Original Article

Leptin increases motility and integrin up-regulation in human prostate cancer cells

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Running title: Leptin directs migration of prostate cancer

Abstract

Prostate cancer is the most commonly diagnosed malignancy in men and shows a predilection for metastasis to distant organs. Leptin, an adipocyte-derived cytokine that is closely associated with obesity, has recently been shown to be involved in carcinogenesis and cancer progression. The aim of this study was to investigate whether leptin is associated with the motility of prostate cancer cells. We found that leptin increased the migration of human prostate cancer cells and expression of αvβ3 integrin on these cells. Leptin-mediated migration and increased integrin expression were attenuated by OBRl receptor antisense oligonucleotide. Activation of insulin receptor substrate (IRS-1), phosphatidylinositol 3–kinase (PI3K), Akt, and NF-κB pathways after leptin treatment was demonstrated. Furthermore, leptin-induced integrin expression and migration activity were inhibited by specific inhibitors; siRNAs; and mutants of the IRS-1, PI3K, Akt, and NF-κB cascades. Therefore, this study shows that leptin stimulates the migration of human prostate cancer cells, one of the mechanisms underlying leptin-directed migration was transcriptional up-regulation of αvβ3 integrin expression through the OBR1/IRS-1/PI3K/Akt/NF-κB signal transduction pathway.

Key Words: Leptin; Prostate cancer; Integirn; Migration; OBRl

Introduction

Prostate cancer is the most commonly diagnosed malignancy in American men and is second only to lung cancer in terms of cancer mortalities in men (Robinson et al., 2008). Early and localized prostatic tumors are most often successfully treated by surgery alone (i.e. radical prostatectomy). As with many cancers, however, the treatment of advanced disease states requires a systemic intervention to inhibit the growth and spread of secondary metastasis.

Metastasis, the major cause of mortality for cancer patients, is a complex and multi-stage process in which secondary tumors are formed in distant sites (Van't Veer and Weigelt, 2003). Typically, the development of metastasis involves several steps that comprise cellular transformation and tumor growth, angiogenesis and lymphangiogenesis, entry of cancer cells into the circulation by intravasation, anchorage and/or attachment on the target organ, invasion of the target organ by extravasation, and proliferation within the organ parenchyma (Hanahan and Weinberg, 2000). The migratory ability of a cancer cell is important for many of these steps, and therefore is correlated with tumor metastasis.

Integrins play a role in tumor metastasis. They are a family of transmembrane adhesion receptors comprising 19α and 8β subunits that interact noncovalently to form up to 24 different heterodimeric receptors (Giancotti and Ruoslahti, 1999; Humphries, 2000). The combination of different integrin subunits on the cell surface allows cells to recognize and respond to a variety of extracellular matrix proteins including fibronectin, laminin, collagen, and vitronectin (Stupack, 2007; Wang et al., 2008). Activation and increased expression of integrin-coupled signaling effectors is involved in the induction of a wide variety of human cancers, including breast, colon, prostate, and ovary (White et al., 2004). In addition, the expression of integrin on tumor cells correlates with poor prognosis in cutaneous melanoma as well as in lung and pancreatic cancers (Nikkola et al., 2004; Oshita et al., 2004; Yao et al., 2007). Integrin also has been implicated in the metastasis of lung, breast, and prostate cancers (King et al., 2008; Pontier and Muller, 2008; Ritzenthaler et al., 2008).

Leptin, the product of the *ob* gene, is a 16-kDa nonglycosylated peptide hormone synthesized almost exclusively by adipocytes that regulates appetite and energy expenditure at the hypothalamic level (Halaas et al., 1995). It has become increasingly apparent that leptin does, however, have other direct effects on nonneural cells (Chao et al., 2007). In recent years, the effects of leptin on tumorigenesis, angiogenesis, and metastasis have received particular attention (Surmacz, 2007; Vona-Davis and Rose, 2007). Leptin receptors (OBR) are found in many tissues in several alternatively spliced forms (Campfield et al., 1995; Tartaglia et al., 1995). One form of the receptor, a long form (OBRl), is highly expressed in the hypothalamus, whereas a short form (OBRs), is highly expressed in microvessels at the blood-brain barrier (Campfield et al., 1995; Fei et al., 1997). Upon binding to OBRl, leptin activates janus kinase 2 (JAK2), which then initiates downstream signaling pathway that includes members of the STAT (signal transducers and activators of transcription) family of transcription factors (Kloek et al., 2002). The leptin receptor, through the activation of JAK2, is able to phosphorylate insulin receptor substrate (IRS) proteins and induce the IRS-phosphatidylinositol 3-kinase (PI3K) signaling pathway (Niswender et al., 2001). Leptin also activates signaling via JAK2/STAT3, ERK, or JNK pathways in prostate cancer cells (Fruhbeck, 2006; Hoda et al., 2007; Miyazaki et al., 2008).

In recent years, increased body weight has been shown to be associated with increased death rates for multiple types of cancers (Calle et al., 2003). Previous studies have consistently shown a positive association between adiposity and increased risk of cancer of the endometrium, kidney, colon, and breast cancer in postmenopausal women (Bergstrom et al., 2001; Peto, 2001). Leptin also increases the migration of human prostate cancer cells (Deo et al., 2008; Frankenberry et al., 2004). We hypothesized that leptin may regulate migration of prostate cancer cells through the up-regulation of integrin. In this study, we found that leptin increased the migration and the expression of integrin in human prostate cancer cells. In addition, OBRl receptor, IRS-1, PI3K, Akt, and NF-κB signaling pathways may be involved in increasing integrin expression and cell migration by leptin.

