expressing the GFP-Akt-PH construct at an extremely low level, approximately 13 times less than transient expression (Supplementary Figure S2A), was established, which allows the cells to retain invadopodia. In these cells, signals corresponding to GFP-Akt-PH were significantly concentrated at F-actin-rich invadopodia and at the gelatin degradation sites (Figure 1E). This accumulation of GFP signals at invadopodia was not observed when cells expressing GFP alone were examined in the same manner (Supplementary Figure S2B). These results indicate that $PI(3,4,5)P_3$ and $PI(3,4)P_2$ produced as downstream effectors of PI3K have an essential role in invadopodia-mediated ECM degradation.

The class I PI3K catalytic subunit p110α is an essential regulator of invadopodia formation

Mammalian cells contain eight PI3K enzymes, which are further classified into classes I, II, and III (Fruman et al., 1998). In the present study, the expression levels of the PI3K family of proteins were examined in MDA-MB-231 cells by real-time quantitative PCR and standard semi-quantitative PCR analyses performed using different sets of primers specific for the PI3K isoforms (Figure 2A and Supplementary Figure S3A). The class I subunits p110α, p110β, and p110δ; the class II subunit C2α; and the class III subunit Vps34 were abundantly expressed in these cells. Furthermore, the expression of the class II subunit C2β was weak but detectable. However, these cells did not express the class I subunit p110γ or the class II subunit C2γ.

siRNA knockdown experiments were performed to determine the contribution of individual PI3K isoforms to invadopodia formation. MDA-MB-231 cells were transfected with siRNAs targeting each PI3K enzyme and subsequently examined for

invadopodia formation and gelatin degradation. The efficiency and selectivity of the siRNAs in knocking down individual PI3K isoforms were confirmed by RT-PCR analysis (Figure 2B), and the knockdown of class I p110 enzymes was also confirmed by immunoblotting (Figure 2C). Cells with reduced p110α levels showed a significant decrease in invadopodia formation and gelatin degradation activity (Figure 2D–G). Similar results were obtained with three other siRNAs targeting different regions of the p110α gene (Supplementary Figure S4A and B). However, cells transfected with siRNAs targeting other class I PI3K enzymes (i.e., p110β and p110δ) did not show decreased invadopodia formation or gelatin degradation activity (Figure 2D and E). Furthermore, knock down of classes II and III PI3Ks, including C2α, C2β, and Vps34, did not affect gelatin degradation activity (Figure 2D). Examination of the localization of endogenous p110α by immunocytochemistry revealed the presence of strong signals corresponding to endogenous p110α at invadopodia that were enriched with F-actin and were associated with gelatin degradation sites (Figure 2H). To ascertain whether invadopodia formation mediated by p110α reflects the invasiveness of cancer cells, an *in vitro* Matrigel invasion assay was performed. MDA-MB-231 cells transfected with p110α siRNA showed markedly reduced invasion through Matrigel in comparison to cells transfected with control siRNA (Fig. 2I). Collectively, these results indicate that among the PI3K family proteins, p110α is specifically involved in invadopodia-mediated invasion of human breast cancer cells.

The effect of p110α knockdown on invadopodia formation was assessed in other invasive breast cancer cell lines, namely BT-549 and Hs578T. BT-549 cells treated with two different p110α siRNAs showed a significant decrease in invadopodia-mediated gelatin degradation (Supplementary Figure S4C and D). As Hs578T cells were sensitive to siRNA transfection under the present experimental conditions, a

short hairpin RNA (shRNA) targeting the p110α gene was introduced into Hs578T cells by lentiviral transduction. Transduction of Hs578T cells with p110α shRNA resulted in a marked reduction of the expression of p110α and a concomitant decrease in gelatin degradation activity, as compared to cells with control shRNA (Supplementary Figure S4E–G).

The PI3K signaling pathway activation status was determined by measuring the amount of phosphorylated Akt, a major downstream effector of the PI3K signaling pathway. Knockdown of p110α suppressed Akt phosphorylation upon epidermal growth factor (EGF) stimulation (Figure 2J), while knockdown of p110β or p110δ had almost no effect. Thus, p110α is likely the primary mediator of growth factor-stimulated PI3K signaling in this cell type. Importantly, EGF-induced phosphorylation of ERK was not affected by p110α knockdown (Figure 2J). This result suggests that p110α inhibition does not affect MAPK signaling, a pathway that has been implicated in invadopodia formation in human melanoma cells (Tague et al., 2004).

