Down-Regulation of 3-Phosphoinositide–Dependent Protein Kinase-1 Levels Inhibits Migration and Experimental Metastasis of Human Breast Cancer Cells

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Abstract

High expression of 3-phosphoinositide–dependent protein kinase-1 (PDK1) has been detected in various invasive cancers. In the current study, we investigated its role in cancer cell migration and experimental metastasis. Down-regulation of PDK1 expression by small interference RNA markedly inhibited spontaneous migration and epidermal growth factor (EGF)–induced chemotaxis of human breast cancer cells. The defects were rescued by expressing wild-type PDK1. PDK1-depleted cells showed impaired EGF-induced actin polymerization and adhesion, probably due to a decrease in phosphorylation of LIM kinase/cofilin and integrin β1. Confocal microscopy revealed that EGF induced cotranslocation of PDK1 with Akt and protein kinase Cζ (PKCζ), regulators of LIM kinase, and integrin β1. Furthermore, PDK1 depletion dampened EGF-induced phosphorylation and translocation of Akt and PKCζ, suggesting that Akt and PKCζ functioned downstream of PDK1 in the chemotactic signaling pathway. In severe combined immunodeficiency mice, PDK1-depleted human breast cancer cells formed more slowly growing tumors and were defective in extravasation to mouse lungs after i.v. injection. Our results indicate that PDK1 plays an important role in regulating the malignant behavior of breast cancer cells, including their motility, through activation of Akt and PKCζ. Thus, PDK1, which increases its expression in cancer cells, can be used as a target for the development of novel therapies. (Mol Cancer Res 2009;7(6):944–54)

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Introduction

Invasion and metastasis are the major causes of mortality and morbidity in cancer (1). In solid tumors, a small portion of primary tumor cells escape from original sites, intravasate into blood circulation system, follow the circulation, and extravasate into the secondary sites where they propagate there (1, 2). Recent studies suggest that epidermal growth factors (EGF), secreted by macrophages along blood vessel walls, induces intravasation of primary tumor cells into circulation, whereas chemokines, secreted from bone, lymph nodes, and brain, actively attract extravasation of circulating tumor cells (3, 4). Thus, both intravasation and extravasation are mediated by chemotaxis, the capacity of a cell to detect an extracellular gradient of chemical stimuli and to migrate to the higher concentration site (3, 5). Although chemokine receptors and growth factor receptors mediate different signal transduction pathways, they apparently elicit similar cellular responses during chemotaxis, such as cell adhesion and formation of filamental actins (F-actin) in the leading edges of migrating cells, which suggests that these two types of receptors may converge at downstream chemotactic signal transduction pathways (6, 7). In fact, both receptors activate PI3kinases and Rac/Cdc42 during chemotaxis (8, 9). Our studies have revealed that protein kinase Cζ (PKCζ), PTEN, and Akt2 regulate both CXCR4 and EGF receptor–mediated chemotaxis of human breast cancer cells (7, 10). Blocking chemotaxis pathways inhibits cancer cell metastasis (6). Shared chemotactic signaling molecules will provide valuable targets for antimetastasis therapies.

3-Phosphoinositide–dependent protein kinase-1, PDK1, is ubiquitously expressed in human tissues (11). PDK1 was initially discovered to phosphorylate the activation loop of Akt1 in a phosphatidylinositol 3,4,5-triphosphate–dependent

manner (12). Later, a spectrum of PDK1 substrates are identified, including PKC isozymes, p70 and p90 ribosomal S6 kinases, serum and glucocorticoid-induced kinases, and protein kinase A (11, 13). PDK1 plays a pleiotropic role in growth and development (14, 15). PDK1 hypomorphic mice were viable but 40% to 50% smaller than control animals, probably due to general reduction in cell size (16). PDK1 depletion by homologous recombination is embryonic lethal, resulting in multiple abnormalities, such as lack of somites and forebrain (16, 17). PDK1 is also indispensable in tolerance to hypoxia of cardiomyocytes, regulation of glyconeogenesis in the liver, and T-cell development in the thymus (17-19).

PDK1-mediated PI3kinases/Akt pathways have been closely associated with various cancers (20, 21). PDK1 are confirmed to be more highly expressed in a majority of human breast cancer cell lines (22, 23). Over 70% of invasive breast carcinomas express activated PDK1 at a moderate to high level (24). Overexpression of PDK1 is sufficient to transform mammary epithelial cells (25). Inhibition of PDK1 by antisense RNA or small molecule inhibitors induces cancer cell apoptosis and inhibits cell proliferation (26-28). Hypomorphic mutation of PDK1 suppresses spontaneous tumorigenesis in PTEN (±) mice (29). Thus, elevated activation of PDK1 induces tumorigenesis by enhancing cell proliferation and inhibiting apoptosis. Recently, PDK1 has been implicated in cell migration (30). However, its role in cancer cell chemotaxis and metastasis is less clear.

PKCζ is required for cancer cell chemotaxis (7). Akt2 binds and activates PKCζ in human breast cancer cells, resulting in phosphorylation of LIM kinase (LIMK)/cofilin and integrin β1, which in turn regulate actin polymerization and cell adhesion during chemotaxis (31). In this study, we examined the role of PDK1 in the malignant behavior of human breast cancer cells including their migration, tumorigenesis, and experimental metastasis as well as key signaling molecular events coupled to PDK1. We report that PDK1 plays an important role in controlling the level of breast cancer cell malignancy.

Results

Down-Regulation of PDK1 Expression Impaired Breast Cancer Cell Migration

PDK1 expression was examined by Western blotting in three human breast cancer cell lines, MDA-MB-231, MCF-7, and T47D (Supplementary Fig. S1). The plasmid expressing PDK1 small interference RNA (siRNA) was transfected into MDA-MB-231 cells to obtain stable clones with PDK1 depletion, designated as siPDK1/MDA cells. As shown in five representative clones, the level of PDK1 mRNA was reduced (Fig. 1A). Consequently, expression of PDK1 protein was also severely down-regulated (Fig. 1B). The remnant levels of PDK1 in stable clones were lower than 30% of the parental cell line. Down-regulation of PDK1 in MDA-MB-231 cells did not alter the expression of PKCζ as well as cell surface EGF receptor. A siRNA vector containing a scrambled sequence was also transfected to MDA-MB-231 cells to generate control cells. Both siPDK1/MDA and control cells expressed green fluorescent proteins (GFP) encoded in the vector. The rate of cell proliferation was examined in both siPDK1/MDA and MDA-MB-231 cells *in vitro*. The doubling time of siPDK1/MDA cells was moderately reduced, in comparison with that of control cells (Fig. 1C). The change in cell proliferation did not interfere with our study of chemotaxis because it took <4 hours to complete chemotaxis assay, which was much shorter than the doubling time. EGF induced a robust extracellular signal-regulated kinase 1/2 phosphorylation in siPDK1/MDA cells, suggesting that these tumor cells could be in response to EGF (Fig. 1D). An *in vitro* "wound healing" assay was used to characterize cellular migration capacity (8). On sensing a scratch on a confluent cell monolayer, cells rearrange their microtubule organization center and move perpendicularly to fill the gap in the absence of a chemoattractant gradient (8, 32). As shown in Fig. 1E, siPDK1/MDA cells moved at a slower pace compared with control cells, suggesting that down-regulation of PDK1 impaired cell motility.

