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# Stromal cell-derived factor-1 enhances motility and integrin up-regulation through CXCR4, ERK and NF-κB-dependent pathway in human lung cancer cells

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ABSTRACT

The chemokine stromal-derived factor-1α (SDF-1α) and its receptor, CXCR4, play a crucial role in adhesion and migration of human cancer cells. Integrins are the major adhesive molecules in mammalian cells. Here we found that SDF-1α increased the migration and cell surface expression of β1 or β3 integrin in human lung cancer cells (A549 cells). CXCR4-neutralizing antibody, CXCR4 specific inhibitor (AMD3100) or small interfering RNA against CXCR4 inhibited the SDF-1α-induced increase in the migration of lung cancer cells. Stimulation of cells with SDF-1α caused an increase in extracellular signal regulated kinase (ERK) phosphorylation in a time-dependent manner. In addition, treatment of A549 cells with ERK inhibitor (PD98059), NF-κB inhibitor (PDTC) or IκB protease inhibitor (TPCK) inhibited SDF-1α-induced cells migration and integrins expression. Treatment of A549 cells with SDF-1α induced IκB kinase α/β (IKK α/β) phosphorylation, IκBα phosphorylation, IκBα degradation, p65 $Ser^{536}$ phosphorylation, and κB-luciferase activity. The SDF-1α-mediated increases in IKK α/β phosphorylation, p65 $Ser^{536}$ phosphorylation, and κB-luciferase activity were inhibited by PD98059 and ERK2 mutant. Taken together, these results suggest that SDF-1α acts through CXCR4 to activate ERK, which in turn activates IKKα/β and NF-κB, resulting in the activations of β1 and β3 integrins and contributing the migration of lung cancer cell.



## 1. Introduction

Lung cancer ranks as the leading cause of death from cancer worldwide. Approximately 80% of lung cancers can be histologically classified as non-small cell lung cancers (NSCLCs). Most patients present with locally advanced (37%) or metastatic (38%) disease at the time of diagnosis [1]. The average 5-year survival rate of these patients with advanced NSCLC remains extremely poor despite advances in chemotherapy. Thus, the high invasiveness of NSCLC to regional

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*Abbreviations:* SDF-1, stromal-derived factor-1; ERK, extracellular signal regulated kinase; IKKα/β, IκB kinase α/β; NSCLCs, non-small cell lung cancers; ECM, extracellular matrix; siRNA, small interference RNA; RT-PCR, mRNA analysis by reverse transcriptase-polymerase chain reaction.

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lymph nodes, liver, adrenal glands, contralateral lung, brain, and bone marrow, etc. may play a key role in its biological virulence [1,2].

The invasion of tumor cells is a complex, multistage process. To facilitate the cell motility, invading cells need to change the cell–cell adhesion properties, rearrange the extracellular matrix (ECM) environment, suppress anoikis and reorganize their cytoskeletons [3]. 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 [4]. 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 [5]. Integrin has been heavily implicated in tumor development [6,7], was correlated to reduced patient survival in colon carcinoma and melanoma [8,9], and has been associated with breast cancer cell metastasis to bone [10]. In addition, αvβ3 integrin has been implicated in prostate cancer progression with effects on angiogenesis, survival and invasion [11,12]. *In vitro* studies have found that integrins facilitated prostate cancer cell adhesion and migration through several ECM substrates [11], and transendothelial migration [13].

Chemokines, structurally related, small (8–14 kDa) polypeptide signaling molecules, can bind to and activate a family of seven-transmembrane G-protein-coupled receptors, the chemokine receptors [14,15]. Chemokines are expressed by many tumor types and can promote mitosis, modulate apoptosis, survival and angiogenesis [16,17]. Interaction between the chemokine receptor CXCR4 and its ligand, stromal-cell-derived factor 1α (SDF-1α or CXCL12), has been found to play an important role in tumorigenicity, proliferation, metastasis and angiogenesis in many cancers, such as breast cancer, melanoma, glioblastoma, pancreatic cancer, cholangiocarcinoma and basal cell carcinoma cells [18–23]. Although the mechanisms underlying SDF-1α/CXCR4-mediated tumor invasion have been studied in some cancers [18,22,23], the role of SDF-1α/CXCR4 in the process of lung cancer cells migration remains largely unknown.

Previous studies have shown that SDF-1α/CXCR4 interactions modulate cell migration and invasion in several cancer cells [22,23]. SDF-1α-mediated invasion may involve activation of integrins receptors [24,25]. However, the effect of SDF-1α on integrins expression and migration activity in human lung cancer cells is mostly unknown. Here we found that SDF-1α increased the migration and the expression of integrins of human lung cancer cells. In addition, ERK, IKKα/β and NF-κB signaling pathways may be involved in the increase of integrin expression and cells migration by SDF-1α.

## 2. Materials and methods

### 2.1. Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for SDF-1α, p-ERK, ERK, IKKα/β, IκBα, p-IκBα, small interference RNA (siRNA) directed against β1 and β3 integrin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PD98059, TPCK and PDTC were purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal antibody specific for CXCR4 was purchased from R&D Systems (Minneaplis, MN, USA). Rabbit polyclonal antibody specific for phosphor-IKKα/β ($Ser^{180/181}$) and phosphor-p65 ($Ser^{536}$) were purchased from Cell Signaling (Danvers, MA, USA). The recombinant human SDF-1α was purchased from PeproTech (Rocky Hill, NJ, USA). The ERK2 dominant negative mutant was provided by Dr. M. Cobb (South-Western Medical Center, Dallas, TX, USA). The IKKα(KM) and IKKβ(KM) mutants were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA, USA). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. Cell culture

The human lung adenocarcinoma cell lines (A549) were obtained from the American Type Culture Collection. The cells were maintained in RPMI-1640 medium which was supplemented with 20 mM HEPES and 10% heat-inactivated FCS, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C with 5% $CO_2$.