Pharmacological inhibition of p110α blocks invadopodia formation

To confirm that p110α is an essential regulator of invadopodia formation, the effect of selective inhibitors of class I PI3K isoforms was investigated. Cells were cultured on fluorescent gelatin-coated coverslips in the presence of PIK-75, TGX-221, or IC87114, which are selective inhibitors of p110α, β, and δ, respectively (Chaussade et al., 2007; Knight et al., 2006). p110α inhibition by PIK-75 treatment significantly inhibited gelatin degradation in a dose-dependent manner, showing an IC_{50} of 45.2 nM (Figure 3A–C), and suppressed invadopodia formation (Figure 3D and E). A similar inhibition of gelatin degradation was observed when BT-549 and Hs578T

breast cancer cells were treated with PIK-75 (Supplementary Figure S4H and I). However, neither TGX-221 nor IC87114 significantly affected gelatin degradation (Figure 3A and C), despite their use at concentrations well above the IC_{50} values reported previously (Chaussade et al., 2007). PIK-75 treatment also markedly inhibited Matrigel invasion of MDA-MB-231 cells (Figure 3F).

As expected, we found that only p110α inhibition by PIK-75 suppressed EGF-induced Akt phosphorylation (Figure 3G). In addition, EGF-induced phosphorylation of ERK was not affected by PIK-75 treatment (Figure 3G). At the concentrations used in these experiments, PIK-75 should specifically inhibit p110α activity but should not block p110β and p110δ activities, based on results of previous studies (Chaussade et al., 2007; Knight et al., 2006). These results indicate that p110α plays a pivotal role in PI3K signaling and regulates the invadopodia-mediated ECM degradation activity of invasive breast cancer cells.

Activating mutations in the PIK3CA gene promote invadopodia formation

The *PIK3CA* gene, which encodes p110α, is one of the most frequently mutated genes in human breast cancers, and mutations in this gene are associated with invasion and metastasis (Maruyama et al., 2007; Saal et al., 2005). Most of the mutations occur at two hot spots, namely E545K in the helical domain and H1047R in the catalytic domain; these mutations constitutively activate the PI3K signaling pathway (Isakoff et al., 2005; Kang et al., 2005). Accordingly, the effect of these *PIK3CA* mutations on invadopodia formation was investigated in MDA-MB-231 cells, which express wild type p110α (Hollestelle et al., 2007). MDA-MB-231 cell lines stably expressing wild-type, E545K, or H1047R p110α were generated (Figure 4A). The expression levels of the ectopic proteins were approximately 4–5 times

higher than the expression level of the endogenous protein (Figure 4A). The results showed an increase in EGF-induced Akt phosphorylation in cells expressing wild-type p110α and further increase in cells expressing either E545K or H1047R p110α in comparison to control mock-infected cells (Figure 4B). Furthermore, morphological analysis revealed that wild-type p110α cells tended to form more lamellipodia or membrane ruffles than control mock-infected cells (Figure 4C). An additional increase in the protrusive activities in E545K and H1047R expressing cells was observed (Figure 4C), which may reflect enhanced cell motility induced by these p110α mutants as described previously (Pang et al., 2009). Invadopodia formation and gelatin degradation activity were moderately increased in wild-type p110α cells and further enhanced in E545K and H1047R expressing cells (Figure 4D–G). The enhanced gelatin degradation activity in E545K and H1047R expressing cells was still sensitive to PIK-75 treatment, indicating that the enzymatic activity is crucial for invadopodia formation (Figure 4H). Similarly to the behavior of the endogenous protein, the E545K and H1047R p110α mutants also accumulated at gelatin degradation sites (Figure 4I). In addition, E545K and H1047R expressing cells showed enhanced invasion through Matrigel compared to mock-infected cells (Figure 4J). These findings indicate that these activating mutations in the *PIK3CA* gene commonly present in human cancers promote the invadopodia-mediated invasive activity of breast cancer cells.