Down-Regulation of PDK1 Impaired EGF-Induced Chemotaxis of Cancer Cell

EGF elicited a robust chemotaxis of both parental and control cells in a dose-dependent manner (Fig. 2A). Down-regulation of PDK1 severely impaired chemotaxis in these stable clones, but did not alter the dose-response curve (Fig. 2A). Because these clones showed similar defects in subsequent experiments, results from clone 52 was used as representatives. As shown in Table 1, checkerboard analysis revealed that EGF-induced both chemotaxis and chemokinesis of siPDK1/MDA cells, and the cell response was reduced with reduction in PDK1.

To verify that chemotaxis defects in cancer cell migration were associated with down-regulation of PDK1, two additional plasmids of PDK1 siRNA were transiently transfected into MDA-MB-231 cells, which should reduce EGF-induced migration (Supplementary Fig. S2). Down-regulation of PDK1 also impaired chemotaxis of T47D and MCF-7 cells, two additional human breast cancer cell lines (Fig. 2B). To further examine that defects of siPDK1/MDA cells were indeed a result from PDK1 reduction, we transiently expressed a functional wild-type PDK1 plasmid, glutathione *S*-transferase (GST)-Myc–tagged PDK1, into siPDK1/MDA cells to overcome small RNA interference. These cells were designated as rescued cells. As shown in Fig. 2C, control cells displayed a comparable level of PDK1 as to that in MDA-MB-231 cells, whereas siPDK1/MDA showed a reduced level of PDK1. After transfection, an upper band corresponding to the GST-Myc–tagged PDK1 was clearly detected on the top of endogenous PDK1 (Fig. 2C, *top*). Chemotaxis index of rescued cells was recovered, indicating that defects in EGF-induced chemotaxis were due to disruption of PDK1 expression (Fig. 2C, *bottom*). As a control, we transiently transfected PDK1 siRNA plasmid into a normal mammary epithelial cell line, MCF-10, and found these cells were also defective in chemotaxis in response to EGF (Fig. 2D). Our results show that down-regulation of PDK1 impaired the EGF-induced chemotaxis of human normal breast and cancer cells.

Down-Regulation of PDK1 Impaired EGF-Induced Cancer Cell Adhesion

Ligand-induced adhesion is closely associated with cell migration (33, 34). Thus, we examined the role of PDK1 in EGF-induced breast cancer cell adhesion. As shown in Fig. 3A, after stimulation by EGF for 5 minutes, control cells exhibited a marked increase in the number of cells adherent to fibronectin-coated coverslips, whereas 15- or 30-minute stimulation did not

further enhance the adhesion. However, siPDK1/MDA cells failed to respond to EGF, especially at 5 minutes, by increased adhesion (Fig. 3A). Transient transfection with other two siPDK1 plasmids also impaired cancer cell adhesion (Supplementary Fig. S3). On transfection with the GST-Myc–tagged PDK1 plasmid, rescued cells showed a significant recovery of adhesion (Fig. 3B).

Membrane spreading, a conserved polarization process in both *Dictyostelium discoideum* and mammalian neutrophils, increases cell sensitivity to a chemoattractant gradient (35). Down-regulation of PDK1 severely impaired membrane spreading of breast cancer cells in the presence or absence of EGF (Fig. 3C). Because integrin β1 mediates EGF-induced cell adhesion to fibronectin, which in turn regulates membrane spreading (36, 37), we examined EGF-induced phosphorylation of integrin β1 at Ser785 in breast cancer cells (38, 39). In control and siPDK1/MDA cells, a basal level of integrin β1 phosphorylation was detected (Fig. 3D). EGF augmented integrin β1 phosphorylation in control cells, but not in siPDK1/MDA cells (Fig. 3D). Thus, PDK1 plays an important role in EGF-induced cancer cell adhesion to fibronectin, probably through regulating the activation of integrin β1.

Down-Regulation of PDK1 Impaired EGF-Induced Actin Polymerization in Breast Cancer Cells

Actin polymerization at leading edges of a cell is the driving force for chemotaxis (40, 41). As shown in Fig. 4A, EGF elicited a transient actin polymerization at 8 seconds and 1 minute in both control and parental cells, consistent with previous reports (42). In siPDK1/MDA cells, actin polymerization was significantly reduced, suggesting that PDK1 played an important role in regulating cytoskeleton rearrangement.

