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Research Article

Leptin Up-Regulates HECTD1 to Promote Phosphoinositide Metabolism and Cell Migration and Invasion in Breast Cancer Cells

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Abstract

Obesity is associated with increased risk for breast cancer and also correlated with breast cancer recurrence and high mortality. Leptin, an adipocyte-derived cytokine that is closely associated with obesity, has recently been shown to be involved in carcinogenesis and cancer progression. However, the underlying mechanisms remain to be defined. Here we show that leptin promotes cell migration and invasion and up-regulates the expression of HECTD1, an ubiquitin ligase that ubiquitinates Phosphatidylinositol 4 phosphate 5-kinase type I γ (PIPKIγ90) to regulate breast cancer cell migration and invasion, in MDA-MB-231 human breast cancer cells. Meanwhile, leptin down-regulates PIP2 and PIP3 production, but has no influence on mRNA of PIPKIγ90, suggesting that leptin promotes PIPKIγ90 ubiquitination. Furthermore, leptin stimulates the migration and invasion of PIPKIγ90-depleted cells that re-express a codon-modified WT PIPKIγ90, but has little effect on the migration and invasion of the PIPKIγ90-depleted cells that re-express PIPKIγ90^{K97R} Collectively, leptin stimulates breast cancer cell migration andinvasion through regulating HECTD1 expression consequently PIPKIγ90 ubiquitination. This study provides a new mechanism for leptin-stimulated breast cancer cell migration and invasion.

INTRODUCTION

Cancer metastasis is the leading cause of mortality in patients with breast cancer [1]. Metastasis is multistep process, which includes detachment of cancer cells from primary tumor, adhesion, migration, and invasion of cancer cells into the blood or lymphatic vessels [2]. Adipocytes secrete adipokines, including leptin , adiponectin and serum amyloid A (SAA) which are known to promote tumor metastasis [3]. Clinical and experimental studies have demonstrated that leptin stimulates different cancer cell proliferation, invasion and metastasis [4,5]. Leptin is a 16 kDa polypeptide encoded by the obese (OB) gene [6]. Leptin exerts its physiological and path physiological effects by binding to the trans-membrane leptin receptor [7]. Leptin and its receptors are over expressed in breast cancer tissues and correlate with poor prognosis [8]. Also, High leptin level associated with obese state is a major cause of breast cancer progression and metastasis [9]. When binds its receptor and leptin activates multiple signaling pathways, including PI3K/Akt, MAPK, STAT3 and Cdk2 [10]. However, it remains to be elucidated how leptin regulates breast cancer cell migration and invasion.

Phosphatidylinositol 4 phosphate 5-kinase type I γ (PIPKIγ90) is a key enzyme that is responsible for the production of phosphatidylinositol 4,5-bisphosphate (PIP2), a signaling molecule that is implicated in a variety of cellular functions. PIPKIγ90 interacts with talin [11,12] and regulates focal adhesion (FA) dynamics [13,14], a key step during cell migration. It is essential for cell migration and cancer invasion [13-16].

It has just been recognized that E3 ubiquitin ligases regulates focal adhesion dynamics, thus modulating cell migration [14,17,18]. We have demonstrated that HECTD1, a 280 kDa HECT domain E3 ubiquitin ligase that regulates cell migration and neural tube closure [19,20], ubiquitinates PIPKIγ90 at K97, resulting in its degradation, thus regulating FA dynamics [14]. This pathway is essential for breast cancer cell migration, invasion and metastasis. Because of the central role of FA dynamics in regulating cell migration and invasion [21], it is likely that the HECTD1-PIPKIγ90 pathway play a pivotal role in adipokines-stimulated breast cancer cell migration and invasion.

In this study, we investigate the regulation of the HECTD1-PIPKIγ90 pathway by leptin and examine the role of this pathway in regulating leptin-stimulated breast cancer cell migration and invasion.