PDK1 and Akt are involved in invadopodia formation

To determine the downstream target of p110α associated with invadopodia formation, the role of PDK1 was examined. PDK1 has been shown to translocate to the plasma membrane upon activation of PI3Ks, and phosphorylate downstream

targets including Akt (Toker and Newton, 2000). PDK1 expression in MDA-MB-231 cells was confirmed by immunoblotting and suppressed by two different siRNA sequences that target different regions of the *PDK1* gene (Figure 5A). PDK1 downregulation clearly impaired invadopodia formation in these cells and the related gelatin matrix degradation (Figure 5B–D).

The role of Akt in invadopodia formation was then examined. The expression of all Akt isoforms (i.e., Akt1, Akt2, and Akt3) was detected in MDA-MB-231 cells by real-time quantitative PCR (Supplementary Figure S3B). To avoid possible functional redundancy, all Akt isoforms were simultaneously knocked down. In cells transfected with two different sets of siRNAs, the expression of total Akt was efficiently suppressed (Figure 5E). Akt knockdown significantly decreased invadopodia formation and gelatin degradation (Figure 5F–H). Furthermore, knockdown of PDK1 or Akt markedly decreased invadopodia formation in both E545K and H1047R p110α cells (Figure 5I). Examination of the localization of endogenous Akt and PDK1 proteins revealed that these proteins accumulated at invadopodia-mediated gelatin degradation sites in MDA-MB-231 cells (Figure 5J) and BT549 cells (Supplementary Figure S4J). These results indicate that the role of PDK1 and Akt as downstream targets of p110α is essential for invadopodia formation.

Pharmacological inhibition of PDK1 and Akt blocks invadopodia formation

To further confirm the involvement of PDK1 and Akt, cells were treated with OUS-03012 and the Akt inhibitor VIII, which are inhibitors of PDK1 and Akt, respectively. Although its specificity may need better characterization, OSU-03012 was shown to potently inhibit PDK1 activity by competing with ATP (Zhu et al., 2004). The Akt inhibitor VIII is a PH-domain dependent specific Akt inhibitor and

blocks activation of Akt (Barnett et al., 2005). Treatment of cells with these inhibitors resulted in a decrease in the levels of phosphorylated Akt (Figure 6A and B). These inhibitors markedly blocked gelatin degradation activity (IC_{50} = 3.6 μM for OSU-03012, 2.2 μM for Akt inhibitor VIII: Figure 6C–F) and invadopodia formation (Figure 6G–J). We also examined the effect of a PKC inhibitor on invadopodia formation because PKC is another major substrate of PDK1 (Toker and Newton, 2000). When treated with the broad-range PKC inhibitors calphostin and GF109203X, MDA-MB-231 cells showed no obvious changes in gelatin degradation activity (Figure 6C). Moreover, OSU-03012 and the Akt inhibitor VIII significantly blocked gelatin degradation activities of cells expressing the activating mutants of p110α (Figure 6K).

Overexpression of Akt constructs affects invadopodia formation

The effect of the ectopic expression of various Akt constructs was examined by generating MDA-MB-231 cell lines stably expressing wild-type (WT), kinase-dead (KD), or a membrane-targeted constitutively active form (Myr) of Akt1 (Figure 7A). Akt phosphorylation increased in cells expressing WT Akt1 but decreased in cells expressing KD Akt1 in comparison to control mock-infected cells (Figure 7A). Myr Akt1 expression robustly enhanced Akt phosphorylation (Figure 7A). Invadopodia formation and gelatin degradation activity were increased in WT Akt1 cells but decreased in KD Akt1 cells, consistent with the changes in Akt phosphorylation (Figure 7B–E). Unexpectedly, however, cells expressing Myr Akt1 showed a marked decrease in invadopodia formation and gelatin degradation (Figure 7B–E). Ectopically expressed WT Akt1 accumulated at invadopodia in a similar manner to endogenous protein (Figure 7F). In contrast, Myr Akt1 uniformly distributed throughout the

plasma membrane and showed no specific localization (Figure 7F). We also generated MDA-MB-231 cell lines expressing other constitutively active forms of Akt1, namely E17K and E40K, which have a higher affinity for phosphoinositides (Aoki et al., 1998; Carpten et al., 2007). Although the expression of these Akt1 mutants markedly increased Alt phosphorylation, it abrogated invadopodia-mediated gelatin degradation activity (Supplementary Fig. S5A and B). Taken together, these results confirm the role of Akt in invadopodia formation and suggest that site-specific and proper activation of Akt is necessary for efficient assembly of invadopodia.