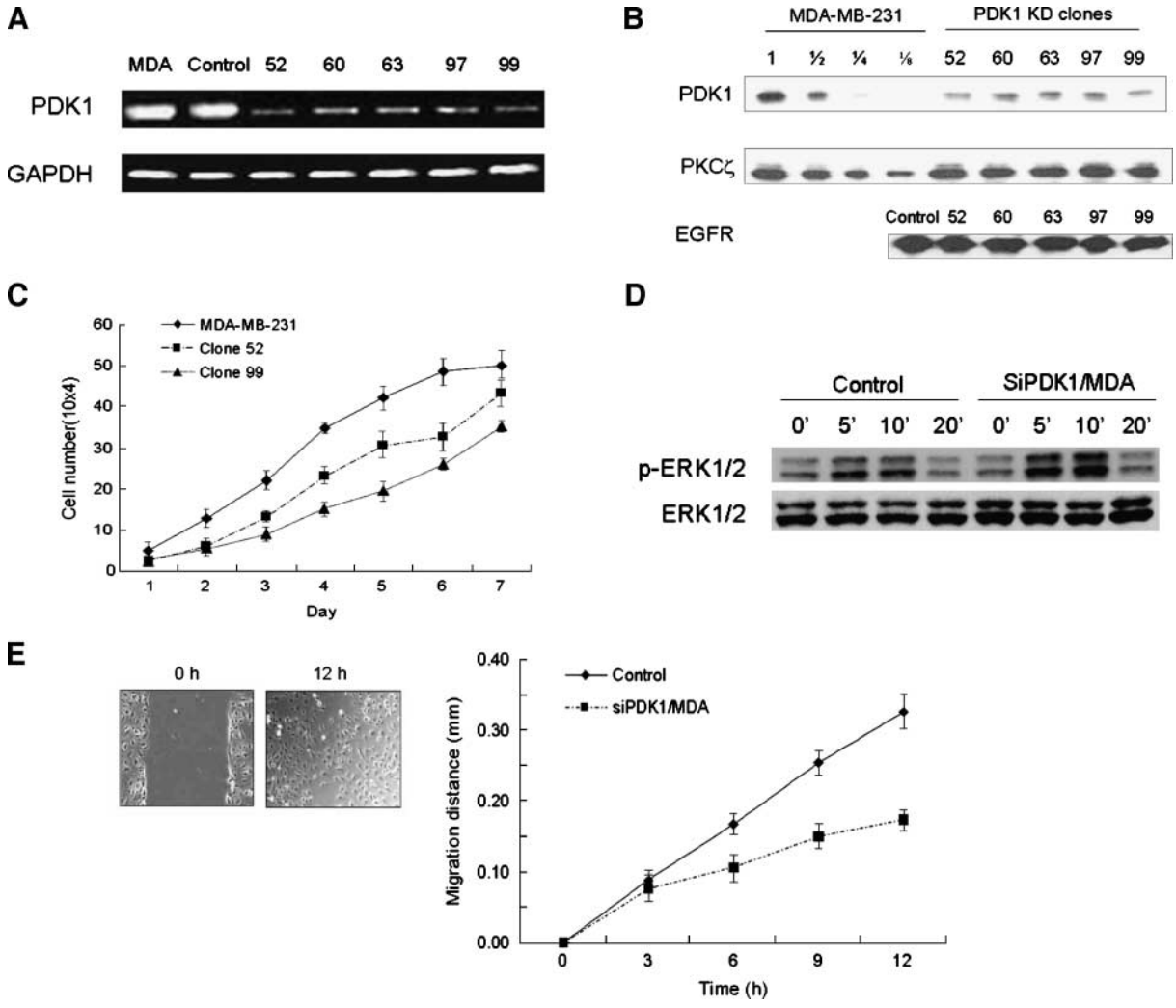

FIGURE 1. Down-regulation of PDK1 impaired cancer cell migration. **A.** RT-PCR analysis of PDK1 in MDA-MB-231 cells, control cells, and five clones of siPDK1/MDA cells. The control cells were transfected with a siRNA plasmid containing a scrambled sequence in MDA-MB-231 cells. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. **B.** PDK1 expression in MDA-MB-231 cells and five siPDK1/MDA clones. The cell lysate of MDA-MB-231 was diluted into a series of different concentrations. In **A** and **B**, each result was a representative result from at least three repeated experiments. **C.** Comparison of cell proliferation in MDA-MB-231 cells and two clones of siPDK1/MDA cells. Points, average of triplicate assays; bars, SD. **D.** Extracellular signal-regulated kinase 1/2 (*ERK1/2*) phosphorylation by 10 ng/mL EGF stimulation in control and siPDK1/MDA cells. **E.** *In vitro* wound healing assay on control and PDK1-knockdown cells. The distance of cell migration during different incubation time was measured after wound was created. Bars, an SD of five wound width along one scratch.

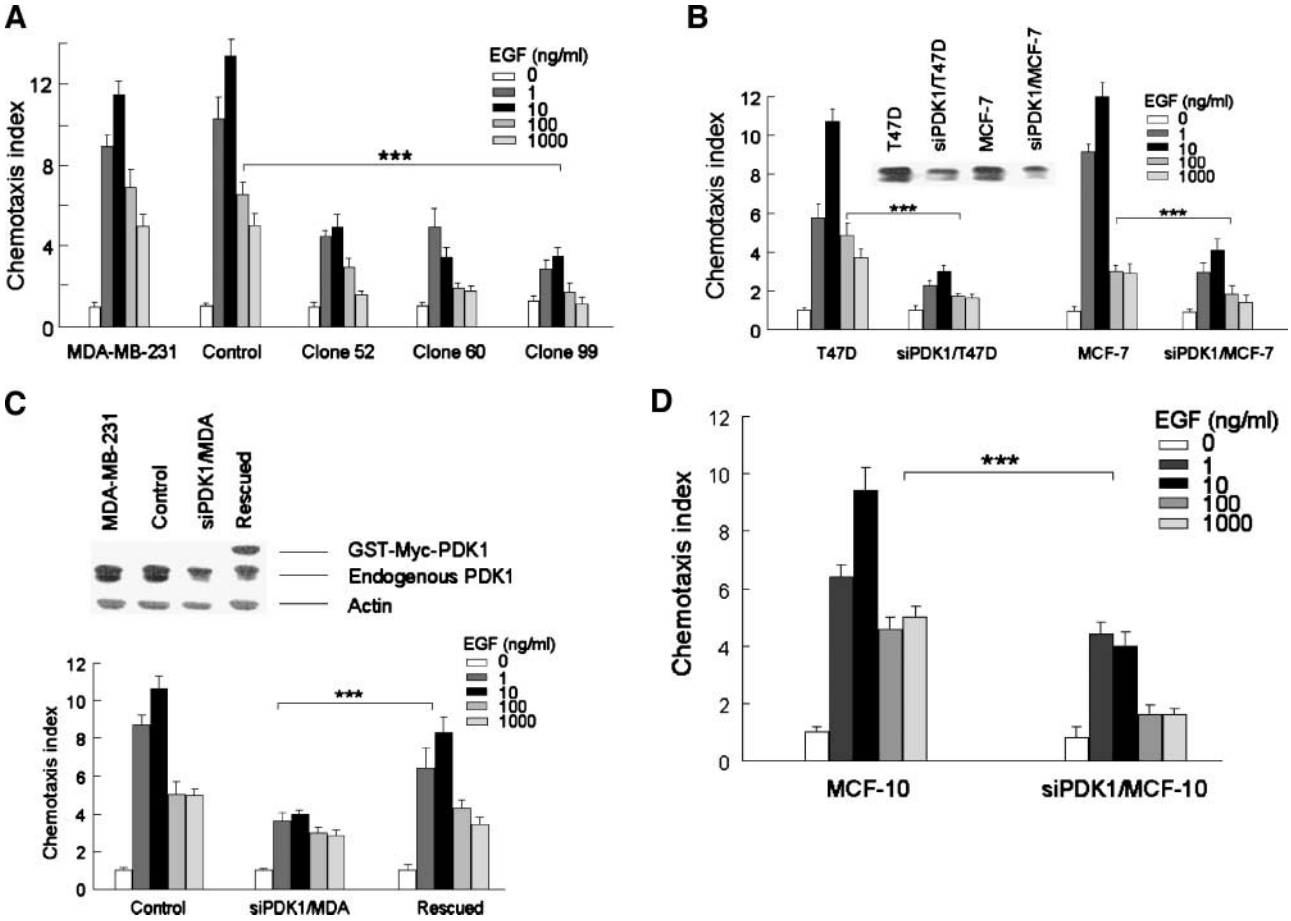

FIGURE 2. Down-regulation of PDK1 inhibited EGF-induced chemotaxis and chemokinesis. **A.** Comparison of EGF-induced chemotaxis of MDA-MB-231 cells, control cells with that of three siPDK1/MDA clones. **B.** PDK1 disruption also reduced the chemotactic responses on EGF stimulation in another two human breast cancer cell lines, T47D and MCF-7. Both cell lines were transiently transfected with one effective siRNA plasmid, respectively. **C.** Recovered chemotactic capacity on EGF in PDK1-rescued cells transiently transfected to express GST-Myc-PDK1. **D.** PDK1 down-regulation also reduced the chemotactic responses on EGF stimulation in normal mammary epithelial cells MDF-10. Data collected in this set of figures were from a representative of at least three repeated experiments. Columns, mean of triplicate measurements; bars, SD. ***, $P < 0.0001$, two-way ANOVA.

Immunofluorescent staining of F-actin showed that at 1 minute, EGF induced an increase in F-actin content in control cells, but not in siPDK1/MDA cells (Fig. 4B). It has been documented that F-actin dynamics is regulated by LIMKs, which phosphorylate cofilin at Ser3, an event critical for cell migration (43, 44). We tested the capacity of LIMK/cofilin in regulating actin polymerization in EGF-activated breast cancer cells. As shown in Fig. 4C, LIMKs were rapidly activated in tumor cells by EGF at 1 minute, resulting in phosphorylation of cofilin at the same time frame. In siPDK1/MDA cells, EGF-induced phosphorylation of LIMK/cofilin was decreased although the levels of both LIMKs and cofilin were comparable with those in control cells (Fig. 4C). We also examined the phosphorylation of these two proteins after a prolonged stimulation by EGF in tumor cells (Fig. 4D). At 60 minutes, EGF-induced LIMK/cofilin phosphorylation was reduced in siPDK1/MDA cells. These results show that PDK1 mediated EGF-induced actin polymerization in cancer cells through regulating LIMK activation and cofilin phosphorylation/inactivation.