### 2.3. Migration assay

The migration assay was performed using Transwell (Costar, NY; pore size, 8-μm) in 24-well dishes. Before performing the migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the CXCR4 neutralizing antibody 12G5, isotype control antibody, AMD 3100, PD98059, PDTC, TPCK or vehicle control (0.1% DMSO). Approximately $2 \times 10^4$ cells in 100 μl of serum-free RPMI-1640 medium were placed in the upper chamber, and 500 μl of the same medium containing 100 ng/ml SDF-1α 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 the SDF-1α treatment (corrected invading cell number = counted invading cell number/percentage of viable cells) [26]. In addition, the average of invading cells in each experiment (control group) was $525 \pm 42$ (cells/well).

### 2.4. Flow cytometric analysis

Human lung cancer 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 rinsed in PBS, the cells were

incubated with rabbit anti-human antibody against β1, β3 integrin or CXCR4, (1:100) for 1 h at 4 °C. Cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:150; Leinco Tec. Inc., St. Louis, MO, USA) for 45 min and analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences) [27]. The values given are crude fluorescence intensity recording by flow cytometry.

### 2.5. Western blot analysis

The cellular lysates were prepared as described previously [28]. 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 IκBα, p-IκBα, IKKαβ or p-ERK (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

### 2.6. Generation of DNA constructs encoding a siRNA against human CXCR4

Oligonucleotides against human *CXCR4* genes were generated and cloned into a pSilencer 3.1-H1 vector (Ambion, Austin, TX, USA), as described [26,29]. We used the Lipofectamine 2000 reagent to transfect the cells with pSilencer 3.1-H1-siCXCR4 or pSilencer 3.1-H1-siCXCR4-mut. Twenty-four hours after transfection, cells were replated in RPMI-1640 with 10% fetal calf serum. siCXCR4 but not siCRCR4-mut specific inhibited the expression of mRNA and protein level of CXCR4 in our previously reported [29].

### 2.7. Transfection and reporter gene assay

Human lung caner cells were co-transfected with 0.8 μg κB-luciferase plasmid, 0.4 μg β-galactosidase expression vector. A549 cells were grown to 80% confluent in 12 well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. RPMI-1640 containing 20% FCS was added 4 h later. After 24 h transfection, the cells were then incubated with the indicated agents. After further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector.

### 2.8. mRNA analysis by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from A549 cells using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reversely transcribed into cDNA using oligo(dT) primer, then amplified for 33 cycles using two oligonucleotide primers:

| |
|---|
| β1 integrin sense AGGATTACTTCGGACTTCAGA and anti-sense CTTTGGCATTCACATTCA |
| β3 integrin sense TGCTCATTGGCCTTGCCGCCCTGC and anti-sense ACTATTCGTCAGTAGGAGTCTAGT |
| GAPDH sense ACCACAGTCCATGCCATCAC and anti-sense TCCACCACCCTGTTGCTGTA |

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

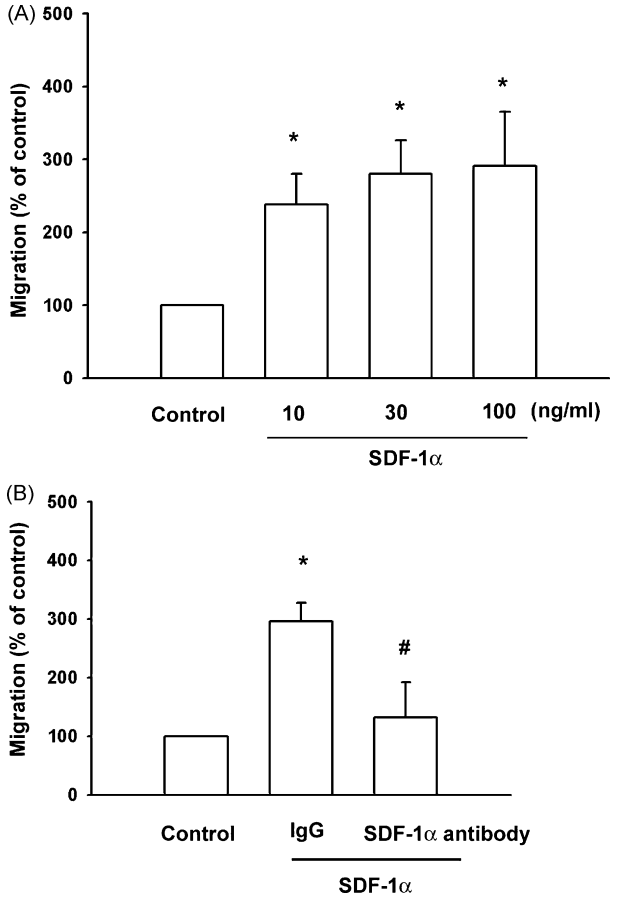


**Fig. 1 – SDF-1α induced the migration activity of human lung cancer cells. A549 cells were incubated with various concentrations of SDF-1α, and *in vitro* migration activities measured with the Transwell after 24 h showed all supported the A549 cell migrations in a dose-dependent way (A). Cells were pretreated with SDF-1α antibody or IgG (10 μg/ml) for 30 min followed by stimulation with SDF-1α (100 ng/ml). The *in vitro* migration activity measured after 24 h showed that SDF-1α antibody could inhibit the activities (B). Results are expressed as the mean ± S.E. *$p < 0.05$ compared with control; #$p < 0.05$ compared with SDF-1α-treated group.**