MATERIALS AND METHODS

Reagents

Anti-PIPKIγ90 polyclonal antibody was from Epitomics. Anti-HECTD1 rabbit polyclonal antibody was custom made by Syd Labs. Anti-tubulin antibody, leptin and pLKO1 lentivirus shRNA that target PIPKIγ90 were from Sigma; PIPKIγ90 shRNA clones are TRCN0000037668 (A1), were from Sigma. Fibronectin were from Akron Biotech; Growth factor reduced Matrigel was from BD Bioscience. DNA primers were synthesized by Integrated DNA Technologies.

Cell culture and infection

MDA-MB-231 human breast cancer cells were from the American Type Culture Collection and were maintained in DMEM medium (Sigma, Inc.) containing 10% fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml). PIPKIγ90-depleted MDA-MB-231 cells that re-express codon-modified the WT PIPKIγ90 and PIPKIγ90^{K97R}, respectively,were prepared as described previously. Briefly, cells were infected with that pLKO1 lentiviral shRNA viruses (clone TRCN0000037668) that target PIPKIγ90, selected with 1 μg/ml puromycin. The resulting cells were infected with pBabe retroviruses, stabilized by growing infected cells in the presence of 0.7 mg/ml neomycin for 10 days.

Real-time Q-PCR

Total RNA was extracted from cells with PureLink RNA kit (Ambion). cDNA was synthesized with SuperScript First Strand Synthesis kit (Invitrogen) from 0.5 to 1.0 μg RNA samples according to the manufacturer's instructions. Quantitative reverse transcriptase PCR (RT-PCR) reactions were carried out using SYBR Green PCR master mix reagents on an ABI Onestep Plus Real-Time PCR System (Applied Biosystems). The relative quantification of gene expression for each sample was analyzed by the Δ*C*t method. The following primers were used to amplify PIPKIγ90 : 5'- CGT CTG GAC AGG ATG GCA GGC -3' and 5'- TGT GTC GCT CTC GCC GTC GGA -3'; HECDT1: 5'- CCC AGC TCC ATT AGC CAA GCA TGG-3' and 5'- CC AAA CGC ATA GTA TCA AGC ACA CAT CTT TC-3';18S rRNA: 5′-ACC TGG TTG ATC CTG CCA GT-3′ and 5′-CTG ACC GGGTTG GTT TTG AT-3.

Western blot analysis

After leptin treatment, cells were washed with ice-cold PBS and harvested in 2×SDS sample buffer (with 2-Mercaptoethanol) containing protease inhibitor cocktail and Bortezomib/ Carfilzomib and boiled immediately. The cell lysates were analyzed by SDS-PAGE and then were electro-transferred to nitrocellulose membrane. The expression levels of HECTD1, PIPKIγ90, and tubulin were detected by incubating the membrane with the specific antibodies and then with Dylight 680-labeled secondary antibodies, and visualizing with Odyssey Infrared Imaging System (LI-COR).

Cell migration assays

Cells were treated with trypsin and resuspended in DMEM medium containing 1% FBS and 100 ng/ml Leptin, plated at low densities on glass-bottomed dishes (MatTek) coated with 5 μg/ml fibronectin and cultured for 2 h in a CO2 incubator. Cell motility was measured with a Nikon Biostation IMQ. Cell migration was tracked for 6 h; images were recorded every 10 min. The movement of individual cells was analyzed with NIS-Elements AR (Nikon) as described previously [14].

Invasion assays

One hundred microliters of Matrigel (1:30 dilution in serum-free DMEM medium) was added to each Transwell polycarbonate filter (6 mm diameter, 8 μm pore size, Costar) and incubated with the filters at 37 °C for 6 h. Breast cancer cells were trypsinized and washed 3 times with DMEM containing 1% FBS. The cells were resuspended in DMEM containing 1% FBS at a density of 5×105 cells/ml. The cell suspensions (100 μl) were seeded into the upper chambers, and 600 μl of DMEM medium containing 1% FBS and 10 μg/ml fibronectin with or without leptin were added to the lower chambers. The cells wereallowed to invade for 12 h (or as indicated) in a CO2 incubator, fixed, stained and the invasive cells on the lower surface of the membrane filter were counted as described previously [13].