Table 1. Chemokinesis Assay of Control and siPDK1/MDA Cells

EGF	Upper Wells (Control Cells)			
Lower Wells	0 ng/mL	1 ng/mL	10 ng/mL	100 ng/mL
0 ng/mL	8 ± 1	15 ± 1	18 ± 3	33 ± 1
1 ng/mL	44 ± 2	34 ± 5	36 ± 2	42 ± 6
10 ng/mL	73 ± 3	52 ± 7	45 ± 5	42 ± 3
100 ng/mL	39 ± 2	32 ± 5	30 ± 5	37 ± 5
EGF	Upper Wells (siPDK1/MDA Cells)			
0 ng/mL	7 ± 1	7 ± 1	12 ± 1	16 ± 2
1 ng/mL	17 ± 3	16 ± 1	18 ± 3	19 ± 3
10 ng/mL	28 ± 3	25 ± 2	24 ± 1	25 ± 2
100 ng/mL	24 ± 3	24 ± 2	25 ± 4	20 ± 3

Down-Regulation of PDK1 Impaired EGF-Induced Activation of Akt and PKCζ in Tumor Cells

We speculate that PDK1 is an upstream regulator of Akt2 and PKCζ during cell chemotaxis (7, 31). To test this hypothesis, we

examined the activation of these signaling molecules by confocal microscopy. The activation hallmark of PDK1, Akt, and PKCζ is their translocation from cytosol to plasma membrane (45-47). As shown in Fig. 5A, PDK1 and Akt were mainly distributed in the cytosol region of resting MDA-MB-231 cells. On EGF stimulation, fluorescent signals of PDK1 were enriched in the plasma membrane, overlapping with Akt (Fig. 5A, *top*). Cytosolic regions in proximity to plasma membrane were depleted of fluorescent signals. EGF also elicited translocation and colocalization of PDK1 and PKCζ in the plasma membrane (Fig. 5A, *bottom*). These results raised the possibility that PDK1 may activate Akt and PKCζ by their close proximity.

We then investigated the effect of PDK1 down-regulation on EGF-induced activation of Akt. It has been shown that PDK1 phosphorylates Akt at Thr308 in the activation loop, whereas Ser473 is phosphorylated by rictor–mammalian target of rapamycin (mTOR) complex (12, 48). As shown in Fig. 5B, in control cells, phosphorylated Akt was detected at 1 minute after EGF stimulation, and peaked at 5 minutes at both Thr308 and Ser473. Reduction of PDK1 severely decreased EGF-induced Akt phosphorylation at Thr308, with concomitant reduction in the phosphorylation of Akt at Ser473 (Fig. 5B). Akt activation in siPDK1/MDA cells was further examined by confocal microscopy (Fig. 5D, *left*) and cells showing membrane fluorescent signals of Akt were designated at positive cells. In the absence of EGF, both control and siPDK1/MDA cells showed minimal number of positive cells (10.9 ± 0.6% and 9.2 ± 0.5%, respectively; Fig. 5D, *right*). EGF simulation markedly increased the number of positive cells to 37.8 ± 1.6% in control cells. In siPDK1/MDA cells, EGF-induced Akt translocation occurred in only 16.7 ± 2.6% cells. The results show that down-regulation of PDK1 impaired EGF-induced activation of Akt in human breast cancer cells.

PDK1 down-regulation was also associated with a reduced and delayed PKCζ phosphorylation on EGF stimulation in breast cancer cells compared with control cells (Fig. 5C). In

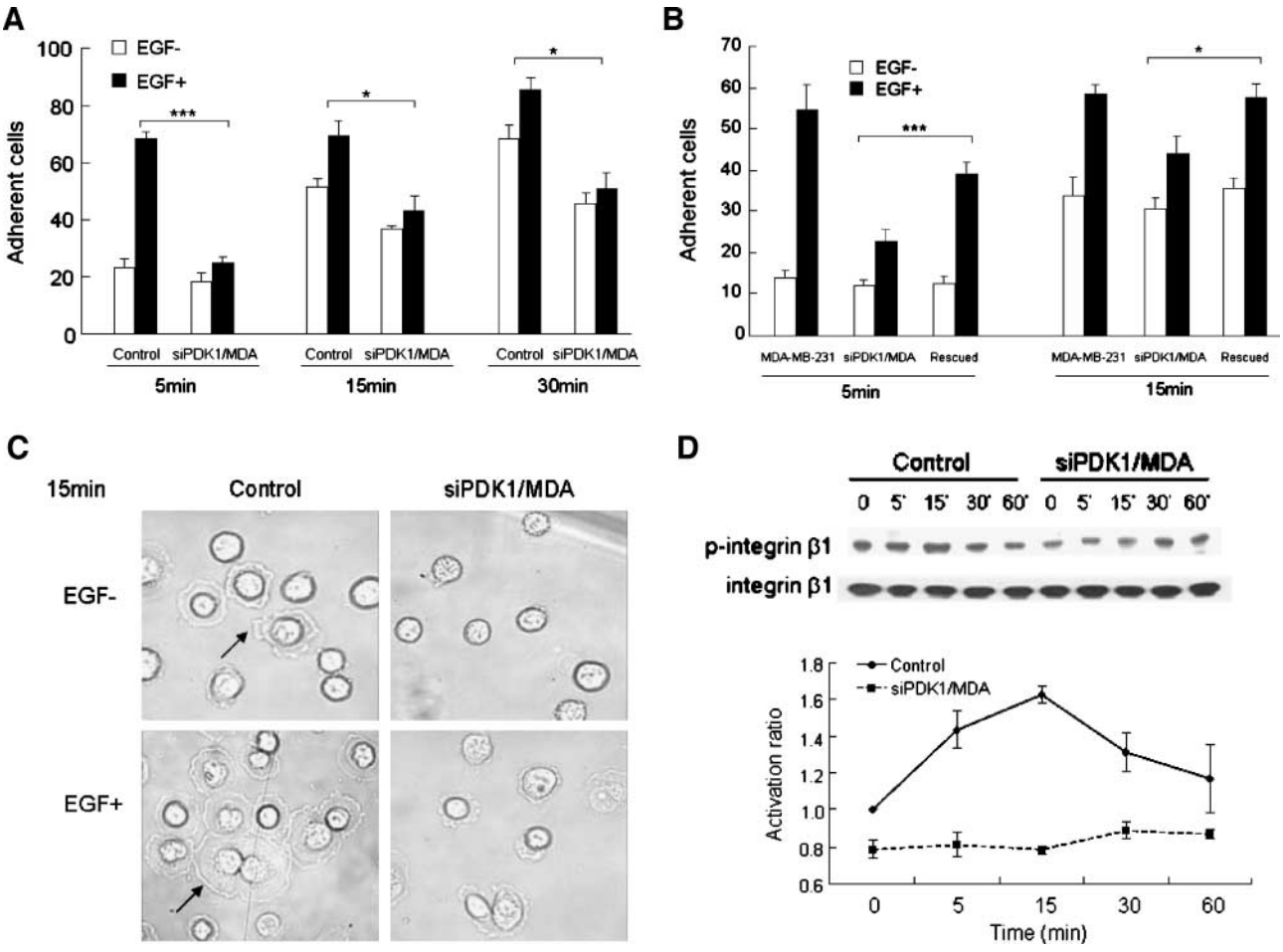

FIGURE 3. Disruption of PDK1 expression impaired ligand-induced cell adhesion. **A.** Comparison of 10 ng/mL EGF-induced cell adhesion at 5, 15, and 30 min of control cells to that of siPDK1/MDA cells on fibronectin-treated coverslips. Cell numbers were counted in five fields on every coverslips under the light microscopy (×200); ***, $P < 0.0001$; *, $P_{(15\ min)} = 0.0172$; *, $P_{(30\ min)} = 0.0103$, two-way ANOVA. **B.** Recovered cell adhesion induced by EGF at 5 and 15 min in PDK1-rescued cells. Data collected in this set were from a representative of at least three repeated experiments. ***, $P < 0.0001$; *, $P = 0.0139$, two-way ANOVA. **C.** The morphologic examination of control and siPDK1/MDA cells at 15 min after EGF stimulation. **D.** Western blotting analysis of integrin β1 phosphorylation in total cell lysates from control and PDK1-depleted cells on stimulation with 10 ng/mL EGF for 0, 5, 15, 30, and 60 min. Total integrin β1 amount in each sample was also shown.