Discussion

In the present study, the PI3K inhibitors LY294002 and wortmannin were shown to effectively inhibit invadopodia formation in MDA-MB-231 human breast cancer cells. This result is consistent with the previous reports describing that the formation of invadopodia in human cancer cells and podosomes in Src-transformed fibroblasts requires the activity of PI3K (Mandal et al., 2008; Nakahara et al., 2003; Oikawa et al., 2008).

Overexpression of the Akt-PH domain, which sequesters the PI3K products $PI(3,4,5)P_3$ and $PI(3,4)P_2$, effectively blocked invadopodia formation. Although the predominant product of PI3K is $PI(3,4,5)P_3$, several evidence raise the possibility that $PI(3,4)P_2$ also plays a significant and redundant role in invadopodia formation in parallel with $PI(3,4,5)P_3$ (Fig. 8). Chuang et al reported that siRNA knockdown of synaptojanin-2, which generates $PI(3,4)P_2$ via dephosphorylation of $PI(3,4,5)P_3$, blocks invadopodia formation in glioma cells (Chuang et al., 2004). Moreover, Oikawa et al. reported that $PI(3,4)P_2$ regulates podosome formation by recruiting Tks5 and N-WASP, essential components of podosomes (Oikawa et al., 2008). Therefore, although further studies are required to precisely define the individual roles of $PI(3,4,5)P_3$ and $PI(3,4)P_2$, our results indicate that these D3-phosphoinositides produced by PI3K activity play an essential role in invadopodia biogenesis.

We and other researchers have previously reported that invadopodia formation is initiated with the assembly of actin core structures, followed by the accumulation of matrix metalloproteinases (MMPs) for ECM degradation (Artym et al., 2006; Oser et al., 2009; Yamaguchi et al., 2005a). The finding that treatment of cells with PI3K inhibitors blocked the formation of F-actin and cortactin structures of invadopodia suggests that PI3K signaling is involved in the first step of invadopodia formation. In

support of this hypothesis, PI3K inhibitors disassembled the F-actin structures of invadopodia, as shown by time-lapse analysis, and that PI3K products were enriched with F-actin at the invadopodia, as detected with the GFP-Akt PH construct. Consistent with these observations, Mandal et al. recently reported that PI3K is required for the formation of F-actin cores of invadopodia induced by TGF-β stimulation (Mandal et al., 2008).

An important finding of the present study was that among the PI3K isoforms, the class I PI3K catalytic subunit p110α is specifically involved in invadopodia formation. We showed that pharmacological inhibition of p110α blocked invadopodia-mediated ECM degradation and invasion in human breast cancer cell lines. Several inhibitors that target PI3Ks are currently being tested in clinical trials for the treatment of human cancers (Engelman, 2009). However, these broad-spectrum PI3K inhibitors can cause significant side effects due to the multiple roles of the PI3K signaling pathway in basic cellular functions. Therefore, current research is extensively focused both on understanding the isoform-specific functions of PI3Ks and on developing isoform-specific inhibitors of the PI3K family proteins (Engelman, 2009; Jia et al., 2009; Zhao and Vogt, 2008).

Recent studies have delineated distinct functions of class I PI3K isoforms (Engelman, 2009; Jia et al., 2009). The p110α subunit was shown to predominantly mediate PI3K signaling activity in receptor tyrosine kinase signal transduction, whereas p110β responds to G protein-coupled receptors (Guillermet-Guibert et al., 2008; Zhao et al., 2006). In addition, it has been reported that immune system function is largely dependent on p110δ and p110γ (Rommel et al., 2007). Moreover, unlike *PIK3CA*, which encodes p110α, cancer-specific mutations have not been reported for genes encoding other class I PI3Ks (Jia et al., 2009). Based on these

findings and the specific role of p110α in invadopodia formation, we hypothesize that p110α is a promising therapeutic target for the treatment of cancer invasion and metastasis with minimal side effects.