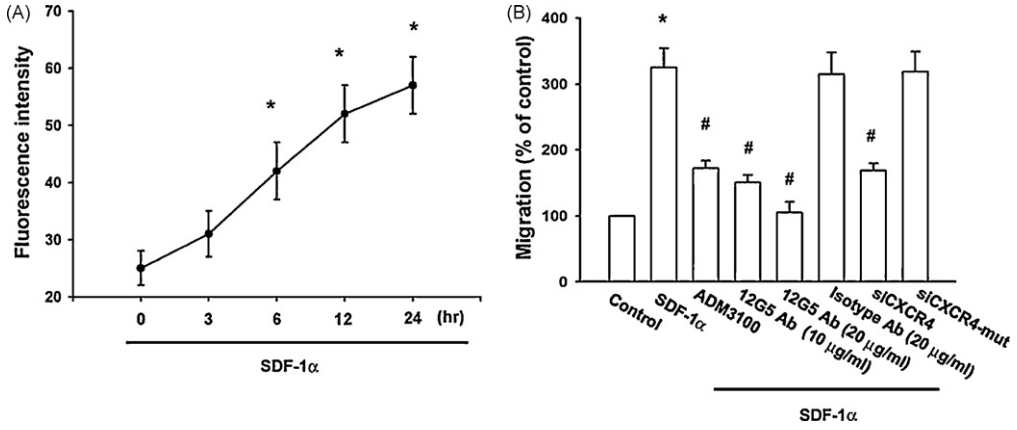


**Fig. 2 – SDF-1α/CXCR4-directed migrated of human lung cancer cells. Cells were incubated with SDF-1α (100 ng/ml) for indicated time intervals, and the cell surface expression of CXCR-4 was determined using flow cytometer (A). A549 cells were pretreated with CXCR-4-neutralizing 12G5 antibody, AMD3100 and isotype antibody for 30 min or transfected with siCXCR4 or siCXCR4-mut for 24 h followed by stimulation with SDF-1α (100 ng/ml). The *in vitro* migration activity measured after 24 h showed that CXCR-4-neutralizing 12G5 antibody, AMD3100 and siCXCR4 siRNA could inhibit the activities (B). Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with SDF-1α-treated group.**

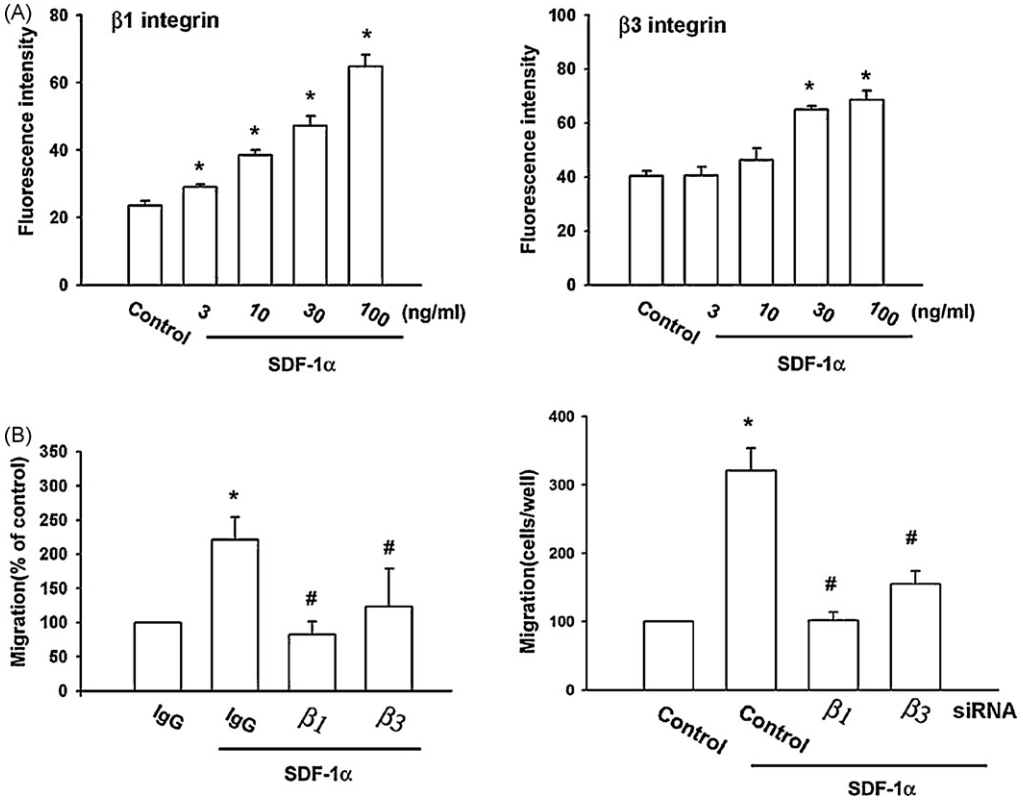


**Fig. 3 – SDF-1α/CXCR4-directed migration activity of human lung cancer cells involves up-regulation of β1 and β3 integrins. Cells were incubated with SDF-1α (100 ng/ml) for various concentrations, and the cell surface expression of β1 or β3 integrin was determined using flow cytometer (A). A549 cells were pretreated with β1 or β3 monoclonal antibodies and IgG (10 μg/ml) for 30 min or transfected with β1, β3 integrin or control siRNA for 24 h followed by stimulation with SDF-1α (100 ng/ml). The *in vitro* migration activity measured after 24 h showed that β1 or β3 monoclonal antibodies and siRNA could inhibit the activities (B). Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with SDF-1α-treated group.**

PCR products were then separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide.