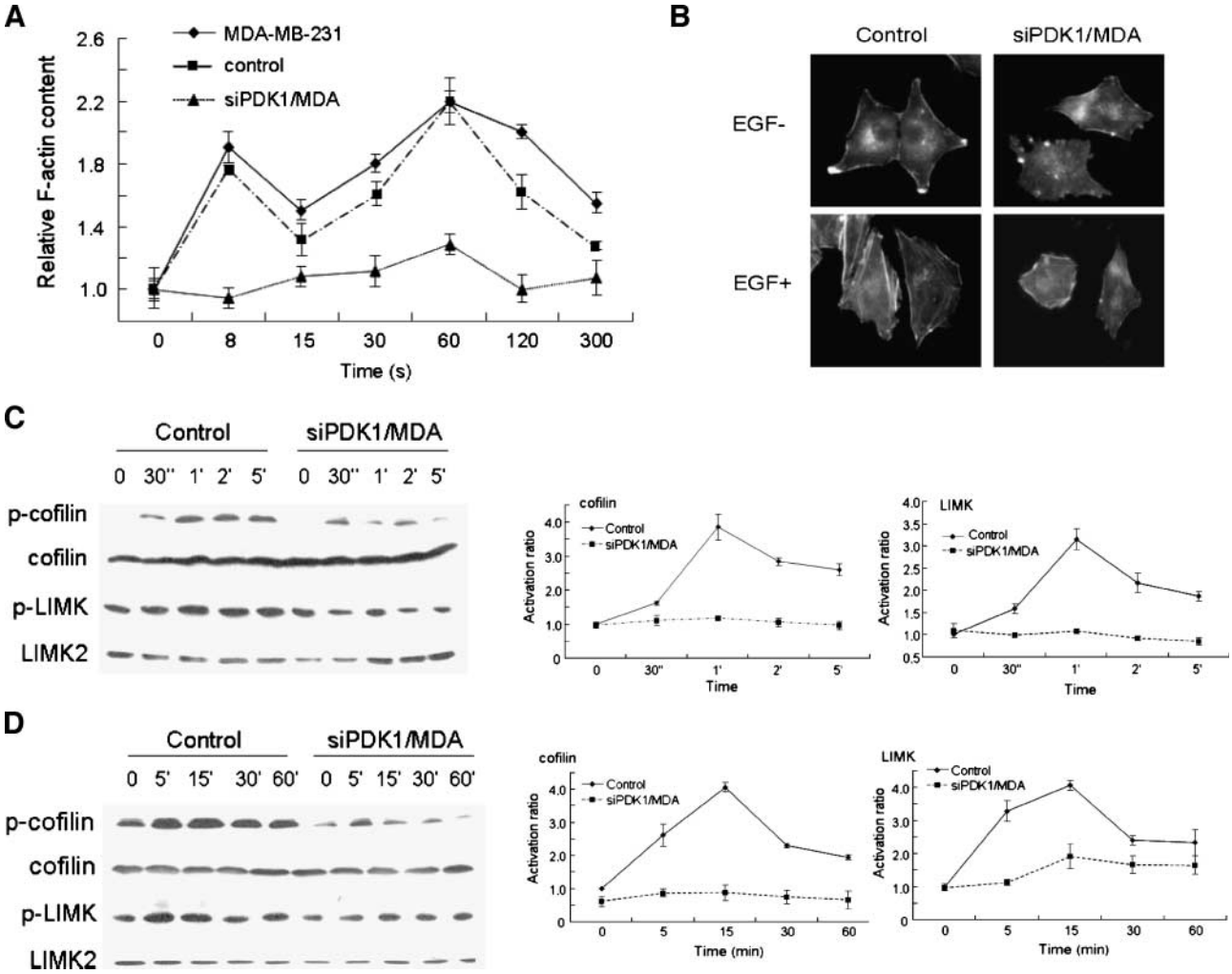

FIGURE 4. Down-regulation of PDK1 blocked actin polymerization in MDA-MB-231 cells. **A.** Time course of EGF-induced F-actin polymerization in MDA-MB-231, control, and siPDK1/MDA cells. EGF, 50 ng/mL. The data were collected from one representative of three repeated experiments. Each data point was an average of triplicate assays. **B.** Cytoskeleton rearrangement in control and PDK1-knockdown cells by immunofluorescence assay. Figures showed representative images from three repeated experiments. Scale bar, 10 μm. **C.** Western blotting analysis of the phosphorylation of cofilin and LIMK in total cell lysates from control and siPDK1/MDA cells on 10 ng/mL EGF stimulation for 0, 30 s, 1, 2 min, and 5 min. **D.** Western blotting analysis of the phosphorylation of cofilin and LIMK in total cell lysates from control and siPDK1/MDA cells on 10 ng/mL EGF stimulation for 0, 5,15, 30, and 60 min. In **C** and **D**, cofilin and LIMK2 were used as a loading control, respectively. The results of Western blotting were from a representative of at least three repeated experiments.

the absence of EGF, control and siPDK1/MDA cells showed a basal level of positive cells (12.4 ± 0.8% and 10.2 ± 1.7%, respectively; Fig. 5D, *right*). Treatment with EGF increased the number of positive cells up to 45.4 ± 2.5% in control cells, but only 22.2 ± 2.1% in siPDK1/MDA cells. Thus, PDK1 regulates EGF-induced PKCζ activation in human breast cancer cells.

Down-Regulation of PDK1 Impaired Lung Colonization of Human Breast Cancer Cells in Severe Combined Immunodeficient Mice

We first examined the role of PDK1 in cancer cell invasiveness by using *in vitro* Matrigel assay. Down-regulation of PDK1 inhibited the invasive capacity of siPDK1/MDA cells compared with control cells (Fig. 6A). We then injected fluorescence-labeled control and siPDK1/MDA cells into tail veins of severe combined immunodeficient (SCID) mice. After 28 days, fluorescent tumor cell colonies in mouse lungs were examined under inverted fluorescent microscope (Fig. 6B, *top*). In mice injected with control cells, 39 ± 16 colonies were detected in lung slices, but only 7 ± 5 colonies were found in mouse lungs from those injected with siPDK1/MDA cells (Fig. 6B, *bottom*). Slices of mouse lung were further analyzed by immunohistochemical staining. Tumor cell colonies were easily identified in the lungs of SCID mice injected with control cells, but not with siPDK1/MDA cells (Fig. 6C). To further evaluate lung colonization potential of tumor cells, mRNA level of a human gene, hypoxanthine phosphoribosyltransferase (HPRT), was determined by semiquantitative reverse transcription-PCR (RT-PCR) in frozen tissue specimens of mouse lungs. Control and siPDK1/MDA cells expressed a similar level of HPRT