Materials and Methods

Materials

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p85, Akt, phospho-Akt (Ser473), p-IRS-1, IRS-1, IKK, p-IκBα, IκBα, p65 and the small interfering RNAs (siRNAs) against IRS-1 and control (negative control for experiments using targeted siRNA transfection; each consisted of a scrambled sequence that would not lead to the specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies specific for IKKα/β phosphorylated at $Ser^{180/181}$ and p65 phosphorylated at Ser^{536} were purchased from Cell Signaling and Neuroscience (Danvers, MA). Ly294002, Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-[(R)-2-O-methyl-3-Ooctadecylcarbonate], PDTC and TPCK were obtained from Calbiochem (San Diego, CA). A selective αvβ3 integrin antagonist, cyclic RGD (cyclo-RGDfV) peptide, and the cyclic RAD (cyclo-RADfV) peptide were purchased from Peptides International (Louisville, KY). Recombinant human leptin was purchased from PeproTech (Rocky Hill, NJ). Mouse monoclonal antibody (mAb) specific for αvβ3 integrin was purchased from Chemicon (Temecula, CA). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA). The p85 (p85; deletion of 35 amino acids from residues 479-513 of p85) and Akt (Akt K179A) dominant-negative 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). pSV-β-galactosidase vector and the luciferase assay kit were purchased from Promega (Madison, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cells and cell cultures

The human prostate cancer cell lines (PC3, DU145 and LnCaP) 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 in 5% CO2.

Migration assay

The migration assay was performed using Transwell inserts (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 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 leptin was placed in the lower chamber. The plates were incubated for 24 hr at 37°C in 5% CO_2, and then cells were fixed in 1% formaldehyde for 5 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 leptin treatment (corrected number of invading cells = number of counted invading cells/percentage of viable cells) (Tan et al., 2009).

Flow cytometric analysis

Human prostate cancer cells were plated in 6-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 rinsing in PBS, the cells were incubated with mouse anti-human antibody against αvβ3 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

Cellular lysates were prepared, and proteins were then resolved by SDS–PAGE and transferred to Immobilon polyvinyldifluoride membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against p-IRS-1, IRS-1, p-IKK, IKK, p-p65, p65, or β-actin (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 with enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Oligonucleotide (ODN) transfection

Prostate cancer cells were cultured to confluence, and the complete medium was replaced with OPTI-MEM containing the antisense phosphorothioate oligonucleotides (5 μg/ml) that had been preincubated with Lipofectamine 2000 (10 μg/ml) for 30 min. The OPTI-MEM containing 20% FCS was added 4 h later. The cells were washed after 24 h incubation at 37 °C. All ODNs were synthesized and high pressure liquid chromatography purified by MDBio (Taipei, Taiwan). The sequences used are as follows: OBRl antisense ODN (AS-ODN), AGACCGAGCGGGCGTTAA and missense ODN (MM-ODN), AGCCCGCGCGAGTGTTCA (Yang et al., 2009).

Quantitative Real Time PCR

qPCR analysis was carried out using TaqMan® one-step PCR Master Mix (Applied Biosystems, Foster City CA). Total cDNA (100 ng/25-μl reaction) was added along with sequence-specific primers and TaqMan® probes. Sequences for all target gene primers and probes were purchased commercially, and β-actin was used as an internal control (Applied Biosystems, CA). Quantitative RT-PCR assays were carried out in triplicate on StepOnePlus sequence detection system. The cycling conditions were

10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. 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 as C_T).

Reporter assay

Prostate cancer cells were transfected with reporter plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Twenty-four hours after transfection, the cells were treated with inhibitors for 30 min and, and then leptin or vehicle was added for 24 hr. Cell extracts were then prepared, and luciferase and β-galactosidase activities were measured (Tang and Lu, 2009).

Statistics

For statistical analysis, the Mann-Whitney *U*-test for non-Gaussian parameters and the Student's *t*-test for Gaussian parameters (including Bonferroni correction) were used. The difference was considered significant when the *P* value was <0.05.

Results

Involvement of αvβ3 up-regulation in the leptin-directed migration of prostate cancer cells

Leptin stimulates directional migration and invasion of human cancer cells (Aloulou et al., 2008; Yang et al., 2009). Leptin-triggered migration of prostate cancer cells was examined using the Transwell assay. Leptin induces cancer proliferation in some cancer cells (Lautenbach et al., 2009; Uddin et al., 2009). Therefore, to exclude possible cell proliferation effects of leptin in prostate cancer cells in the calculation of the number of cells that migrated, results of cell migration experiments were corrected (Yang et al., 2009). Leptin induced the migration of androgen-independent prostate

cancer cells (PC3 and DU145 cells) in a concentration-dependent manner (Fig. 1A). Furthermore, leptin also induced the migration of androgen-dependent prostate cancer cells (LnCaP cells; Fig. 1A). There is significant expression of integrins in human prostate cancer cells (Bisanz et al., 2005; Tang and Lu, 2009). We therefore hypothesized that integrins may be involved in the leptin-mediated migration of prostate cancer cells. Flow cytometry analysis showed that leptin induced the cell surface expression of αvβ3 and β3 but not α2, α5, β1, or α5β1 integrin (Fig. 1B). In addition, leptin also increased the protein and mRNA expression of αv and β3 integrin (Fig. 1C). Pretreatment of cells with anti-αvβ3 monoclonal antibody (mAb) (10 μg/ml) for 30 min markedly inhibited the leptin-induced migration of prostate cancer cells (Fig. 1D). The cyclic RGD peptide (cyclo-RGDfV) at low concentration binds αvβ3 with high affinity and effectively blocks its function (Tang et al., 2007). Treatment of cells with cyclic RGD inhibited the leptin-induced migration of prostate cancer cells; this effect was not seen in the presence of an inactive RGD analog, cyclic RAD (Fig. 1D). These data suggest that leptin-induced cancer migration may occur via activation of the αvβ3 integrin receptor.

Involvement of OBR1 receptor in leptin-mediated migration of prostate cancer cells

Leptin exerts its effects through interactions with specific leptin receptors (OBRl and OBRs) (Campfield et al., 1995). OBRl mediates leptin-enhanced cell migration in chondrosarcoma cells (Yang et al., 2009). Therefore, we next examined whether OBR1 was involved in leptin-mediated cell migration in human prostate cancer cells. Transfection of PC3 cells with OBRl AS-ODN but not OBRl MM-ODN reduced the mRNA expression of OBRl (data not shown). Transient transfection of cells with OBRl AS-ODN effectively inhibited the migration activity of prostate cancer cells (Fig. 2A). In addition, OBRl AS-ODN also reduced leptin-enhanced integrin up-regulation (Fig. 2B). These data suggest that leptin/OBR1 receptor interactions play a key role in the migration of prostate cancer cells.