Quantitation of polyphosphoinositides in cells

Polyphosphoinositides were extracted and derivative using trimethylsilyl diazomethane as described [22]. Quantitation of Polyphosphoinositides in MDA-MB-231 cells were measured as their TMSdiazomethane derivatives using a Shimadzu UFLC equipped with a Vydac 214MS C4, 5 u,4.6×250 mm column, coupled with an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode as described previously [13].

RESULTS AND DISCUSSION

Leptin stimulate breast cancer cell migration and invasion

To verify the effect of leptin on breast cancer cells migration, MDA-MB-231 cells were plated on glass-bottomed dishes coated with 5 μg/ml fibronectin, and treated with 100ng/ml Leptin, and the migration was determined by time-lapse cell migration assays. Leptin increased the velocity and directionality of cell migration, and strongly increased the net distance of cell migration (Figure

1A and 1B), indicating that leptin stimulates the migration of MDA-MB-231 cells by enhancing velocity and directionality.

The effect of leptin on the invasion of MDA-MB-231 cells was measured by examining the functional capacities of the cells penetrating through transwell filters coated with 0.35 mg/ml Matrigel, and different concentrations of leptin were added to the lower chamber. Leptin significantly promoted the invasion of MDA-MB-231 cells, with a peak concentration at100ng/ml (Figure 2A and 2B).

Obesity is associated with increased risk for breast cancer and also correlated with breast cancer recurrence and high mortality [23]. It is generally thought that obesity stimulates cancer cell invasion and metastasis through adipokines secreted by adipocytes.Multiple adipocyte-derived cytokines have been implicated in obesity-related breast carcinogenesis including leptin [24]. Leptin is an adipokine produced and secreted mainly by the adipose tissue in proportion to fat stores. Many adipokines and/or their receptors are now recognized to be expressed ectopically by cancer cells [25]. In turn, cancer cells may respond to adipokines in an endocrine, paracrine, or