### 2.9. Statistics

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

## 3. Results

### 3.1. SDF-1α/CXCR4 interaction directs the migration of lung cancer cells

SDF-1 is a powerful chemoattractant cytokine that stimulates directional migration and invasion of human cancer cells. The SDF-1α for lung cancer cells migration was examined using the Transwell assay with correction of SDF-1α induced proliferation effects on human lung cancer cells [26]. SDF-1α dose-dependently directed lung cancer cells (A549 cell) migration (Fig. 1A). In addition, pretreatment of cells with the SDF-1α-neutralizing antibody (10 μg/ml) inhibited SDF-1α-induced cells migration (Fig. 1B). Interaction of SDF-1 with its specific receptor CXCR4 on the surface of basal cancer cell has been reported to induce cancer invasion [26]. We then examined whether SDF-1/CXCR4 interaction is involved in the signal-transduction pathway leading to cancer migration caused by SDF-1α. Human lung cancer cells were treated with SDF-1α for different time intervals, and the results from flow cytometry indicated that SDF-1α significantly increased the cell surface expression of CXCR4 time-dependently (Fig. 2A). Pretreatment of A549 cells for 30 min with CXCR4-specific chemical inhibitor AMD3100 (500 ng/ml), CXCR-4-neutralizing antibody (12G5) (10 μg/ml) but not mouse monoclonal immunoglobulin isotype control (isotype Ab) (20 μg/ml) antagonized the SDF-1α-induced cancer cell migration (Fig. 2B). High concentration of 12G5 (20 μg/ml) completely inhibited the SDF-1α-induced cancer cell migration (Fig. 2B). In addition, treatment of cells with AMD3100 (500 ng/ml), 12G5 (20 μg/ml) or isotype Ab (20 μg/ml) did not affect cell viability, which was assessed by the using MTT assay (data not shown). Transient transfection of small interfering RNA against CXCR4

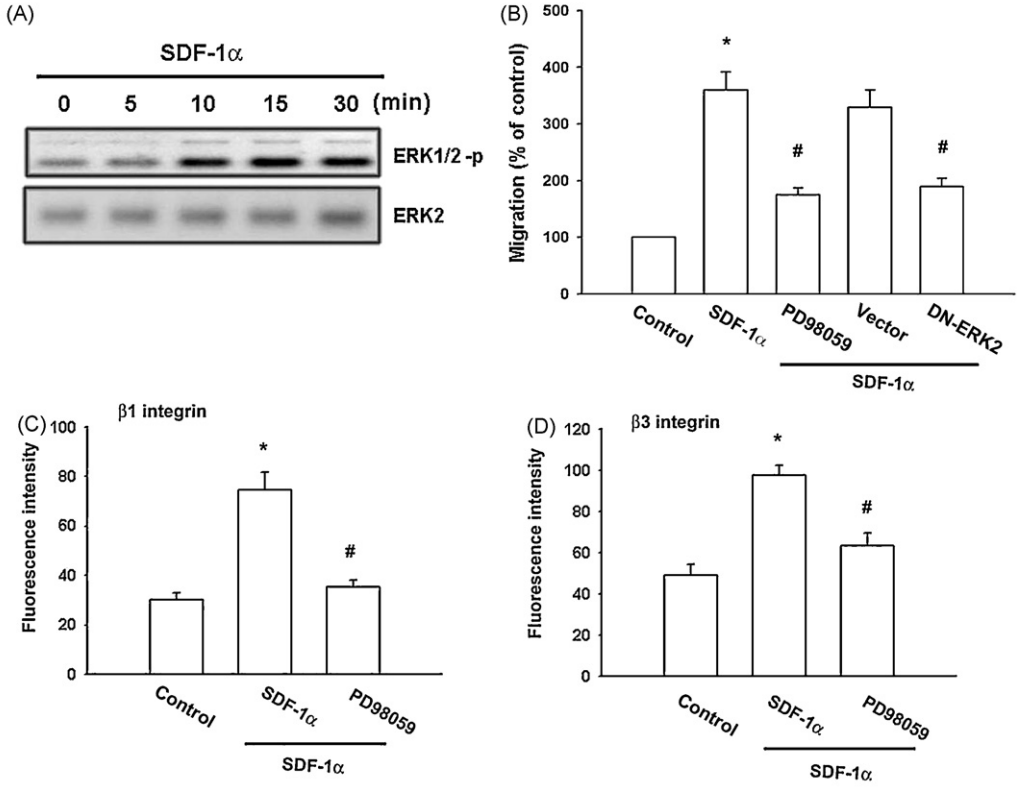


**Fig. 4 – ERK is involved in SDF-1α-mediated migration and integrin up-regulation in human lung cancer cells. (A) Cells were incubated with SDF-1α (100 ng/ml) for indicated time intervals, and p-ERK expression was determined by Western blot analysis. SDF-1α activated the extracellular signal-related kinase 1/2 (ERK1/2) pathway in A549 cells, as evidenced by the increase in phosphorylated p42 and p44 (p-ERK1/2) at 5 min and peaked at 30 min. (B) Cell were pretreated for 30 min with PD98059 (30 μM) or transfected with dominant negative (DN) mutant of ERK for 24 h followed by stimulation with SDF-1α (100 ng/ml), and *in vitro* migration was measured with the Transwell after 24 h. (C and D) Cell were pretreated for 30 min with PD98059 (30 μM) followed by stimulation with SDF-1α (100 ng/ml) for 24 h, and the cell surface β1 or β3 integrin was measured by using flow cytometry. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with SDF-1α-treated group.**

(siCXCR4), but not a mutant form of siCXCR4 (siCXCR4-mut), effectively inhibited the migration of lung cancer cell directed by SDF-1α (Fig. 2B). These data suggest that SDF-1/CXCR4 interaction plays a key role in lung cancer cell migration.