The signaling pathways of IRS-1/PI3K, Akt are involved in the potentiating action of leptin stimulation

Leptin-induced signaling via JAK2 and phosphorylation of STAT3 or other pathways such as IRS-1 and PI3K has been reported (Niswender et al., 2001). We therefore directly measured the phosphorylation of IRS-1 in response to leptin treatment. Treatment of PC3 cells with leptin induced an increase in IRS-1 phosphorylation in a time-dependent manner (Fig. 2C). To further examine whether IRS-1 activation is involved in the signal transduction pathway leading to cell migration by leptin, IRS-1 siRNA was used. IRS-1 siRNA specifically inhibited the expression of IRS-1 (Fig. 2D; upper panel). Leptin-induced cell migration and αvβ3 integrin up-regulation were also inhibited by IRS-1 siRNA (Fig. 2D&E). Therefore, IRS-1 is very important in leptin-induced migration in prostate cancer cells. We next examined whether leptin activated PI3K, a critical downstream target of IRS-1 (Niswender et al., 2001). Treatment of PC3 cells with leptin for 10-60 min increased phosphorylation of the p85 subunit of PI3K (Fig. 3A). Pretreatment of cells with PI3K inhibitor (Ly294002) or transfection with a dominant negative mutant of p85 attenuated leptin-induced migration and αvβ3 integrin expression (Fig. 3B-E). Therefore, PI3K is involved in leptin-mediated cell migration in human prostate cancer cells. We next measured Akt phosphorylation in response to leptin. Treatment of PC3 cells with leptin resulted in a time-dependent phosphorylation of Akt (Fig. 4A). Pretreatment of prostate cancer cells for 30 min with Akt inhibitor or transfection with Akt mutant for 24 h markedly attenuated the leptin-induced cell migration and αvβ3 integrin up-regulation (Fig. 4B-E). These results indicate that the IRS-1/PI3K/Akt pathway is involved in leptin-induced migration of human prostate cancer cells.

NF-κB signaling pathways is involved in the leptin-mediated integrin up-regulation and migration activity

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human cancer cells (Fong et al., 2009; Huang et al., 2009). To examine

whether NF-κB activation is involved in leptin-induced cancer migration, an NF-κB inhibitor, PDTC, was used. Pretreatment with PDTC (10 μM) inhibited leptin-induced migration of prostate cancer cells (Fig. 5A). In an inactivated state, NF-κB is normally held in the cytoplasm by the inhibitor protein IκB. Upon stimulation, IκB proteins become phosphorylated, which subsequently targets IκB for ubiquitination, and then are degraded by the 26S proteasome. Therefore, the IκB protease inhibitor TPCK was further used. Cells pretreated with TPCK (3 μM) also reduced leptin-induced migration of cancer cells (Fig. 5A). Treatment of cells with PDTC or TPCK also antagonized leptin-induced expression of αvβ3 integrin (Fig. 5B). We further examined the upstream molecules involved in leptin-induced NF-κB activation. Stimulation of cells with leptin induced IKKα/β phosphorylation in a time-dependent manner (Fig. 5C). Furthermore, transfection with IKKα or IKKβ mutants markedly inhibited the leptin-induced cancer cells migration (Fig. 5D). These data suggest that IKKα/β activation is involved in leptin-induced migration of human prostate cancer cells. Treatment with prostate cancer cells with leptin also caused IκBα phosphorylation in a time-dependent manner (Fig. 5C). Previous studies showed that p65 Ser^{536} phosphorylation increases NF-κB transactivation (Madrid et al., 2001), and the antibody specific against phosphorylated p65 Ser^{536} was used to examine p65 phosphorylation. Treatment of cells with leptin for various time intervals resulted in p65 Ser^{536} phosphorylation (Fig. 5C). These results indicated that NF-κB activation is important for leptin-induced cancer cell migration and the expression of αvβ3 integrin. In addition, pretreatment of cells with Ly294002 or Akt inhibitor for 30 min reduced leptin-increased p65 phosphoryation (Fig. 6A). Therefore, these results indicated that NF-κB may function as a downstream signaling molecule of PI3K and Akt in the leptin signaling pathway.

To directly measure NF-κB activation after leptin treatment, prostate cancer cells were transiently transfected with κB-luciferase as an indicator of NF-κB activation. Leptin treatment of prostate cancer cells for 24 h caused an increase in κB-luciferase activity (Fig. 6B). Transfection of cells with OBRl AS-ODN or IRS-1 siRNA

inhibited leptin-increased κB-luciferase activity (Fig. 6B). In addition, Ly294002, Akt inhibitor, PDTC and TPCK also reduced leptin-mediated NF-κB activity (Fig. 6C). Moreover, co-transfection of cells with a dominant-negative p85, Akt, IKKα, or IKKβ mutant antagonized leptin-induced NF-κB activity (Fig. 6D). Taken together, these data suggested that activation of OBR1 and the IRS-1, PI3K and Akt signaling pathways are required for leptin-induced NF-κB activation in prostate cancer cells.

Discussion

Prostate cancer cells have a striking tendency to metastasize (Robinson et al., 2008; Van't Veer and Weigelt, 2003). The analysis of trophic signals that control metastasis during prostate cancer is crucial for the identification of new molecular targets for anti-metastasis therapy. Although leptin expression was shown in a previous study to enhance tumorigenesis and metastasis of human cancer cells, its role in prostate cancer invasion was not elucidated (Gainsford et al., 1996). We hypothesized that leptin would help to direct the migration of prostate cancer cells. This study showed that leptin induced migration of human prostate cancer cells, and one of the mechanisms underlying leptin-directed migration was transcriptional up-regulation of αvβ3 integrin and activation of OBRl, IRS-1, PI3K, Akt, and NF-κB pathways. In this study, we found that leptin increased migration and cell surface αvβ3 integrin expression dose-dependently (Fig. 1A; Supplementary Fig S1). The concentration of leptin at 1 μM has most efficiency in cell migration and integrin expression. Therefore, we used leptin (1 μM) to investigate the leptin-mediated signaling pathways. Although this concentration dose not cover the physiologic range in human. However, it's may explain the pathologic condition of leptin in human.