The *PIK3CA* mutations found in human cancers primarily occur at two hot spots: E545K in the helical domain and H1047R in the catalytic domain (Samuels and Ericson, 2006; Zhao and Vogt, 2008). These mutations are known to promote the catalytic activity of p110α, thereby leading to constitutive activation of the PI3K signaling pathway (Kang et al., 2005). We determined that the E545K and H1047R mutations in p110α enhanced invadopodia-mediated ECM degradation and invasion. This finding provides mechanistic insight into the role of p110α mutations in cancer invasion.

Although we clearly showed that basal p110α activity is required for invadopodia formation, mutations of p110α are not sufficient to trigger invadopodia formation. In fact, several breast cancer cell lines that contain p110α mutations, such as MCF-7 and T47D (Hollestelle et al., 2007), are unable to form invadopodia, as reported previously (Coopman et al., 1998; Yamaguchi et al., 2009). Therefore, it is likely that activation of other factors and/or signaling pathways trigger invadopodia formation, and the concurrent activation of p110α by mutations may act as a positive modulator in this process. This concept is supported by the facts that activating p110α mutations are preferentially observed in invasive tumors (Maruyama et al., 2007; Saal et al., 2005) and often associated with other alterations, such as ERBB2 overexpression and *K-ras* mutations (Oda et al., 2008).

In the present study, we demonstrated, for the first time, that PDK1 and Akt are involved in invadopodia formation. Importantly, knockdown and pharmacological inhibition of Akt or PDK1 abolished the enhanced invadopodia formation induced by

E545K and H1047R p110α. Previous studies have shown that PDK1 and Akt are overexpressed and/or mutated in various human cancers and have implicated these proteins in cancer invasion and metastasis (Liu et al., 2009; Pinner and Sahai, 2008; Sheng et al., 2009; Xie et al., 2006). Therefore, our findings may provide a further rationale for targeting PDK1 and Akt, in addition to p110α, in the development of anti-invasion and anti-metastasis strategies.

Additional evidence that Akt is required for invadopodia formation was provided by the overexpression of wild-type and kinase dead forms of Akt. Unexpectedly, however, overexpression of constitutively active forms of Akt markedly blocked invadopodia formation. Because we observed that Akt localized to invadopodia, site-specific and controlled activation of Akt by p110α and PDK1 may be required for proper invadopodia formation and cancer invasion. In agreement with this idea, the constitutively active form of Akt was shown to inhibit invasion of breast cancer cells both *in vitro* and *in vivo* (Hutchinson et al., 2004; Liu et al., 2006). Further studies are necessary to elucidate the exact mechanisms underlying the regulation of invadopodia formation by the p110α/PDK1/Akt pathway.

In conclusion, our results strongly suggest that the PI3K signaling pathway mediated by p110α is a critical regulator of invadopodia-mediated invasion of human breast cancer cells. These findings identified a new cellular function of the well-known oncogene product p110α and provided new insights into the molecular mechanisms of invadopodia formation and cancer cell invasion.

Materials and Methods

Cell culture

Human breast cancer cell lines MDA-MB-231, BT-549, and Hs578T were obtained from the American Type Culture Collection. MDA-MB-231 cells were maintained in a 1:1 mixture of high glucose-DMEM and RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 units/ml of penicillin, and 10 μg/ml of streptomycin. BT-549 and Hs578T cells were maintained in RPMI1640 and DMEM, respectively, supplemented as above.