### 3.2. SDF-1α/CXCR4-directed lung cancer cells migration involves integrin up-regulation

Previous studies have shown significant expression of β1 and β3 integrin in human lung cancer cells [30,31]. We, therefore, hypothesized that integrins may be involved in SDF-1α/CXCR4-directed lung cancer cells migration. Flow cytometry analysis showed that SDF-1α-induced the cell surface expression of β1 and β3 integrin dose-dependently (Fig. 3A). Pretratment of cells for 30 min with anti-β1 and β3 monoclonal antibody (mAb) (10 μg/ml) or transfected with siRNA against β1 and β3 integrin for 24 h markedly inhibited the SDF-1α-induced cancer migration (Fig. 3B). In addition, treatment of cells with β1 or β3 mAb (10 μg/ml) did not affect cell viability, which was assessed by the using MTT assay (data not shown). These data suggest that SDF-1α-induced cancer migration may occur via activation of β1 and β3 integrin receptor.

### 3.3. ERK1/2 and NF-κB signaling pathways are involved in the SDF-1α-mediated integrin up-regulation and migration of lung cancer cells

As SDF-1α/CXCR4 interaction has been shown to activate extracellular signal-related kinase 1/2 (ERK1/2) pathway in various cancer cell lines [22,23,26]. We performed Western blot analysis to elucidate the signal-transduction mechanisms involved in the SDF-1α-induced cancer cells migration. SDF-1α activated the ERK1/2 pathway in A549 cells, as evidenced by the increase in phosphorylated p42 and p44 (p-ERK1/2) at 5 min and peaked at 15–30 min (Fig. 4A). On the other hand, treatment of cells with SDF-1α did not affect the phosphorylation of p38 and JNK (Supplemental data Figure S1). SDF-1α-induced the migration and β1 or β3 integrin expression of A549 cells were greatly reduced by treatment with PD98059 (30 μM), a specific ERK inhibitor (Fig. 4B–D). Treatment of cells with PD98059 (30 μM) did not affect cell viability, which was assessed by the using MTT assay (data not shown). In addition, transfection of cells with ERK2 mutant also inhibited SDF-1α-induced the migration of lung cancer cells (Fig. 4B). As previously mentioned, NF-κB activation is necessary for the migration and invasion of human cancer cells [32]. To examine

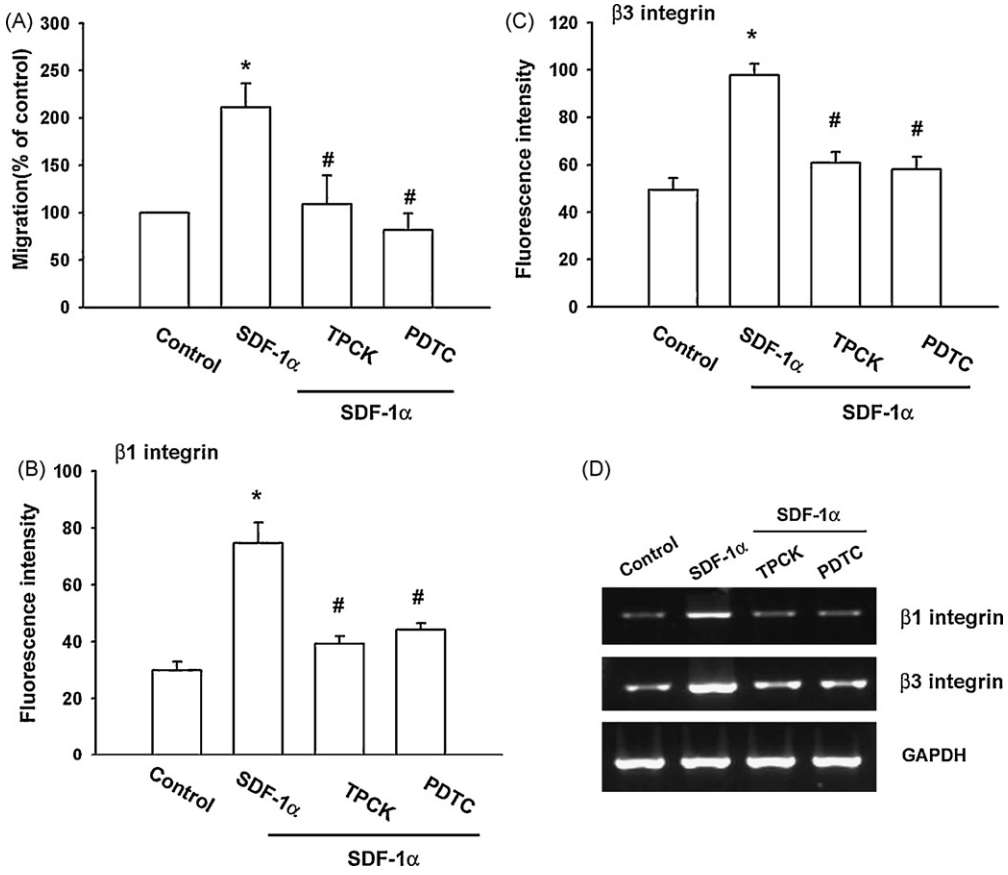


**Fig. 5 – SDF-1α induces cells migration and integrin up-regulation through NF-κB. (A) Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with SDF-1α (100 ng/ml), and *in vitro* migration was measured with the Transwell after 24 h. (B and C) Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with SDF-1α (100 ng/ml) for 24 h, and the cell surface β1 or β3 integrin was measured by flow cytometry. (D) A549 cells were pretrated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with SDF-1α (100 ng/ml) for 24 h, and the mRNA level of β1 or β3 integrin was measured by RT-PCR analysis. Results are expressed as the mean ± S.E. *$p < 0.05$ compared with control; #$p < 0.05$ compared with SDF-1α-treated group.**

whether NF-κB activation is involved in SDF-1α-induced cancer migration, an NF-κB inhibitor, PDTC, was used. Fig. 5A shows that PDTC (10 μM) inhibited SDF-1α-induced lung cancer cell migration. Furthermore, A549 cells pretreated with TPCK (3 μM), an IκB protease inhibitor, also reduced SDF-1α-induced cancer cell migration (Fig. 5A). Treatment of cells with PDTC (10 μM) or TPCK (3 μM) did not affect cell viability, which was assessed by the using MTT assay (data not shown). In addition, treatment of cells with PDTC or TPCK also antagonized SDF-1α-induced the expression of β1 and β3 integrins (Fig. 5B and C). Furthermore, pretreatment of cells with PDTC or TPCK inhibited SDF-1α-induced the mRNA expression of β1 and β3 integrins (Fig. 5D). These results indicated that NF-κB activation is important for SDF-1α-induced cancer cell migration and the expression of β1 and β3 integrins.