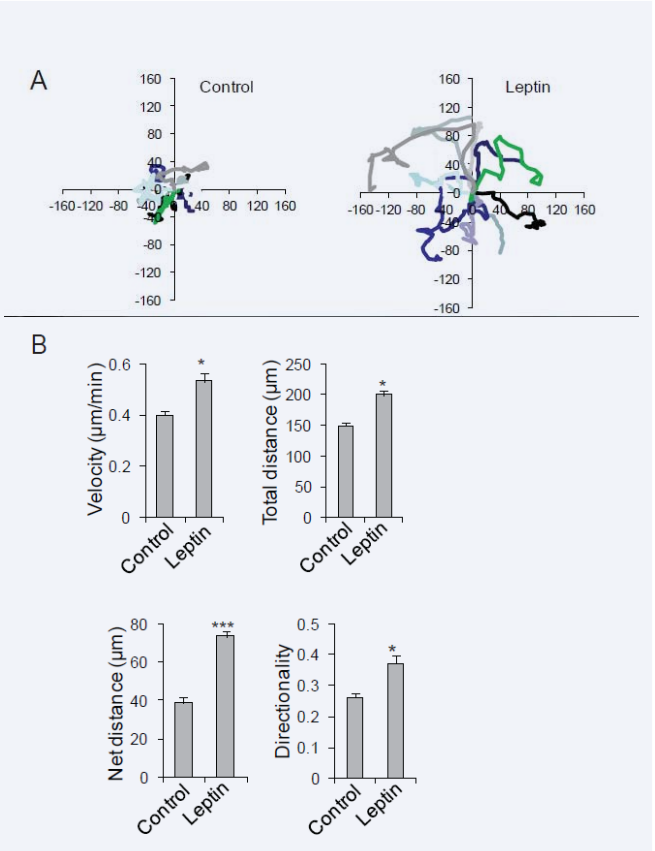

Figure 1 Effect of leptin on MDA-MB-231 breast cancer cell migration. (A) Migration tracks of MDA-MB-231 cells were plated on glass-bottomed dishes coated with 5 μg/ml fibronectin, and treated with 100ng/ml Leptin, compared to Leptin-untreated MDA-MB-231 cells. (B) Statistic results of velocity, net distance, total path and directionality of the cells that control and 100ng/ml Leptin treated. The data are expressed as mean S.E.M. of more than 50 cells from at least three independent experiments. *P<0.05, ***P < 0.001 compared to control cells.

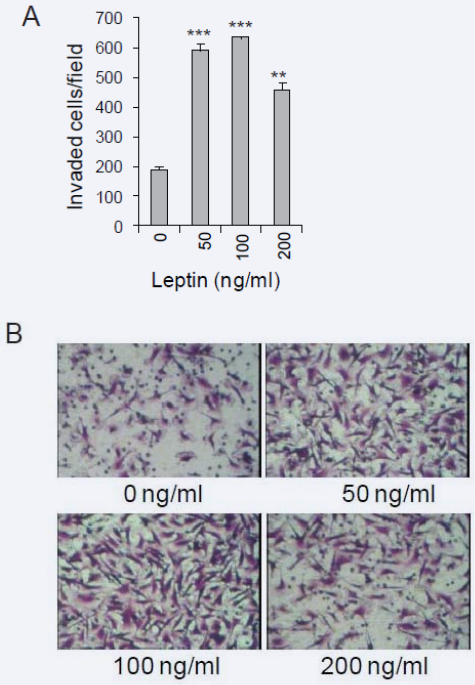

Figure 2 Effect of leptin on MDA-MB-231 breast cancer cell invasion.(A) MDA-MB-231 cells were incubated in 1% serum media containing 0 ng/ml,50 ng/ml,100ng/ml,200 ng/ml of leptin. At indicated time points, cells that had migrated to the bottom surface of the transwell membranes were counted. Data (mean ± SEM) are representative of three to four independent experiments. **P<0.01, ***P < 0.001(vesus no leptin treatment). (B) MDA-MB-231 cells were incubated with a changing concentration of 0 ng/ml,50 ng/ml,100ng/ml,200 ng/ml. After 14 h, cells were observed under microscopy and photographed. Each photograph represents an example of four randomly chosen areas from three to four separate experiments.

autocrine fashion [26]. We showed here that leptin stimulated breast cancer cell migration and invasion. These results are consistent with previous reports [5]. Interestingly, our study here showed that leptin stimulated breast cancer cell migration through steering the directionality (Figure 1). Further studies are needed to understand the role of leptin in regulating breast cancer metastasis.

Leptin up-regulates HECTD1 expression and modulates phosphoinositide metabolism in MDA- MB-231 cells

To dissect the mechanism by which leptin regulates cell migration and invasion, we examined whether leptin regulates HECTD1 and PIPKIγ90 mRNA levels. To this end, MDA-MB-231 cells were treated with 100ng/ml leptin for different concentrations as indicated and mRNA of HECTD1 and PIPKIγ90 were detected by Real-time Q-PCR. Leptin significantly up-regulated the mRNA levels of HECTD1 after exposure to leptin for 2-8 h (Figure 3A), but had little effect on those of PIPKIγ90 (Figure 3B). The expressions of HECTD1 and PIPKIγ90 were further examined by Western blotting. As shown in (Figure 3C), an increase in the steady-state levels of HECTD1 was observed at 1 h after exposure to leptin, reached a plateau at 4 h, and then dampened gradually. On the other hand, the steady-state

levels of PIPKIγ90 decreased after leptin treatment, suggesting that increasing HECTD1 causes PIPKIγ90 ubiquitination and degradation.