Prostate cancer is very common in developed countries and is widely variable in its clinical course. Most cases remain confined to the prostate and adjacent soft tissue and cause no harm. However, approximately one in eight cases metastasize widely,

typically to bone (Mundy, 2002). Like the organ in which it arises, prostate cancer growth and survival are supported by androgenic hormones. Widely metastatic cases are therefore treated by androgen deprivation therapy. Here we found that leptin supported the chemomigration in androgen-dependent (LnCaP) and independent (PC3 and DU145) prostate cancer cells. The inhibitors and mutants of IRS-1, PI3K, Akt and NF-κB pathway reduced leptin-induced migration in three prostate cancer cell lines. Therefore, the same signaling pathway was required for the leptin-induced prostate cancer cell migration.

The leptin receptor belongs to the cytokine receptor superfamily (Campfield et al., 1995). Human cancer cells express OBRl (Bergstrom et al., 2001; Yang et al., 2009); in addition, OBRl is involved in leptin-mediated cell motility (Yang et al., 2009). The role of OBRl in the motility of human prostate cancer cells is, however, largely unknown. We found that the OBRl AS-ODN but not the control OBRl MM-ODN reduced leptin-increased cell migration and integrin expression. Therefore, OBRl is very important in leptin-mediated motility. Collectively, the interpretation of our data would appear to encourage us to conclude that leptin/OBR1 plays a novel role in regulating prostate cancer cell migration in a clinical/experimental setting, and it would also appear to be feasible as a biological marker to predict the relative likelihood/extent of peritoneal metastasis following prostate cancer cells.

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 (Giancotti and Ruoslahti, 1999; Humphries, 2000). Here we found that leptin increased αvβ3 expression but not α5β1, α2, α5, or β1 integrin expression, which plays an important role during tumor metastasis. Furthermore, leptin also increased mRNA and protein levels of αv and β3 integrins. In the present study, blocking αvβ3 integrin inhibited leptin-induced cancer cell migration. Also, cyclic RGD but not cyclic RAD inhibited leptin-induced migration activity, further confirming the involvement of αvβ3 integrin in leptin-mediated induction of cancer

migration.

Several signaling pathways have been implicated in leptin-mediated cell migration and increased integrin expression. Upon leptin binding, OBR1 activates JAK2, which in turn phosphorylates tyrosine residues in the receptor tails, leading to the recruitment and activation of STAT-3 (Szanto and Kahn, 2000). OBR1 is also able to phosphorylate IRS proteins and stimulate the IRS-PI3K signaling pathway via activation of JAK2 (Szanto and Kahn, 2000). Here we report that IRS-1 siRNA inhibited leptin-induced migration and integrin up-regulation, indicating the possible involvement of IRS-1 in these processes. Leptin also increases the association of tyrosine-phosphorylated IRS-1 with p85, the regulatory subunit of PI3K, via its Src homology 2 domains (Wang et al., 1997).

In our study, pretreatment of leptin with PI3K inhibitor Ly294002 antagonized leptin-induced migration. Furthermore, the dominant-negative mutant of p85 also inhibited leptin-mediated migration and αvβ3 integrin expression. Others have shown that PI3K activation leads to phosphorylation of phosphatidylinositides, which then activate the downstream main target, Akt; Akt is a cytoplasmic serine kinase that is important in regulating cell growth, differentiation, adhesion, and inflammatory reactions (Hirsch et al., 2000). In this study, we demonstrated that both leptin-induced migration and integrin expression were inhibited by the Akt inhibitor. Furthermore, the leptin-induced increase in migration activity was also blocked by a dominant-negative Akt mutant. The cytoplasmic serine kinase Akt was found to be activated by leptin in human prostate cancer cells. Together these results provide evidence of IRS-1/PI3K-dependent Akt activation in leptin-mediated migration and integrin up-regulation in prostate cancer cells.

In conclusion, we present evidence of a novel mechanism of leptin-mediated migration of prostate cancer cells by up-regulation of αvβ3 integrin expression and activity via OBRl, IRS-1, PI3K, Akt, IKKα/β, and NF-κB-dependent pathways (Fig. 6E). The discovery of a leptin-mediated signaling pathway helps us to understand mechanisms of human prostate cancer cell metastasis and may help us to develop

effective therapy in the future.

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Figure legends

Fig. 1 Leptin-directed migration of human prostate cancer cells involves up-regulation of αvβ3 integrin.

(A) Cells were incubated with various concentrations of leptin, and *in vitro* migration activities measured with the Transwell. (B) PC3 cells were incubated with leptin (1 μM) for 24 hr, and the cell surface expression of α2, α5, β1, β3, α5β1 or αvβ3 integrin was determined using flow cytometry. (C) PC3 cells were incubated with leptin (1 μM) for 24 hr, and the protein and mRNA levels of αv or β3 integrin were determined using Western blot and qPCR. (D) PC3 cells were pretreated with αvβ3 monoclonal antibody (10 μg/ml), cyclic RGD (100 nM) or cyclic RAD (100 nM) for 30 min followed by stimulation with leptin (1 μM). 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 leptin-treated group.

Fig. 2 Involvement of OBRl in leptin-directed migration of human prostate cancer cells.

PC3 cells were transfected with OBRl AS-ODN or MM-ODN for 24 hr followed by stimulation with leptin (1 μM), and *in vitro* migration (A) and cell surface αvβ3 integrin (B) were measured with the Transwell and flow cytometry after 24 hr. (C) PC3 cells were incubated with leptin (1 μM) for various time intervals, and IRS-1 phosphorylation was determined by Western blot. PC3 cells were transfected with IRS-1 or control siRNA for 24 hr and IRS-1 expression was examined by Western blot (D; upper panel). PC3 cells were transfected with IRS-1 or control siRNA for 24 hr followed by stimulation with leptin (1 μM), and *in vitro* migration (D) and cells surface αvβ3 integrin (E) were measured with the Transwell and flow cytometry after 24 hr. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with leptin-treated group.