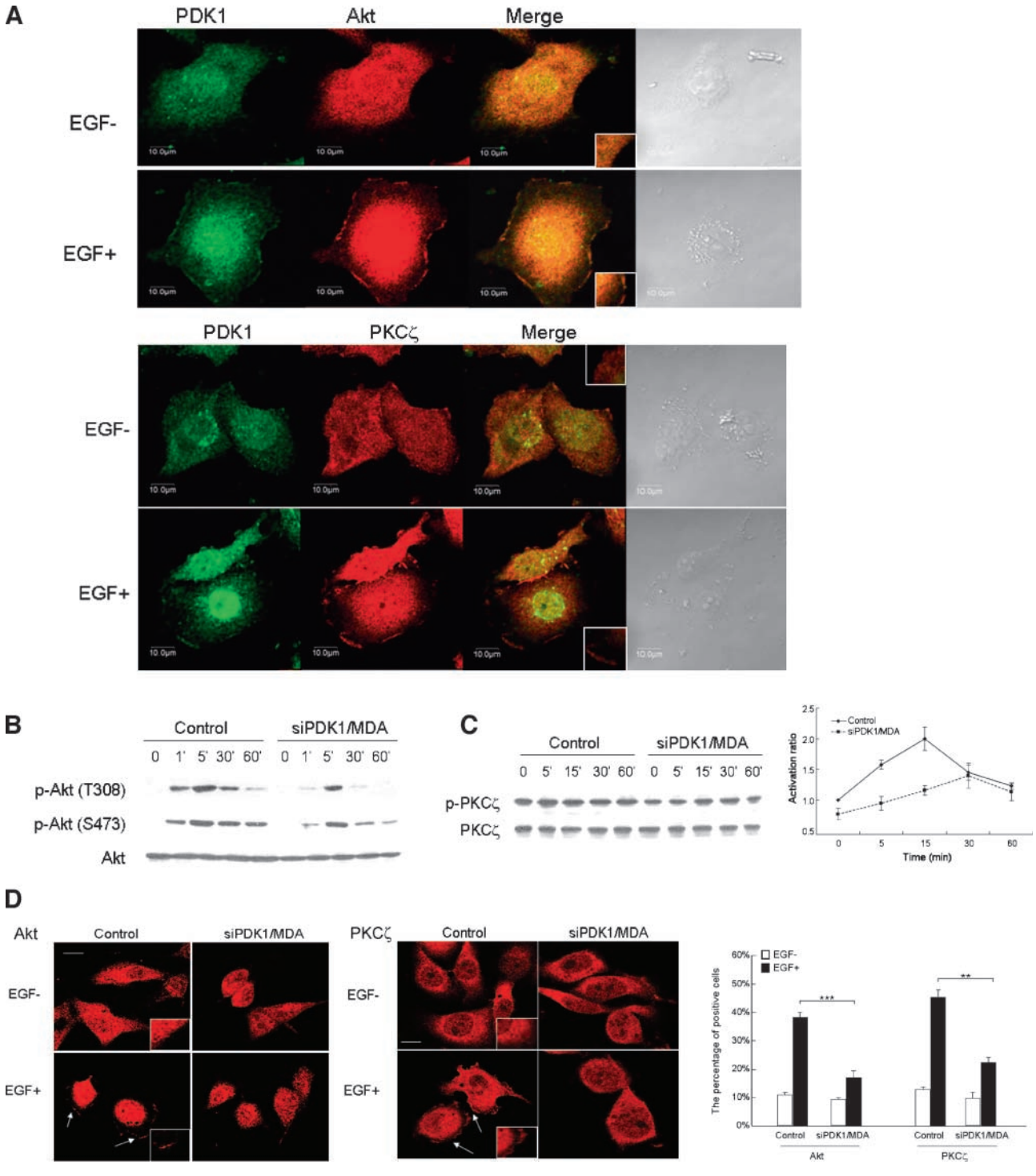

FIGURE 5. The inhibitory effects of PDK1 depletion on activation of Akt and PKCζ. **A.** Colocalization analysis of PDK1 with Akt or PKCζ with or without EGF stimulation by immunofluorescence assay, respectively. **B.** Western blotting analysis of Akt phosphorylation (T308 or S473) in total cell lysates from control and siPDK1/MDA cells on 10 ng/mL EGF stimulation for 0, 1, 5, 30, and 60 min. Total input of Akt in each sample was displayed at the bottom of the panel (the result in this set was from a representative of at least three repeated experiments). **C.** Western blotting analysis of PKCζ phosphorylation in total cell lysates from control and siPDK1/MDA cells on 10 ng/mL EGF stimulation for 0, 5, 15, 30, and 60 min. Total input of PKCζ in each sample was also shown. **D.** The EGF-induced cellular translocation of Akt or PKCζ in control and siPDK1/MDA cells, respectively. In **A** and **D**, images showed the representative cells that exhibited the translocation on EGF stimulation. Arrows, activated Akt or PKCζ on the membrane. Scale bar, 10 μm. Bar graph, the percentage of cells that exhibited Akt or PKCζ translocation of total examined cells under indicated assay conditions. Data were the mean of triplicate experiments (***, $P = 0.001$; **, $P = 0.0014$, two-way ANOVA).