The decrease in the steady-state levels of PIPKIγ90 prompted us to examine the effect of leptin on phosphoinositide levels in the cells. MDA-MB-231 cells were treated with leptin for times as indicated and Polyphosphoinositid were extracted, derivatized using trimethylsilyl diazomethane and measured using mass spectrometry. As shown in Figure 3D, leptin treatment resulted in a significant reduction in PIP,PIP2 and PIP3 levels in MDA-MB-231 cells.

Previously we demonstrated that HECTD1 ubiquitinated PIPKIγ90 at K97, consequently resulting in PIPKIγ90 degradation and reduction in PIP2 production. In this study we found that leptin stimulated the gene expression of HECTD1 in MDA-MB-231 cells. After treatment with leptin for 1h , the levels of HECTD1 mRNA significantly increased (Figure 3A) and the steady- state levels of HECTD1 increased ~4-fold correspondingly Fig.3C; On the other hand, mRNA expression of PIPKIγ90 in MDA-MB-231 cells is not influenced by the treatment of leptin (Figure 3B), but the steady-state levels of PIPKIγ90 decreased with increasing hours of leptin treatment Fig.3C. Furthermore, leptin treatment caused a reduction in the levels of PIP2 and PIP3, the direct and indirect product of PIPKIγ90 Fig. 3D. These results suggest that leptin stimulates the ubiquitination of PIPKIγ90 by regulating the gene expression of HECTD1. Nevertheless, PIPKIγ90 ubiquitination by increasing HECTD1 expression can not explain the reduction of PIP levels after leptin treatment. Also, the decrease in the levels of PIP, PIP2 and PIP3 in time course was much steeper the reduction in steady-state levels of PIPKIγ90 after leptin treatment. These results indicate additional mechanisms involved in leptin-mediated regulation of phosphoinositide metabolism.