### 3.4. *SDF-1a causes an increase in IKK α/β phosphorylation, IκBα phosphorylation and IκBα degradation*

We further examined the upstream molecules involved in SDF-1α-induced NF-κB activation. Stimulation of cells with SDF-1α induced IKKα/β phosphorylation in a time-dependent manner (Fig. 6A). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited the SDF-1α-induced cancer cells migration (Fig. 6B). These data suggest that IKKα/β activation is involved in SDF-1α-induced the migration activity of human lung cancer cells. Treatment with lung cancer cells with SDF-1α also caused IκBα phosphorylation and IκBα degradation in a time-dependent manner (Fig. 6C). Previous studies showed that p65 Ser$^{536}$ phosphorylation increases NF-κB transactivation [33,34], and the antibody specific against phosphorylated p65 Ser$^{536}$ was used to examine p65 phosphorylation. Treatment of A549 cells with SDF-1α for various time intervals resulted in p65 Ser$^{536}$ phosphorylation which began at 10 min and was sustained to 120 min (Fig. 6D, upper panel). The protein levels of p65 were not affected by SDF-1α treatment (Fig. 6D, bottom panel). To directly determine NF-κB activation after SDF-1α treatment, A549 cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 6E, SDF-1α (10–100 ng/ml) treatment of A549 cells for 24 h caused a concentration-dependent increase in κB-luciferase activity.

### 3.5. *ERK signal-transduction-mediated SDF-1a-induced IKK α/β phosphorylation, p65 phosphorylation and κB luciferase activity*

To further investigate whether SDF-1α-induced IKK α/β phosphorylation, p65 Ser$^{536}$ phosphorylation, and NF-κB

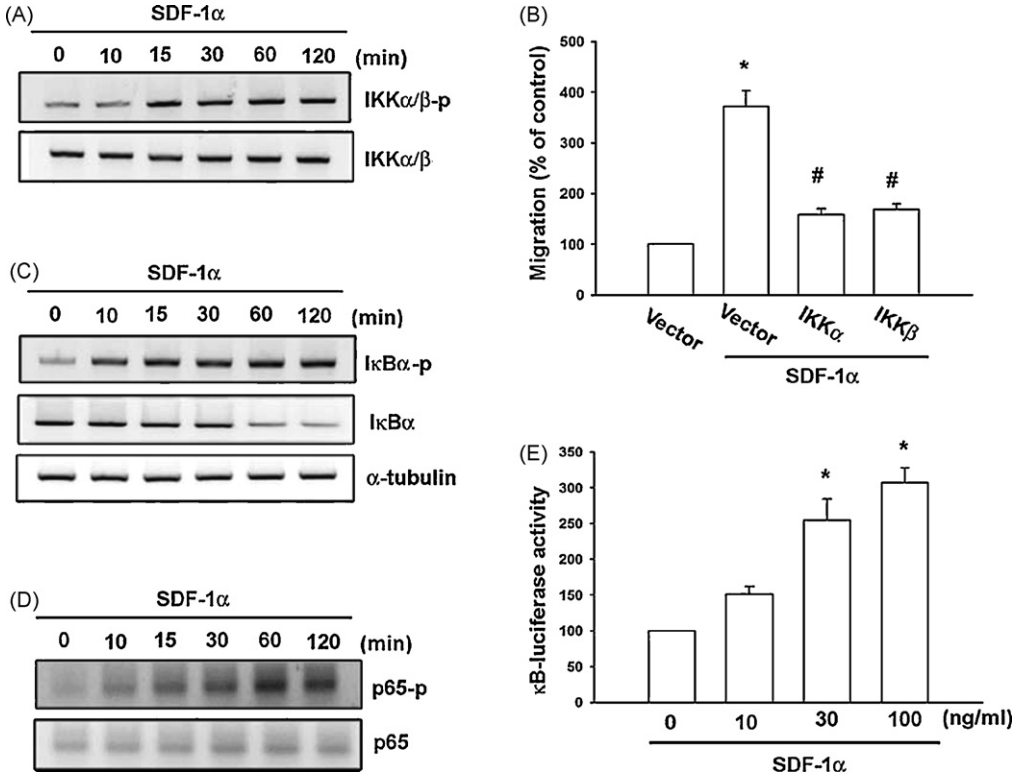


**Fig. 6 – SDF-1α induces IKK α/β phoshorylation, IκBα phosphorylation, IκBα degradation and p65 Ser$^{536}$ phosphorylation in lung cancer cells. (A) Cells were incubated with SDF-1α (100 ng/ml) for indicated time intervals, and p-IKKα/β expression was determined by Western blot analysis. (B) Cells were transfected with dominant negative (DN) mutant of IKKα or IKKβ for 24 h followed by stimulation with SDF-1α (100 ng/ml), and *in vitro* migration was measured with the Transwell after 24 h. (C and D) Cells were incubated with SDF-1α (100 ng/ml) for indicated time intervals, and IκBα, p-IκBα and p65 Ser$^{536}$ expression was determined by Western blot analysis. (E) Cells were transfected with κB promoter plasmid for 24 h, and were then incubated with SDF-1α (10-100 ng/ml) for 24 h. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with SDF-1α-treated group.**