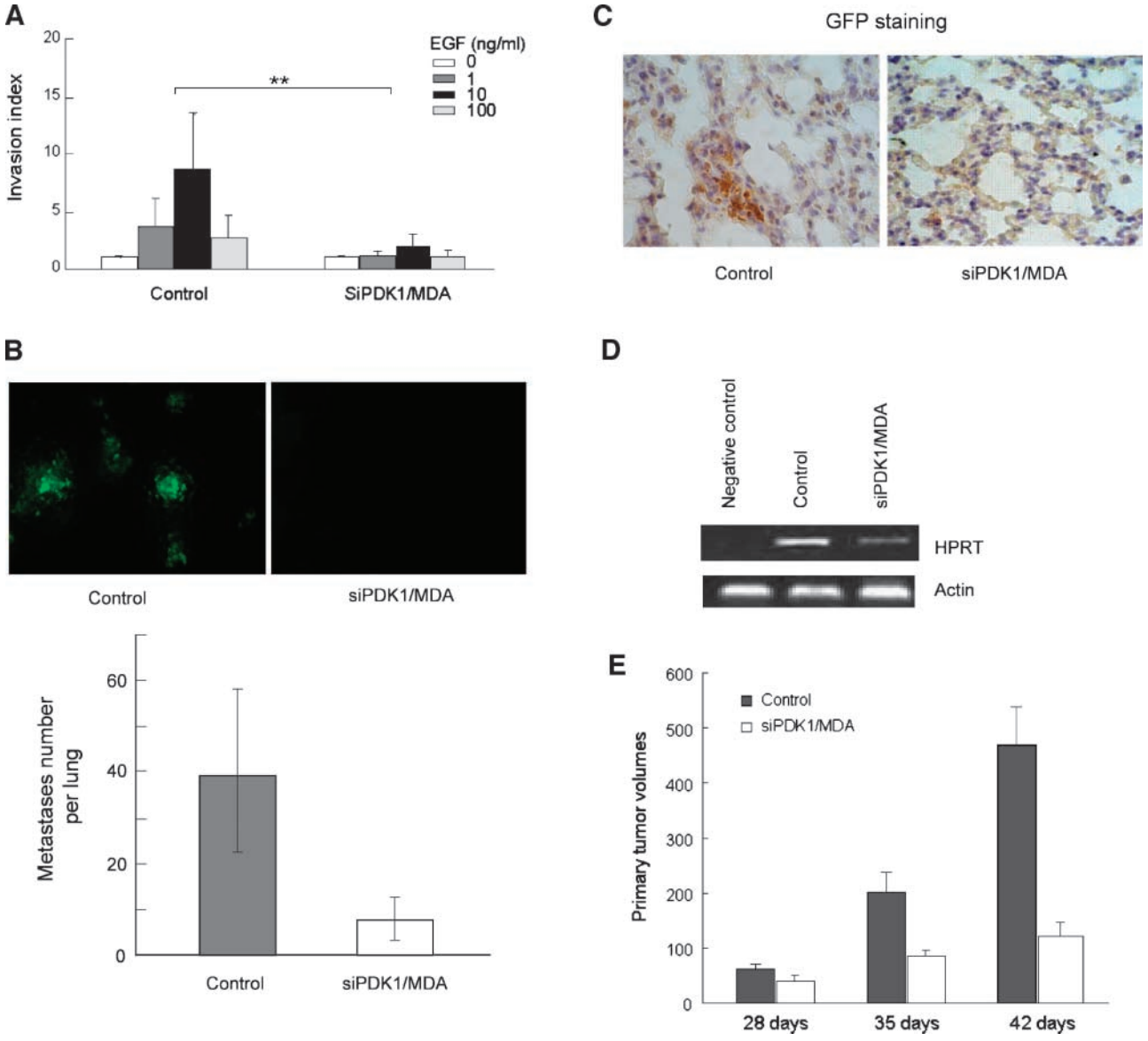

FIGURE 6. Disruption of PDK1 by siRNA inhibited the metastasis of MDA-MB-231 cells to the SCID mouse lungs. **A.** The siPDK1/MDA cells were defective in EGF-induced invasion assay (the result was a representative of three repeats. Each data point was a SD of three transwells. **, $P < 0.01$, two-way ANOVA). **B.** Fluorescent images of lung slices from SCID mice 28 d after tail vein injection with 1×10^6 control or siPDK1/MDA cells and the comparison of green fluorescent tumor colonies on lungs of each SCID mouse injected with control or siPDK1/MDA cells (figures showed the representative data from two repeated experiments. Each group contained six mice. Bars, SD). **C.** Human tumors on mouse lungs were visualized by anti-GFP antibody staining 28 d after tail vein injection. **D.** Expression of human HPRT gene was detected by RT-PCR of mouse lung lysates after tail vein injection of control and siPDK1/MDA cells. The lung lysate from SCID mice without injection was used as the negative control. The murine actin was the control of PCR. **E.** The siPDK1/MDA cells showed the impaired tumorigenesis. Tumor cells were s.c. injected into the abdominal region on both sides of each mouse. Primary tumor volumes were calculated according to the mathematical formula for ellipsoid shapes.

mRNA (data not shown). When equal amount of total RNA from tissue homogenates was used as templates, RT-PCR results revealed a marked decrease in HPRT level in mice injected with siPDK1/MDA cells, indicating that down-regulation of PDK1 inhibited lung colonization of tumor cells (Fig. 6D). In addition, we did s.c. injection of breast cancer cells in SCID mice. siPDK1/MDA cells showed a decrease in tumorigenesis (Fig. 6E). Taken together, the results indicate that PDK1 played an important role in breast cancer cell tumorigenesis and lung colonization in experimental metastasis assays.

Discussion

Our results revealed that PDK1 plays an important role in sustaining the malignant phenotype of human breast cancer cells. Disruption of PDK1 expression by siRNA severely reduced cell response to EGF-induced chemotaxis, their capacity to grow tumors in SCID mice and to form lung colonies when injected intravenously.

A large body of reports have documented that PDK1/Akt regulates cell growth, proliferation, and apoptosis (14, 15). Our studies provide further evidence showing reduced tumor

cell migration in response to EGF after depletion of PDK1 despite normal expression of cell surface EGF receptor. PDK1/Akt/PKCζ axis seems to be key regulator of cancer cell migration in response to EGF. Confocal microscopy showed that EGF induced cotranslocation of PDK1 with Akt and PKCζ to cell membrane, which is required for increased cell migration. Disruption of PDK1 impaired the activation of Akt and PKCζ by EGF, suggesting that PDK1 was an upstream regulator of Akt and PKCζ. Our studies showed that depletion of Akt2 by siRNA blocked EGF-induced PKCζ activation and Akt2 was coimmunoprecipitated with PKCζ (31), suggesting that Akt2 may directly regulate PKCζ activation. Our results also suggest that contribution from PDK1 direct-phosphorylation was minor in comparison with Akt2-mediated activation of PKCζ. Inhibiting PDK1, Akt2, and PKCζ causes similar cellular defects both in actin polymerization and in cell adhesion to fibronectin surface. Recently, PDK1 was reported to be required for phosphorylation of myosin light chain and cell motility by direct binding to ROCK1 in the plasma membrane (49). This mechanism may also be involved in the regulation of PDK1 on EGF-induced phosphorylation of LIMK/cofilin and actin polymerization. We observed that EGF induced coimmunoprecipitation of PKCζ with LIMK1 because their interaction was previously reported (Supplementary Fig. S4; ref. 50). However, integrin β1 was not coimmunoprecipitated with PKCζ, suggesting that PKCζ may indirectly regulate integrin β1. We therefore propose a working model (Supplementary Fig. S5): EGF induces activation and membrane translocation of PDK1/Akt2/PKCζ, which subsequently regulate actin polymerization in tumor cells through LIMK/cofilin, and cell adhesion through integrin β1.

PDK1 phosphorylates Akt at Thr308, which undergoes a conformational change to allow for further activation by phosphorylation at Ser473 (51). Recent studies have identified a rictor-mTOR complex responsible for the phosphorylation of Ser473 (48, 52). Phosphorylation of Ser473 in the hydrophobic motif of Akt may facilitate the phosphorylation of Thr308 in the activation loop. However, it is not clear whether the phosphorylation of Thr308 and Ser473 each is an independent event. We found that down-regulation of PDK1 inhibited EGF-induced Akt phosphorylation at both Thr308 and Ser473, suggesting that PDK1 plays a role in rictor-mTOR–mediated phosphorylation of Ser473. The precise role of rictor-mTOR complex in cancer cell migration is unknown, thus requires further clarification.

To examine the biological significance of PDK1 in the malignant behavior of breast cancer cells, we investigated the tumorigenesis and extravasation capacity of tumor cells depleted of PDK1. Tail vein injection revealed that lung colonization of human breast cancer cells in SCID mice was significantly reduced in siPDK1/MDA cells, suggesting that inhibition of PDK1 activities may effectively blocked cancer cell extravasation, which was attributable to their reduced migratory and adhesive responses to EGF. In addition, siPDK1/MDA cells showed a greatly reduced capacity to form s.c. tumors, indicating a pivotal role of PDK1 in maintaining the degree of malignancy of tumor cells. Because PDK1 is overexpressed by many tumor cell types, breast cancer cells in particular, it constitutes a plausible molecular target for the development of novel anticancer therapy.

Materials and Methods

Cell Lines, Animals, and Reagents

Human breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection; T47D and MCF-7 were from Invitrogen; SCID mice were from Wei Tong Li Hua Experimental Animal Co. Ltd; RPMI 1640 was from Invitrogen; FCS was from Biochrom; Chemotaxis chambers and membranes were from Neuroprobe; 24-well Costar Transwell chambers were from Corning Incorporated; EGF from Peprotech; and Matrigel was from BD Biosciences. Antibodies against Akt, phosph-cofilin, and PKCζ were from Santa Cruz Biotechnology, Inc. Antibodies to PDK1 and integrin β1 were from BD Transduction. Antibodies to LIMK2, cofilin, phosph-Akt, actin, and phosph-PKCζ were purchased from Cell Signaling Technology, Inc. Antibodies to phosph-LIMK1/2 and phosph-integrin β1 were bought from Upstate. Alexa 568-phalloidin, Alexa 546-labeled secondary antibody, and FITC-labeled secondary antibody were from Molecular Probes, Inc.

Cell Culture

Human breast cancer cells MDA-MB-231, T47D, MCF-10, and MCF-7 were cultured in the complete medium containing RPMI 1640 supplemented with penicillin, streptomycin and 10% FCS.