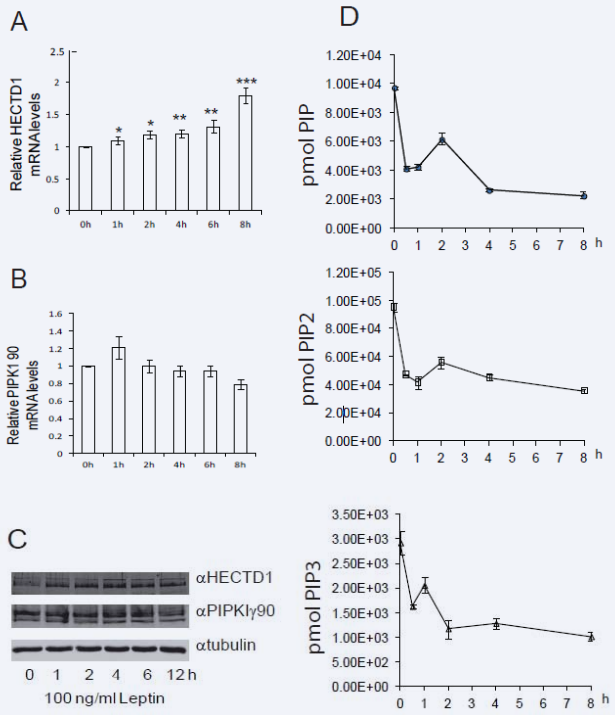

Figure 3 Leptin upregulates HECTD1 and PIPKIγ90 ubiquitination and down-regulates PIP2 production in MDA-MB-231 cells. (A)The relative mRNA levels of HECTD1 in MDAMB-231 cells treated with 100ng/ml leptin 0,1, 2, 4, 6, 8 h were detected by Real-time Q-PCR. Data= mean±S.E.M. *P<0.05, **P<0.01,***P < 0.001 compared to leptin untreated cells. (B)The relative mRNA levels of PIPKIγ90 in MDA- MB-231 cells treated with 100ng/ml of leptin for 0, 1, 2, 4, 6, 8 h were detected by Real-time Q- PCR. Data= mean±S.E.M. (C) The relative steady-state levels of HECTD1 and PIPKIγ90 in MDA-MB-231 cells treated with 100ng/ml leptin for 0, 1, 2, 4, 6, 12 h were detected by Western blotting using anti-HECTD1 antibody or anti-PIPKIγ90 antibody. (D)The PIP ,PIP2 and PIP3 levels in MDA-MB-231 cells treated with 100ng/ml of leptin for different times were detected by mass spectrometry . Data= mean±S.E.M.

Leptin stimulate breast cancer cell migration and invasion by modulating PIPKIγ90 ubiquitination

To further investigate whether PIPKIγ90 ubiquitination is involved in leptin-stimulated breast cancer cell migration, we depleted endogenous PIPKIγ90 of MDA-MB-231 by lentiviral expression of PIPKIγ90 shRNA and then re-expressed codon-modified WT and ubiquitination-deficient mutant of PIPKIγ90^{K97R}, respectively, by retrovirus infection. The cells were plated on fibronectin, and the migration was determined by time-lapse cell migration assays. As shown in Figure 4B, leptin stimulated the migration of PIPKIγ90-depleted cells that re-express codon-modified WT PIPKIγ90, while re-expression of PIPKIγ90^{K97R} counterpart inhibited the cell migration promoted by leptin (Figure 4A and 4B), indicating that leptin stimulates the migration of MDA-MB-231 cells by modulating PIPKIγ90 ubiquitination.

We further examined the role of PIPKIγ90 ubiquitination in the invasion of breast cancer cells by Matrigel invasion assays. The invasion of PIPKIγ90-depleted cells that respectively re-express codon-modified WT PIPKIγ90 and PIPKIγ90^{K97R} with the 100ng/ml of leptin in the lower chamber was compared to the countpart without leptin. Leptin stimulated the invasion of PIPKIγ90-depleted cells that re-express WT PIPKIγ90, but has no effect on that of PIPKIγ90- depleted cells that re-express the ubiquitination-resistant mutant PIPKIγ90^{K97R} (Figure 5). These results indicate that PIPKIγ90 ubiquitination is essential for Leptin-stimulated breast cancer cell invasion.

Our previously study showed that PIPKIγ90 ubiquitination by HECTD1 regulated FA assembly and disassembly, thus modulating breast cancer cell migration, invasion and metastasis [14]. The current studies indicate that PIPKIγ90 ubiquitination at K97 is also essential for leptin- stimulated breast cancer cell migration and invasion.We found that leptin stimulated the migration and invasion of cells rescued with WT PIPKIγ90, but not the migration and invasionof cells expressing PIPKIγ90^{K97R} (Figures 4 and 5). Leptin caused an increase in the net distance and directionality of migration, whereas PIPKIγ90 ^{K97R} inhibited the increase induced by leptin, supporting the conclusion that leptin promotes cell migration through steering the migration directionality.

Taken together, Leptin stimulates breast cancer cell migration and inavsion through up- regulating HECTD1 expression to modulate PIPKIγ90 ubiquitination. This study suggests that blocking the ubiquitination of PIPKIγ90 could be an alternative