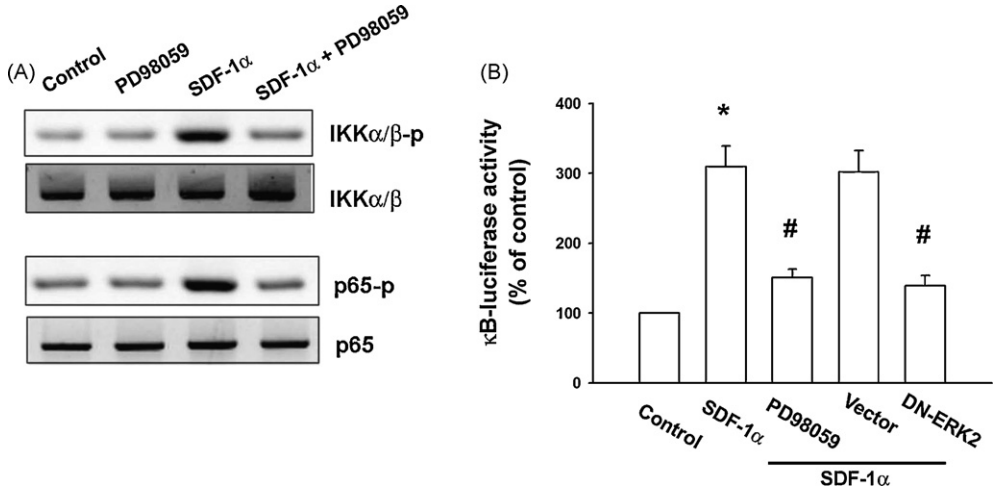


**Fig. 7 – ERK inhibitor antagonized the SDF-1α-induced IKK α/β phoshorylation, p65 Ser$^{536}$ phosphorylation and κB luciferase activity in lung cancer cells. (A) A549 cells were pretreated with PD98059 (30 μM) for 30 min before treatment with SDF-1α (100 ng/ml) for another 60 min, after which IKKα/β phosphorylation and p65 Ser$^{536}$ phosphorylation were determined by immunoblotting with antibodies specific for phospho-IKKα/β and phospho-p65, respectively. Equal loading in each lane is shown by the similar intensities of IKKα/β and p65, respectively. (B) A549 cells transiently transfected with κB-luciferase plasmid for 24 h were either cotransfected with ERK2 mutant or pretreated with PD98059 (30 μM) for 30 min, before incubation with SDF-1α (100 ng/ml) for 24 h. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with SDF-1α-treated group.**

activation occur through the ERK pathway, A549 cells were pretreated for 30 min with PD98059 (30 μM), which inhibited the SDF-1α-induced increase in IKK α/β phosphorylation as shown in Fig. 7A. Moreover, the SDF-1α-induced increase in p65 Ser$^{536}$ phosphorylation was also attenuated by PD98059 (Fig. 7A). In addition, the SDF-1α-induced increase in κB-luciferase activity was also inhibited by treatment with PD98059 and ERK2 mutant, respectively (Fig. 7B). Taken together, these data suggest that activation of ERK is required for SDF-1α-induced IKK α/β phosphorylation, p65 Ser$^{536}$ phosphorylation, and NF-κB activation in lung cancer cells (Fig. 8).

## 4. Discussion

Lung cancer is by far the most common cause of cancer-related death in the world [35]. This high mortality is probably attributable to early metastasis, principally spreading of malignant cells to many tissues including bone, especially for NSCLC [36]. The mean level of SDF-1 (~50 ng/ml) was observed for healthy patients in bone marrow and joint [48]. Fig. 1A shows that the maximum effect of SDF-1α (100 ng/ml) was nearly reached from the low concentration (10 ng/ml) used. Therefore, the concentration of SDF-1α (10 ng/ml) may be the threshold of the migration activity in human A549 cells. The SDF-1α secreted by the injury site (bone marrow and joint) could mediate the attraction of lung cancer cells to bone. It has been reported that osteoblasts-derived growth factor and chemokines play central roles as trophic factors that attract lung cancer to bone [36]. The SDF-1, constitutively secreted by human osteoblasts, has shown its key role for homing of hematopoietic cells to the marrow [49]. We also found that human osteoblasts secreted the high basal level of SDF-1α [385 ± 38 pg/ml/96 h/10,000 cells ($n$ = 3)] and low basal level of other cytokines including: IL-1β [63 ± 15 pg/ml/96 h/10,000 cells ($n$ = 3)] and TNF-α [95 ± 16 pg/ml/96 h/10,000 cells ($n$ = 3)]. Therefore, the osteoblasts-derived-SDF-1α is more important then IL-1β or TNF-α in the chemomigration of human lung cancer to bone. We hypothesized that SDF-1 and its CXCR4 receptor would help to direct the migration of lung cancer cells. We found that human lung cancer cells express CXCR4. One of the mechanisms underlying SDF-1α/CXCR4 directed migration was transcriptional up-regulation of β1 and β3 integrin and activation of CXCR4, ERK, IKKα/β and NF-κB pathways. Here we only used the *in vitro* migration model to examine the migration activity of human lung cancer cells. Whether the similar pathways are involved in SDF-1α-induced tumor metastasis *in vivo* needs further examination.