Plasmid Construction and Transfection

GST-Myc-PDK1 plasmid was a present from Dr. Gail Fraser and Dr. Maria Deak in the University of Dundee (Dundee, United Kingdom). Two PDK1 siRNA plasmids were constructed using pRNAT-U6.1/Hygro as a vector obtained from Genescript Corp. The targets of siRNA were *GAAGGATACGGACCTCTTAAA* and *CACdGCCTAACAGGACGTATTA*. Another PDK1 siRNA plasmid was a gift from Dr. Rahil Rahim (Temple University, PA). A scrambled sequence inserted into pRNAT-U6.1/Hygro was directly purchased from Genscript Corp.

siRNA Transfection

MDA-MB-231 cells were planted in 35-mm dish 1 d before transfection. Transfection was done with Lipofectamine2000 (Invitrogen) based on manufacture's protocol. To establish stable cell lines, transfected cells were enriched by using 0.8 mg/mL Hygromycin B from BD Biosciences. Then GFP-expressing single cells were sorted by fluorescence-activated cell sorting, followed by Western blotting analysis. For transient transfection, cells were harvested 24 h after transfection and used for experiments.

RT-PCR

Total RNA from cells or tissue samples was extracted using Trizol (Invitrogen) Specific primers for glyceraldehyde-3-phosphate dehydrogenase (F: *5′-ACCACAGTCCATGCCATCAC;* R: *5′-TCCACCACCCTGTTGCTGTA*), PDK1 (F: *5′-CATCCAGTCCAGCGTGGTGTT;* R*: 5′-ACAGCCTAACCGCTTTGTGG*), human HPRT (F: *5′-TTCCTTGGTCAGGCAGTATAATCC;* R: *5′-AGTCTGGCTTATATCCAACACTTCG*), and murine actin (F: *5′-GCATGTGCAAAGCCGGCTTC;* R:

5′-CTGCTCGAAGTCTAGAGCAAC) were synthesized from TaKaRa Biotechnology Co. Ltd. PDK1 was amplified 25 cycles with one-step PCR kit (TaKaRa), and the melting temperature was set at 51°C. Human HPRT was amplified 29 cycles, and the melting temperature was set at 58°C.

Cell Proliferation Assay

Equal amount of MDA-MB-231 and siPDK1/MDA cells were planted in 12-well plates, respectively. In every 24 h, cells were harvested and counted. At least two wells were used for each data point.

Western Blotting Assay

Western blotting assay was done as described previously (7). Cell lysates were loaded on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with primary antibodies at various dilution, such as anti-PDK1 at 1:1,500, anti-actin at 1:1,000, anti-Akt at 1:1,000, anti–phosph-Akt at 1:1,000, anti–phospho-PKCζ at 1:1,000, anti-PKCζ at 1:3,000, anti–phosph-LIMK1/2 at 1:1,500, anti-LIMK2 at 1:1,000, anti–phospho-integrinβ1 at 1:1,500, anti-integrinβ1 at 1:2,000, anti-cofilin at 1:1,500, and anti–phospho-cofilin at 1:3,000.

Chemotaxis Assay

Chemotaxis assay was done as described previously (7). The chemotaxis index was calculated by the ratio of cell numbers responding to a chemoattractant gradient versus that in a medium control. In checkerboard assay, cells were suspended in medium containing various concentrations of EGF before addition to the upper chambers.

Matrigel Invasion Assay

The cell *in vitro* invasiveness was measured by using 24-well Costar Transwell chambers with a filter membrane coated with 30 μL 3.5 mg/mL Matrigel in cell culture medium. The gel was solidified at 37°C for 1 h. The lower compartment contained 300 μL medium with or without EGF at various concentrations. Cells were placed in the upper compartment and incubated for 42 h at 37°C in 5% CO_2. After incubation, the membranes were fixed with 5% glutaraldehyde in PBS for 10 min at room temperature, and stained with 0.5% Toluidine Blue in 2% Na_2CO_3 for 15 min for quantification.

Adhesion Assay

The assay was carried out as described (7). Cell suspension in the presence or absence of 10 ng/mL EGF was loaded onto a 35-mm dish containing a glass coverslip pretreated with fibronectin at 37°C for 2 h. In 5, 15, or 30 min, suspension was removed, and adhesive cells were gently washed, fixed, and counted by a coulter counter under an inverted microscope (Olympus, Inc.).

Wound Healing/Scratch Assay

Cells were cultured for 2 d in 35-mm dishes to form a fluent monolayer. Then, the complete medium was replaced by a RPMI supplied with 0.1% of bovine serum albumin. A linear scratch was formed by using a pipette tip. Then, the width of the "wound" was recorded by the number of grids in the left ocular of a microscope at various time points. Every grid equals to 0.1 mm in length.

F-Actin Content Assay

F-actin content was analyzed as described previously (7). In brief, after 18 h of culture, cells were starved for 3 h followed by stimulation 50 ng/mL EGF at 37°C for different time courses. Cells were then fixed and permeablized with 0.1% Triton X-100 in F-actin buffer [10 mmol/L HEPES, 20 mmol/L KH_2PO_4, 5 mmol/L EGTA, 2 mmol/L $MgCl_2$, Dulbecco's PBS (pH 6.8)] followed by incubation with Alexa 568-phalloidin. After washing, the labeled phalloidin bound to the F-actin in cells were extracted by methanol for 1 h. Fluorescence in each sample was analyzed at Ex/Em 578/603 nm and normalized against total protein content. The relative F-actin content was calculated by the following equation:

$$\text{F-actin } \Delta \text{ t / F-actin0} = (\text{fluorescence } \Delta \text{ t / mg/ml}) / (\text{fluorescence 0 / mg/ml}).$$

Immunofluorescence Microscopy

Cells were starved for 3 h after 1-d culture. After stimulation with 10 ng/mL EGF at 37°C, cells were fixed and permeablized with 0.1% Triton X-100 at 4°C. To stain actin, cells were incubated with Alexa 568-phalloidin. Cells were then kept in PBS and visualized with Olympus inverted fluorescent microscope. For staining Akt or PKCζ, cells were incubated with anti-Akt (1:100) or anti-PKCζ (1:100) antibody overnight at 4°C. Then cells were probed by the Alexa 546–secondary antibody (1:800) before taken images by Olympus FV500 Spectrum inverted fluorescent confocal microscope (Olympus, Inc.). For double staining, FITC-labeled secondary antibody (1:500) and Alexa 546–labeled secondary antibody (1:800) were used.

Experimental Metastasis in SCID Mice

Lung colonization of cancer cells was analyzed *in vivo* by a tail-vein injection model as described previously (6). Control and siPDK1/MDA cells (10^6) were injected into the tail vein of SCID mice, respectively. After 28 d, lungs were collected for visualization by inverted fluorescent microscopy and for RT-PCR. Meanwhile, lungs were also isolated for immunohistochemical staining by using anti-GFP antibodies. The GFP-positive regions in the lungs were counted as the number of metastases. A group of at least six mice were used for each data point.

Tumorigenesis was measured by injecting 2×10^7 cells/mL s.c. into the abdominal region on both sides of each mouse (5 mice × 2 sides → $n = 10$). Primary tumor volumes were calculated according to the mathematical formula for ellipsoid shapes.

Statistical Analysis

Statistical analysis was carried out to determine the significance of chemotactic response, Matrigel invasion assay, and adhesion assay by using Prism 3.0, two-way ANOVA analysis. Western blotting results were quantitated and analyzed by ImageJ.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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