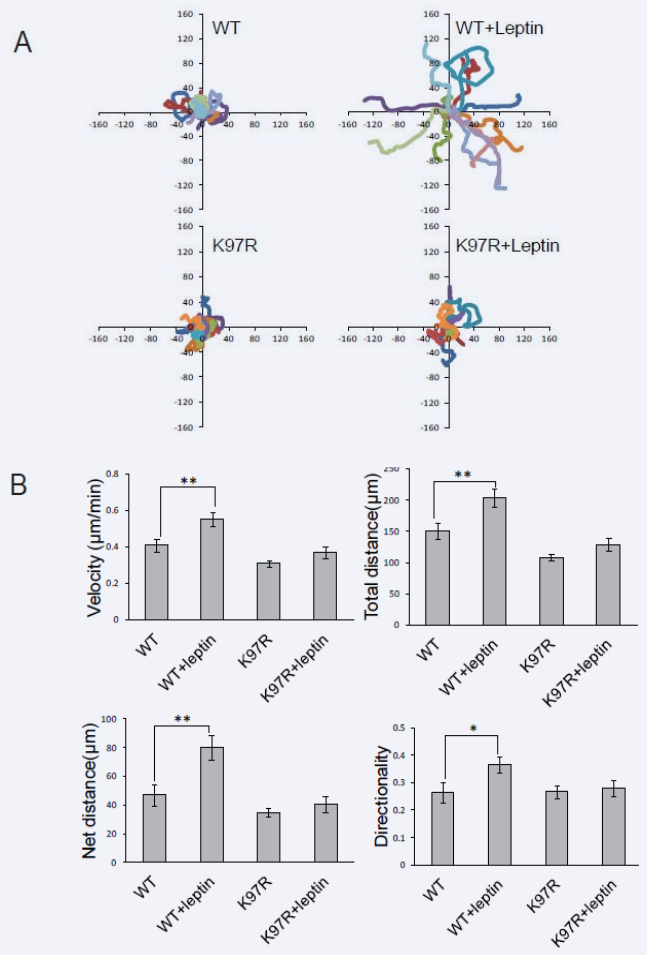

Figure 4 PIPKIγ90 ubiquitination is essential for leptin-stimulated breast cancer cell migration (A) Migration tracks of PIPKIγ90-depleted MDA-MB-231 cells that stably express ZZ- PIPKIγ90 or ZZ-PIPKIγ90K97R in the absence and presence of 10 ng/ml of leptin. (B) Statistic results of velocity, net distance, total path and directionality of PIPKIγ90-depleted cells that stably express ZZ-PIPKIγ90 or ZZ-PIPKIγ90K97R with or without 100ng/ml of leptin. The dataare expressed as mean S.E.M. of more than 50cells from three independent experiments. *P <0.05 compared to leptin untreated ZZ-PIPKIγ90 cells. (C).

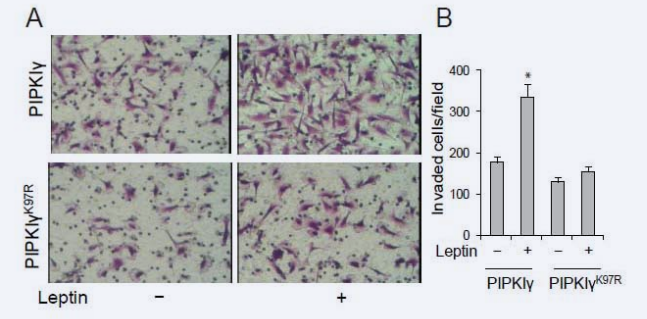

Figure 5 Leptin stimulate breast cancer cell invasion by modulating PIPKIγ90 ubiquitination. (A) leptin stimulates the invasion of PIPKIγ90-depleted cells that re-express a codon-modified WT PIPKIγ90, but has little effect on the invasion of PIPKIγ90-depleted cells that re-express PIPKIγ90K97R. (B) Quantification of Experiment "(A)". Data are presented as mean ± S.E.M., n= 5. *P<0.051.

therapeutic strategy for breast carcinoma in obese patients with high leptin levels.

CONCLUSIONS

The study demonstrates that leptin promotes breast cancer cell migration and invasion, up- regulates HECTD1 expression and enhances PIPKIγ90 ubiquitination, and that PIPKIγ90 ubiquitination is essential for leptin-stimulated breast cancer cell migration and invasion. our findings suggest that inhibition of leptin-HECTD1-PIPKIγ90 pathway could be a potential therapeutic strategy for targeting obesity-related breast cancer.

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