Fig. 3 PI3K is involved in leptin-mediated migration and integrin up-regulation in

human prostate cancer cells.

(A) PC3 cells were incubated with leptin (1 μM) for various time intervals, and p85 phosphorylation was determined by Western blot. (B-E) Cells were pretreated for 30 min with Ly294002 (10 μM) or transfected with dominant negative (DN) mutant of p85 for 24 hr followed by stimulation with leptin (1 μM), and *in vitro* migration and cells surface αvβ3 integrin were measured with the Transwell and flow cytometry after 24 hr. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with leptin-treated group.

Fig. 4 Akt pathway is involved in leptin-mediated migration in human prostate cancer cells.

(A) PC3 cells were incubated with leptin (1 μM) for various time intervals, and Akt phosphorylation was determined by Western blot. (B-E) 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 leptin (1 μM), and *in vitro* migration and cells surface αvβ3 integrin were measured with the Transwell and flow cytometry after 24 hr. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with leptin-treated group.

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

(A&B) Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with leptin (1 μM), and *in vitro* migration and cells surface αvβ3 integrin were measured with the Transwell and flow cytometry after 24 hr. (C) PC3 cells were incubated with leptin (1 μM) for indicated time intervals, and p-IKK, p-IκBα and p-p65 expression was determined by Western blot analysis. (D) Cells were transfected with DN-IKKα, DN-IKKβ or vector for 24 hr, followed by stimulation with leptin (1 μM) for 24 hr, and cell migration was measured with the Transwell after 24 hr. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p <$

0.05 compared with leptin-treated group.

Fig. 6 OBRl receptor, IRS-1, PI3K and Akt pathway is mediated leptin-mediated NF-κB activation.

(A) PC3 cells were pretreated for 30 min with Ly294002 or Akt inhibitor followed by stimulation with leptin (1 μM) for 60 min, and p-p65 expression was examined by Western blot. Cells transiently transfected with κB-luciferase plasmid for 24 hr and then pretreated with Ly294002, Akt inhibitor, PDTC and TPCK (C) for 30 min or cotransfected with OBR1 AS-ODN and IRS-1 siRNA (B) or cotransfected with p85, Akt, IKKα and IKKβ mutant (D) before incubation with leptin (1 μM) for 24 hr. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity. Results are expressed as the mean ± S.E.M. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with leptin-treated group. (E) Schematic presentation of the signaling pathways involved in leptin-induced migration and αvβ3 integrin expression of prostate cancer cells. Leptin activates OBRl, IRS-1, PI3K and Akt pathways, which in turn induces NF-κB activation, which lead to αvβ3 integrin expression and increases the migration of human prostate cancer cells.

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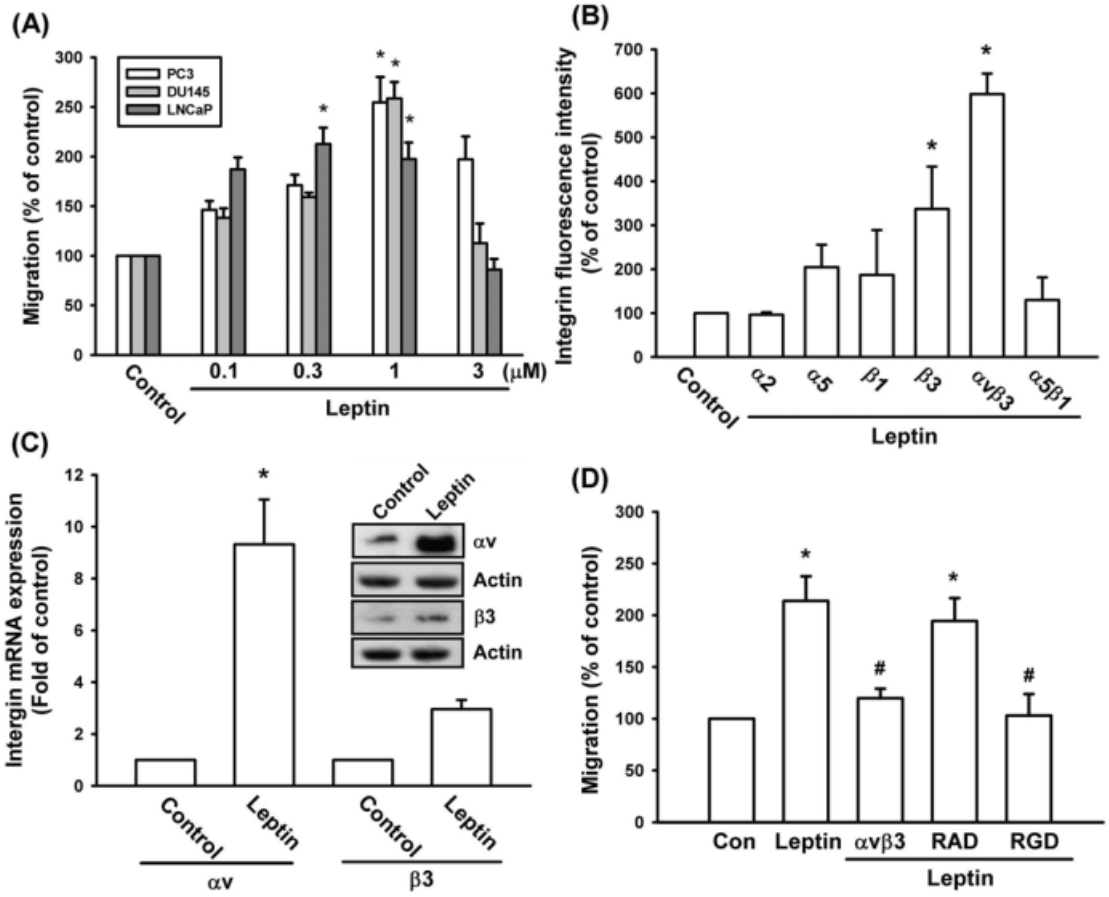