Antibodies, reagents, and constructs

Alexa dyes, fluorescently labeled phalloidin, and secondary antibodies were purchased from Invitrogen. LY294002, wortmannin, anti-p110α, anti-p110β, anti-ERK, and anti-Akt antibodies were purchased from Cell Signaling Technology. Anti-p110δ antibody, calphostin, and Akt inhibitor VIII were purchased from Calbiochem. Anti-β-actin antibody and recombinant human EGF were purchased from Millipore. Anti-HA antibody was purchased from Covance. PIK-75 and IC87114 were purchased from Symansis. TGX-221 was purchased from Cayman Chemical. OSU-03012 was purchased from Echelon Biosciences. GF109203X was purchased from Enzo Life Sciences. Gelatin and other chemicals were purchased from Sigma-Aldrich. For GFP-Akt-PH domain constructs, the cDNA that encoded the mouse Akt-PH domain (1–111aa) was subcloned into the pEGFP-C1 vector (Clontech). pBabe-puro constructs for HA-tagged wild-type, E545K, and H1047R forms of p110α, and pLNCX constructs for HA-tagged wild-type, kinase-dead, and constitutively active myristoylated forms of Akt have been previously described (Ramaswamy et al., 1999; Zhao et al., 2005) and were obtained from Addgene. The PrimeSTAR Mutagenesis

Basal Kit (Takara) and QuickChange Lightning Site-directed Mutagenesis Kit (Agilent Technologies) were used to generate the Akt-PH domain R25C mutant, and Akt1 E17K and E40K mutants.

Plasmid transfection, retroviral infection, lentiviral infection, and generation of stable cell lines

MDA-MB-231 cells were transfected with indicated plasmids using Lipofectamine 2000 (Invitrogen) or Lipofectamine LTX (Invitrogen), according to the manufacturer's instructions. To generate stable cell lines, transfected cells were selected with G418 at 1 mg/ml, and resistant clones were isolated. For retroviral infection, cDNAs were inserted into the pMXs-IP or pLNCX vector and recombinant retroviruses were produced with the Plat-A packaging cell line, as previously described (Kitamura et al., 2003). Cells were infected with the recombinant retroviruses and selected with 1 μg/ml of puromycin or 1 mg/ml of G418. Control and p110α shRNA lentiviral particles were purchased from Santa Cruz Biotechnology. Lentiviral infection was carried out according to the manufacturer's instructions and infected cells were selected with 1 μg/ml of puromycin.

Immunofluorescence analysis

Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 5 min. To detect the localization of GFP-Akt PH construct and PDK1, cells were fixed and permeabilized in 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.075 mg/ml of saponin for 1 h at 37°C. The cells were blocked in 1% BSA and 1% goat serum for 30 min. The cells were incubated with primary antibodies for 1 h, and then with fluorophore-conjugated secondary antibodies and

phalloidin (Molecular Probes) for 30 min. Samples were observed with an IX81-ZDC-DSU confocal microscope (Olympus) equipped with a cooled CCD camera (ORCA-ER; Hamamatsu) and the imaging system was driven by Metamorph software (Universal Imaging). All images were acquired using 60× (PLAPON60XO, NA 1.42) or 100× (UPLSAPO100XO, NA 1.4) oil objectives. Images were analyzed and processed with various software packages, including Metamorph, ImageJ version 1.41o (National Institutes of Health, USA, http://rsbweb.nih.gov/ij/), and Adobe Photoshop CS4.

Time-lapse microscopic analysis

In brief, time-lapse series of cells were taken at 37°C using the above described Olympus IX81-ZDC-DSU microscope equipped with a humidified CO_2 chamber. Digital images were converted in ImageJ 1.41o, and the fluorescence intensity of GFP-actin at the invadopodia was calculated.

RNA interference

All RNA interference (RNAi) experiments were carried out using Stealth RNAi molecules (Invitrogen). The Stealth RNAi molecules used in this study are shown in Supplementary Table S1. Cells were transfected with 30 nM siRNA using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. The cells were cultured for 48–72 h and used for invadopodia formation assay and other assays.

RT-PCR

Total RNA was isolated with an RNeasy Plus Mini Kit (Qiagen). Template cDNAs were synthesized with SuperScript III (Invitrogen). Quantitative RT-PCR was performed with Thunderbird qPCR Mix (TOYOBO) in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). For standard PCR amplification, KOD Plus DNA polymerase (TOYOBO) and puReTaq Ready-To-Go PCR Beads (GE Healthcare) were used. The sequences of primer pairs used are shown in Supplementary Table S2.

