig. 1 MIF-directed migration of human chondrosarcoma cells involves up-regulation of αvβ3 integrin

(A) JJ012 cells were incubated with MIF for 24 h, and *in vitro* migration was measured by Transwell after 24 hr. (B) JJ012 cells were incubated with MIF (30 ng/ml) for 24 h, and the mRNA levels of αv, α2, α5, β1, β3, or β5 integrin was determined using qPCR. (C) Cells were incubated with MIF (30 ng/ml) for 24 h, and the cell surface expression of αvβ3 integrin was determined using flow cytometry. (D) Cells were pretreated with αvβ3, α2β1 or α5β1 monoclonal antibody (10 μg/ml) for 30 min followed by stimulation with MIF. The *in vitro* migration activity measured after 24 h. (F) Cells were pretreated with cyclic RGD (100 nM) or cyclic RAD (100 nM) for 30 min followed by stimulation with MIF. The in vitro migration activity was measured after 24 h. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with MIF-treated group.

Fig. 2 PI3K is involved in MIF-induced migration and integrin up-regulation in human chondrosarcoma cells.

(A) JJ012 cells were incubated with MIF (30 ng/ml) for indicated time intervals, and p-p85 was examined by Western blot analysis. (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 h followed by stimulation with MIF. The *in vitro* migration and integrin expression was examined by Transwell, qPCR, and flow cytometry analysis. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with MIF-treated group.

Fig. 3 Akt is involved in MIF-induced migration and integrin up-regulation in human chondrosarcoma cells.

(A) JJ012 cells were incubated with MIF (30 ng/ml) for indicated time intervals, and p-Akt was examined by Western blot analysis. (B-D) 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 MIF. The *in vitro* migration and integrin expression was examined by Transwell, qPCR, and flow cytometry analysis. (E) Cells were transfected with p85 mutant for 24 h followed by stimulation with MIF for 30 min. The p-Akt expression was examined by Western blotting. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with MIF-treated group.

Fig. 4 MIF induces cell migration and integrin up-regulation through NF-κB.

(A-C) Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with MIF. The *in vitro* migration and integrin expression was examined by Transwell, qPCR, and flow cytometry analysis. (D) JJ012 cells were incubated with MIF (30 ng/ml) for indicated time intervals, and p-p65 was examined by Western blot analysis. (E) Cells were pretreated for 30 min with Ly294002, wortmannin, and Akt inhibitor followed by stimulation with MIF for 60 min, and p-p65 expression was examined by Western blotting. JJ012 cells were pretreated with Ly294002, wortmannin, and Akt inhibitor for 30 min (F) or transfected with mutant of p85 and Akt (G) before exposure to MIF. 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 MIF-treated group.

Fig. 5 Upregulation of MIF and αvβ3 integrin in migration-prone cells.

(A&C) After 10 rounds of selection of JJ012 or SW1353 cells by cell culture

insert system, migration-prone subline (S10) exhibited more migration than original JJ012 or SW1353 cells (S0). (B&D) S10 expressed more MIF, αv, and β3 integrin protein expression than original JJ012 or SW1353 cells (S0). Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control.

Fig. 6 Knockdown of MIF inhibited the migratory ability in chondrosarcoma cells. (A) The *in vitro* migration activity of JJ012/control-shRNA and JJ012/MIF-shRNA cells was measured with the Transwell. (B) The protein levels of MIF, αv and β3 integrin in JJ012/control-shRNA and JJ012/MIF-shRNA cells was examined by western blot analysis. Results are expressed as the mean ± S.E.

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