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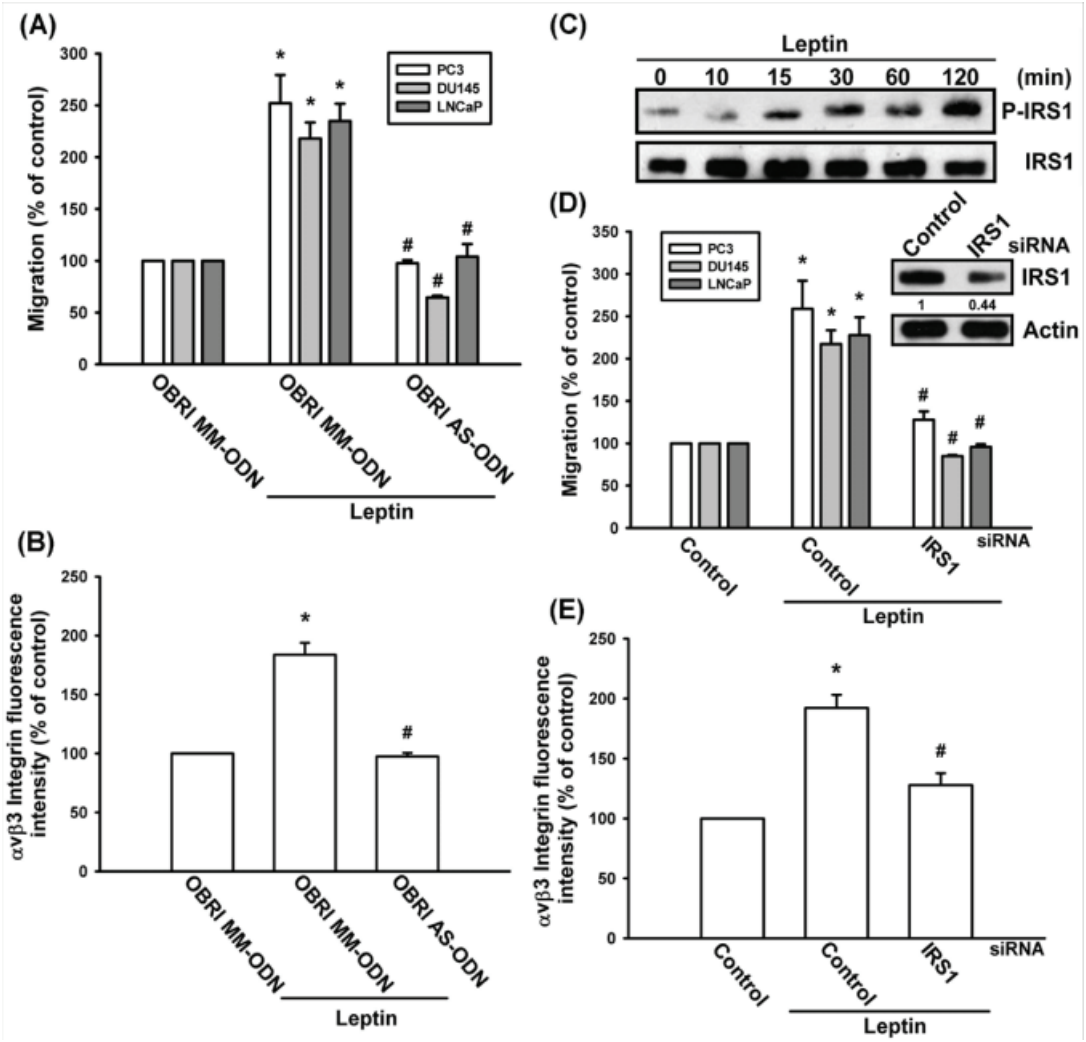

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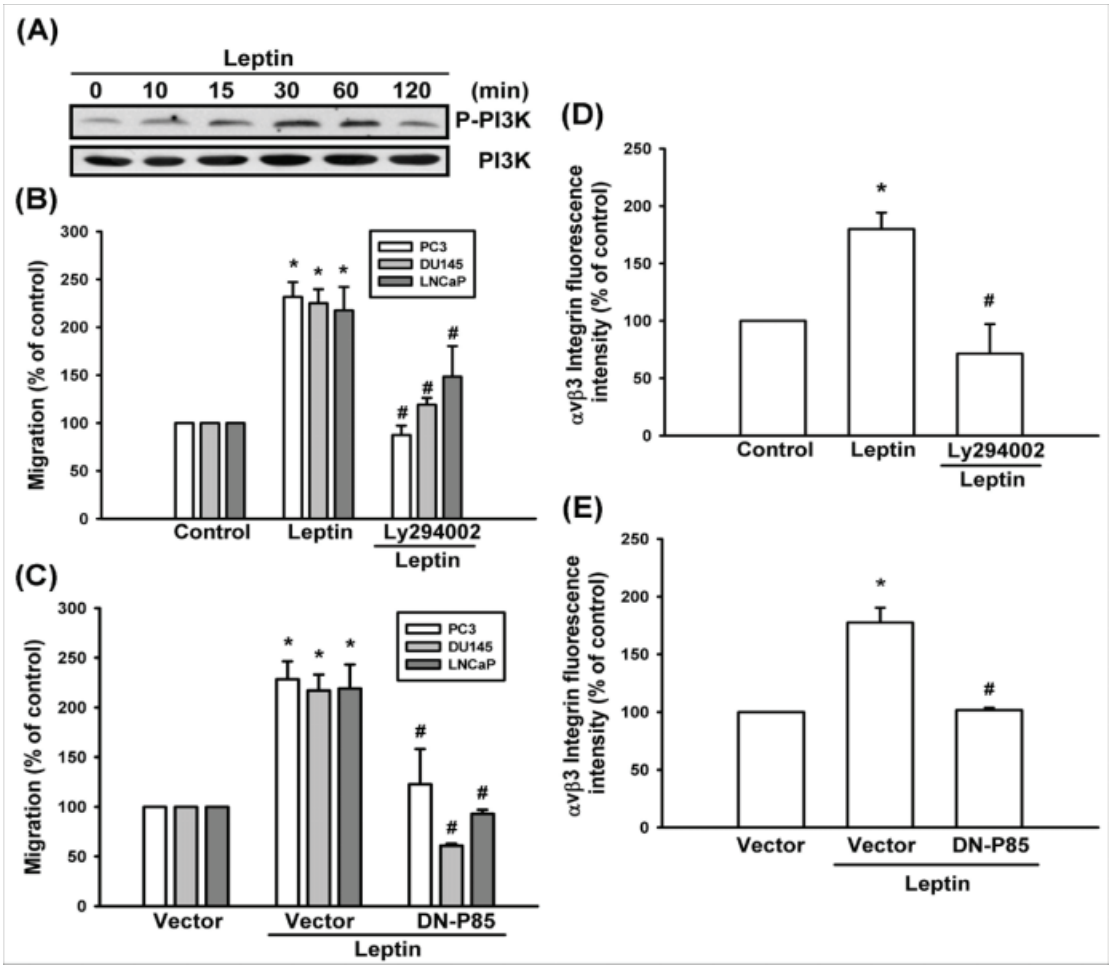