Immunoblotting

Cells were washed with ice-cold PBS twice before direct extraction in SDS-PAGE sample buffer or lysis in a buffer containing 25 mM Tris-HCl (pH7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail (Roche). The samples were resolved by SDS-PAGE, transferred to PVDF membranes, and blocked with 5% nonfat dried milk. The membranes were incubated first with primary antibodies for 1h, then with peroxidase-conjugated secondary antibodies for 30 min. The antibodies were diluted in Can Get Signal Immunoreaction Enhancer Solution (TOYOBO). Immunoreactive bands were detected using an ECL-plus kit (GE Healthcare).

Invadopodia assay

Fluorescent matrix-coated dishes were prepared as previously described (Bowden et al., 2001). Gelatin was labeled with TRITC in a buffer containing 40 mM NaCl and 50 mM $Na_2B_4O_7$ (pH 9.3) and unbound dyes were removed by extensive dialysis against PBS at 37°C. Coverslips (12-mm, circular) were coated with 100 μl of 25 mg/ml fluorescent gelatin and 20 mg/ml sucrose in PBS and then crosslinked with

0.5% glutaraldehyde on ice for 15 min followed by 30 min at room temperature. After extensive washing with PBS, the coverslips were treated with 5 mg/ml sodium borohydrate for 5 min to quench autofluorescence of residual glutaraldehyde. The coverslips were then sterilized with 70% ethanol for 15 min. MDA-MB-231 cells were cultured on the gelatin-coated coverslips for 3–7 h. To quantitate the gelatin degradation activity of invadopodia, we calculated the degradation area observed in images with the ImageJ 1.41o software and normalized the measurements to the total number of cells in each image. Ten randomly selected fields, usually containing 30-50 cells in total, were imaged with a 60× objective and analyzed for each experiment. The values of control cells were set to 100% and the relative values of other cells were then calculated accordingly. The relative number of invadopodia and the % of cells with invadopodia were also calculated from the microscopy images.

EGF stimulation

Cells were serum-starved overnight in medium containing 0.35% BSA and stimulated with 8 nM EGF for 10 min at 37°C. The cells were subsequently washed twice with ice-cold PBS and lysed with a lysis buffer containing 50 mM Tris-HCl (pH7.5), 1% NP-40, 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, and a protease inhibitor cocktail. The lysates were separated from cell debris by centrifugation and used for immunoblotting.

Invasion assay

Matrigel invasion assay was performed with BD BioCoat Tumor Invasion System (BD Biosciences), composed of BD Falcon FluoroBlok 24-multiwell inserts plates (8 μm pore size) coated with BD Matrigel matrix. The insert wells were rehydrated with

500 μl of PBS for 2 h at 37°C. Cells were labeled with CellTracker Green CMFDA (Invitrogen) and resuspended at 1×10^5/ml in serum-free medium. 500 μl of the labeled cell suspension was added to the upper chambers and 750 μl of growth medium containing 10% FBS was added to the lower chambers as chemoattractant. After 24 h of incubation at 37°C, cells that invaded onto the lower surface of the filters were directly imaged with an IX81-ZDC-DSU confocal microscope (Olympus), using a 10× objective (UPLFLN10X2PH, NA 0.3). Invaded cells were counted in 5 randomly selected fields per filter and average number of control cells were set to 100% and the relative values of other cells were then calculated in each experiment.

Statistical analysis

Data are representative of at least three independent experiments. Statistical analysis was performed using Student's t-tests. All p values shown are vs control cells.

Online supplemental material

Fig. S1 shows the effects of PI3K inhibitors, LY294002 and Wortmannin, on invadopodia formation. Fig. S2 shows the expression levels of GFP-Akt-PH and the localization of GFP in MDA-MB-231 cells. Fig. S3 shows RT-PCR analysis of the expression of PI3K and Akt isoforms in MDA-MB-231 cells. Fig. S4 shows the effects of p110α knockdown and PIK-75 treatment on invadopodia formation and localization of PDK1 and Akt at invadopodia in human breast cancer cell lines. Fig. S5 shows the effects of expression of E17K and E40K Akt1 constructs on invadopodia formation. Table S1 and Table S2 shows siRNA and primer sequences, respectively, used in this study. Video 1 and Video 2 shows disassembly of

invadopodia by LY294002 treatment in GFP-actin and Venus-cortactin cells, respectively.

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