Integrins link the extracellular matrix to intracellular cytoskeletal structures and signaling molecules and are implicated in the regulation of a number of cellular processes, including adhesion, signaling, motility, survival, gene expression, growth and differentiation [37,38]. Here we found that SDF-1α increased β1 and β3 integrins expression by using flow cytometry analysis, which plays an important role during tumor migration. Furthermore, SDF-1α also increased the mRNA levels of β1 and β3 integrins. Pretreatment of cells with β1 and β3 mAb or transfection with β1 and β3 antagonized the SDF-1α-increased cell migration. The increase of tumor migration may result from the up-regulation of β1 and β3 integrins (the mRNA and protein levels). Therefore, whether SDF-1α also increased the expression of the ligands (fibronectin, vitronectin or collagen) of integrins needs further

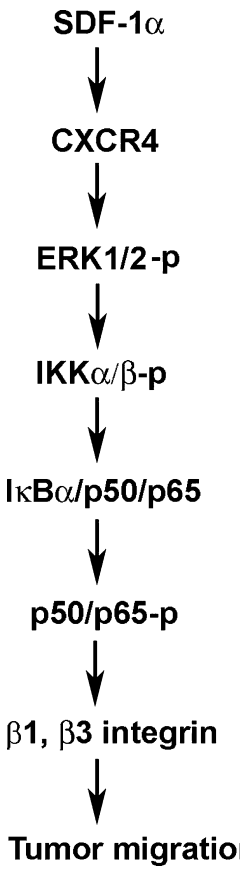


**Fig. 8 – Schematic presentation of the signaling pathways involved in SDF-1α-induced migration and integrins expression of lung cancer cells. SDF-1α activates CXCR4 receptor and ERK pathway, which in turn induces IKKα/β phosphorylation, p65 Ser$^{536}$ phosphorylation, and NF-κB activation, which leads to β1 and β3 integrins expression and increases the migration of human lung cancer cells.**

investigation. Integrins play particularly pivotal roles in cell migration and invasion [38]. The flow cytometry revealed high level expression of β1 and β3 integrins on A549 cells, and lower level of human lung bronchial epithelial cells (NL-20) (Supplemental data Figure S2). Compared with A549 cells, SDF-1α only slightly increased the cells surface expression of β1 and β3 integrins in NL-20 cells. Therefore, the high levels of β1 and β3 integrin expression are correlated with transformation and tumor migration in lung cells. Previous studies have shown that SDF-1α/CXCR4 interactions modulate cell migration and invasion in several cancer cells [22,23,39]. In the present study, we used CXCR4-specific chemical inhibitor AMD3100 and CXCR-4-neutralizing antibody to determine the role of CXCR4 and found that it inhibited SDF-1α-induced cell migration, indicating the possible involvement of CXCR4 in SDF-1α-induced migration and integrin expression in lung cancer cells. This was further confirmed by the result that the small interfering RNA against CXCR4 inhibited the enhancement of migration activity by SDF-1α, indicating the involvement of SDF-1/CXCR4 interaction in SDF-1α-mediated induction of chemomigration and integrins expression.

A variety of growth factors stimulate the expression of integrin via signal-transduction pathways that converge to activate NF-κB complex of transcription factors. ERK1/2 pathway induces the expression of NF-κB transcription factors [40]. We found SDF-1α-enhanced ERK1/2 phosphorylation in human lung cancer cells. Previous studies have revealed that SDF-1α treatment activates ERK1/2 in human basal cell carcinoma cells, astrocytes and glioblastoma cells [19,20,21,41]. The SDF-1α-directed lung cancers migration and integrin expression were effectively inhibited by PD98059. This was further confirmed by the results that the dominant negative mutant of ERK inhibited the enhancement of migration activity by SDF-1α. Recently, P2Y2 receptor was shown to induce redistribution of integin through the activation of the ERK pathway [42]. Matsuo et al. [43] suggested ERK pathway was required for the fibronectin-induced αv integrin expression. Our data indicate that ERK might play an important role in the expression of integrin and migration of human lung cancer cells.

Many NF-κB activation pathways have been reported, and all of them rely on sequentially activated kinase cascades [34]. The classical pathway is triggered by various proinflammatory cytokines such as IL-1β and TNF-α [34]. These extracellular signals activate the IKK complex which phosphorylates IκBα at Ser$^{32}$ and Ser$^{36}$ and signals for ubiquitin-related degradation. The released NF-κB is then translocated into the nucleus where it promotes NF-κB-dependent transcription [34]. Besides the phosphorylation and degradation of the IκB signal pathway, an IκB-independent pathway such as p65 phosphorylation for optimal NF-κB activation has been defined [34]. p65 Ser$^{276}$ is phosphorylated by the protein kinase A catalytic subunit and mitogen- and stress-activated protein kinase-1, and this phosphorylation increases p65 transcriptional activity [44,45]. In addition, p65 is phosphorylated at Ser$^{536}$ by a variety of kinases through various signaling pathways, and this enhances the p65 transactivation potential. TNF-α induces rapid p65 phosphorylation at Ser$^{536}$ through IKKs, resulting in increased transcriptional activity of p65 [46]. ERK also mediates phosphorylation of p65 Ser$^{536}$ via IKKs pathways [47]. The results of this study showed that the ERK pathway contributed to SDF-1α-induced p65 Ser$^{536}$ phosphorylation in A549 cells. SDF-1α-induced IKKα/β phosphorylation as well as an increase in p65 phosphorylation at Ser$^{536}$ which began at 10 and 120 min, respectively, and PD98059 inhibited SDF-1α-induced p65 phosphorylation at Ser$^{536}$. These results indicate that ERK may act through IKKα/β to increase p65 phosphorylation at Ser$^{536}$ and enhance NF-κB transactivation.

In conclusion, we present here a novel mechanism of SDF-1α/CXCR4-directed migration of lung cancer cells by up-regulation of both β1 and β3 integrins. SDF-1α increases cells migration and integrins expression by binding to the CXCR4 receptor and activation of ERK, IKKα/β, and NF-κB-dependent pathway.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.08.025.

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