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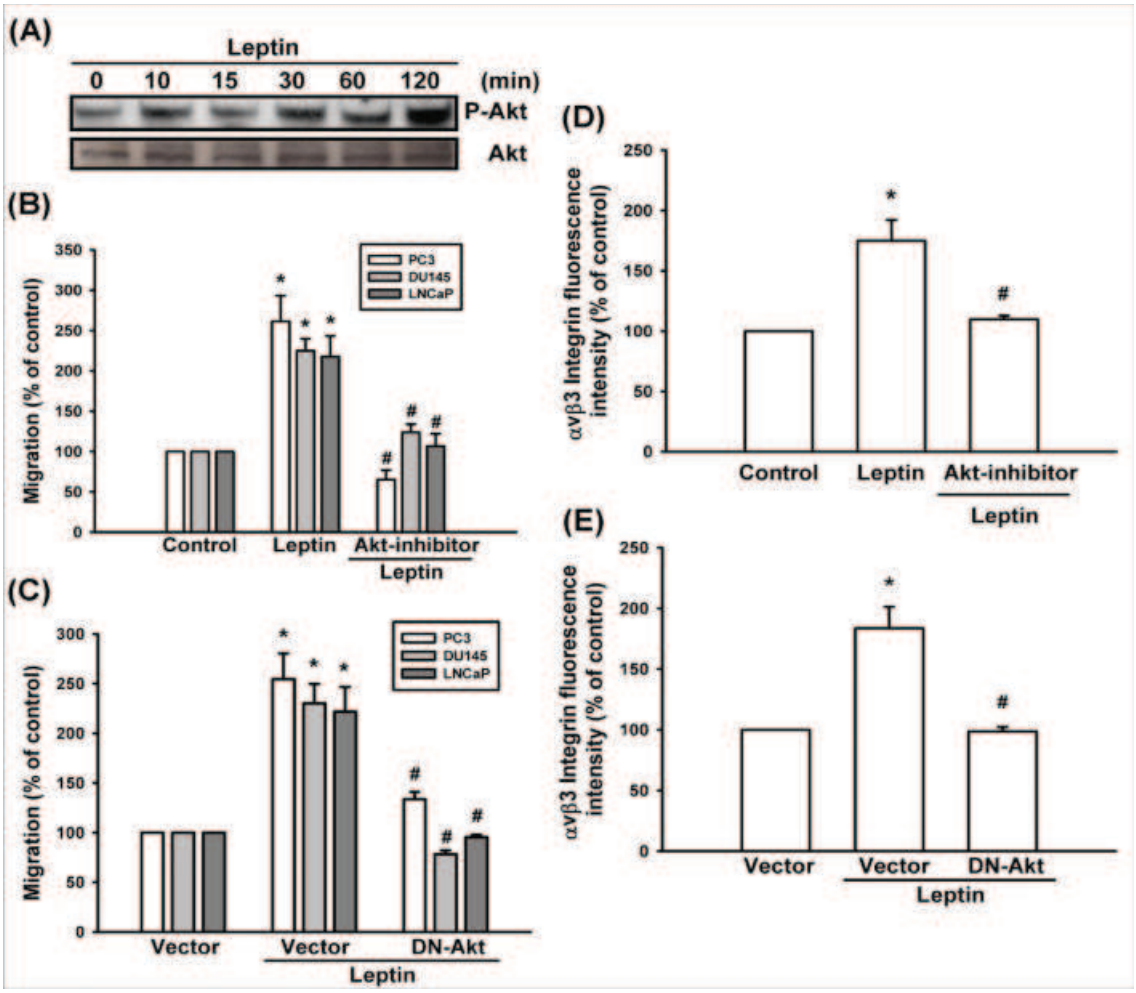

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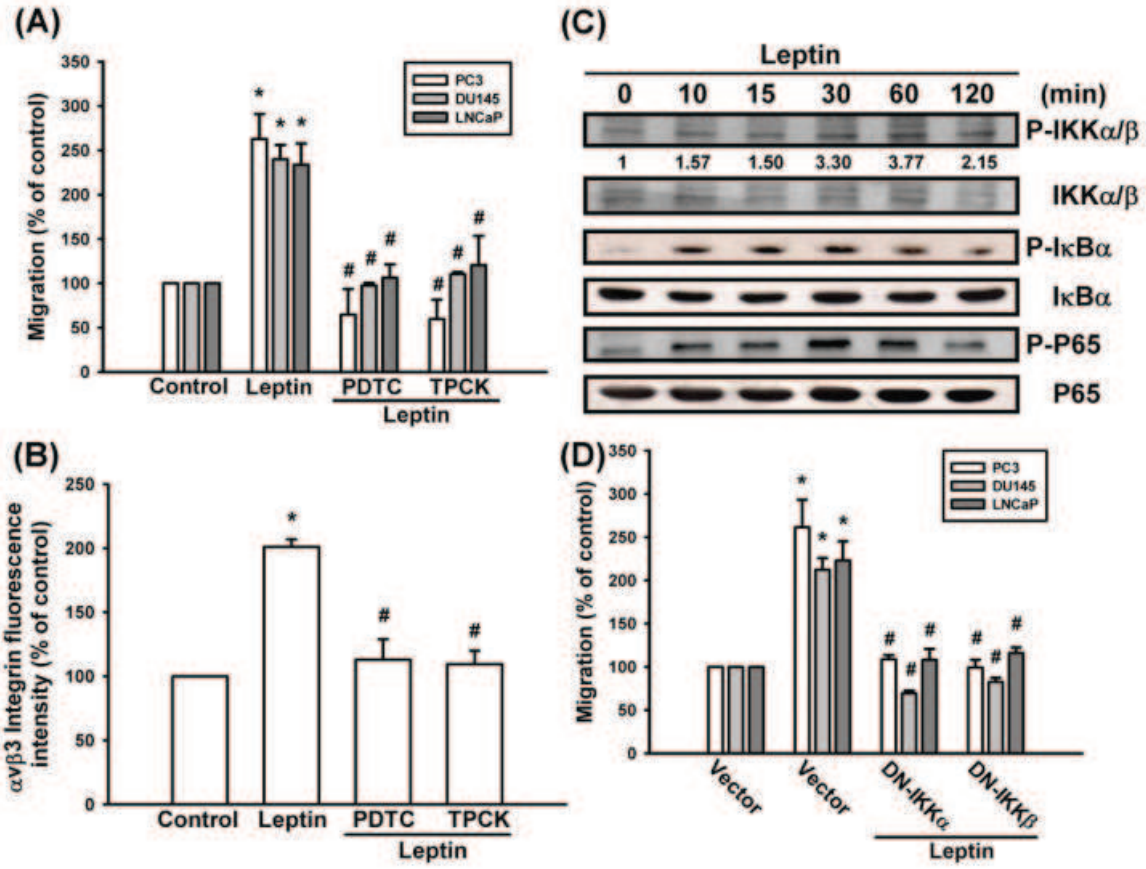

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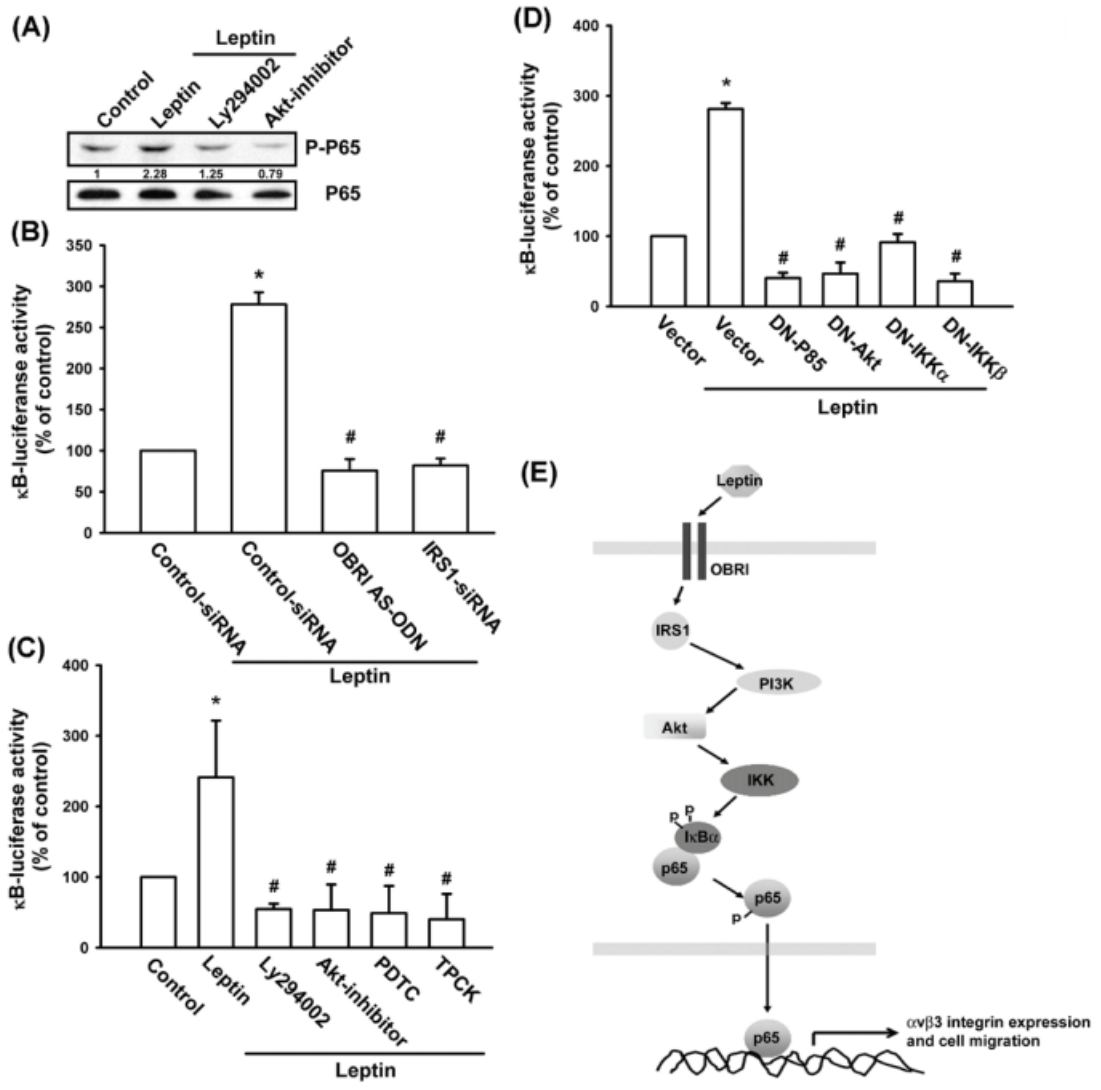

Fig 6.