Essential role of PDK1 in regulating endothelial cell migration

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The serine/threonine protein kinase phosphoinositide-dependent kinase 1 (PDK1) plays a central role in cellular signaling by phosphorylating members of the AGC family of kinases, including PKB/Akt. We now present evidence showing that PDK1 is essential for the motility of vascular endothelial cells (ECs) and that it is involved in the regulation of their chemotaxis. ECs differentiated from mouse embryonic stem cells lacking PDK1 completely lost their ability to migrate in vitro in response to vascular endothelial growth factor-A (VEGF-A). In addition, PDK1$^{-/-}$ embryoid bodies exhibit evident developmental and vascular defects that can be attributed to a reduced cell migration. Moreover, the overexpression of PDK1 increased the EC migration induced by VEGF-A. We propose a model of spatial distribution of PDK1 and Akt in which the synthesis of phosphatidylinositol 3,4,5 triphosphate at plasma membrane by activation of phosphoinositide 3-kinase recruits both proteins at the leading edge of the polarized ECs and promotes cell chemotaxis. These findings establish a mechanism for the spatial localization of PDK1 and its substrate Akt to regulate directional migration.

Introduction

The phosphoinositide 3-kinase (PI3K) signaling pathway mediates a multitude of cellular responses after extracellular stimulation by peptide growth factors and hormones (Cantley, 2002). Deregulation of this pathway is associated with human diseases such as cancer and diabetes (Vivanco and Sawyers, 2002). PI3K and its product, phosphatidylinositol 3,4,5-trisphosphate (PtdIns[3,4,5]P_3), are key signaling molecules in cell motility, particularly in chemotaxis, which is a process involved in a wide range of cellular responses, including morphogenesis, wound healing, immune response, angiogenesis, and metastasis of tumor cells (Stephens et al., 2002; Dormann and Weijer, 2003; Van Haastert and Devreotes, 2004). Upon chemoattractant stimulation, a PtdIns(3,4,5)P_3 gradient is created and maintained at the leading edge of cells with amoeboid motility, such as leukocytes and *Dictyostelium discoideum* (Merlot and Firtel, 2003). This process involves both localized accumulation and activation of PI3Ks, which generate PtdIns(3,4,5)P_3/PtdIns(3,4)P_2, and the phosphatase PTEN, which removes them (Servant et al., 2000; Funamoto et al., 2002). PtdIns(3,4,5)P_3 serves as a docking site for a subclass of PH domain–containing proteins that are recruited at the leading edge. However, it is not clear which PI3K downstream effectors lead to activation of the actin polymerization machinery required for cell migration. Akt is one of the candidate molecules through which PI3K regulates chemotaxis, but the role of Akt in the control of cell polarity and chemotaxis has been established only in *D. discoideum*, where Akt phosphorylates PAKa, regulating its subcellular localization and myosin II assembly (Chung et al., 2001). Unfortunately, PAK1, the mammalian homologue of PAKa, lacks the Akt phosphorylation site, suggesting the existence of a different signaling pathway.

One important downstream effector of PI3K is the 3-phosphoinositide–dependent protein kinase-1 (PDK1; Alessi et al., 1997). PDK1 phosphorylates and activates a group of related protein kinases belonging to the AGC family (Vanhaesebroeck and Alessi, 2000). These include isoforms of Akt (Brazil and Hemmings, 2001), p70 ribosomal S6 kinase (S6K; Volarevic

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Abbreviations used in this paper: E, embryonic day; EB, embryoid body; EC, endothelial cell; ES, embryonic stem; MEF, murine embryonic fibroblast; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5 triphosphate; SGK, serum- and glucocorticoid-induced protein kinase; shRNA, short hairpin RNA.

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and Thomas, 2001), p90 ribosomal S6 kinase (RSK; Jensen et al., 1999), PKC (Newton, 2003), and serum- and glucocorticoid-induced protein kinase (SGK; Kobayashi and Cohen, 1999). Evidence has indicated that PDK1 is constitutively active, and that regulation involves the conversion of substrates to forms that can be phosphorylated by PDK1 in agonist-treated cells (Toker and Newton, 2000; Mora et al., 2004). For example, the phosphorylation of Akt by PDK1 is regulated by the conformational change induced by engagement of the PH domain to the membrane by PtdIns(3,4,5)P_3/PtdIns(3,4)P_2, which relieves autoinhibition of the active site, allowing PDK1 to access T308 on the activation loop (Stokoe et al., 1997; Filippa et al., 2000). PDK1 also contains a PH domain that binds PtdIns(3,4,5)P_3 with high affinity and has a crucial role in activation of Akt (Anderson et al., 1998; McManus et al., 2004). Other PDK1 substrates, such as S6K and SGK, which lack a PH domain and are phosphorylated by PDK1 at the same ratio in the presence or absence of PtdIns(3,4,5)P_3, interact with a pocket in the kinase domain of PDK1, called the PIF-binding pocket (Biondi et al., 2001). The prior phosphorylation of S6K and SGK at their hydrophobic motif promotes their interaction with the PIF-binding pocket of PDK1 and their T-loop phosphorylation (Collins et al., 2003). The key role that PDK1 plays in activating certain AGC kinase members was substantiated by the finding that mouse embryonic stem (ES) cells lacking PDK1 fail to activate Akt, S6K, and RSK in response to stimuli that trigger the activation of these enzymes in wild-type ES cells (Williams et al., 2000). Unexpectedly, although Akt and RSK have been reported to play important roles in regulating survival and proliferation, ES cells lacking PDK1 were viable (Williams et al., 2000).

Nevertheless, PDK1 is required for normal embryo development, as mice embryos lacking PDK1 die at day E9.5 displaying multiple abnormalities, including lack of somites, forebrain, and neural crest–derived tissues (Lawlor et al., 2002). PDK1 hypomorphic mice, in which a general and extensive reduction of PDK1 expression was obtained by intron insertion of a neomycin resistance gene, were viable and fertile, but were 40–50% smaller than control animals, and their organ and cell sizes were also proportionately reduced. Interestingly, activation of Akt and S6K1 by insulin was normal in the PDK1 hypomorphic mice, showing that regulation of cell size by PDK1 is independent of insulin's ability to activate Akt and S6K (Lawlor et al., 2002). Moreover, PDK1 knock-in mouse embryos, in which the PH domain was disrupted, die at embryonic day (E) 11.5 (McManus et al., 2004). In these knock-in cells, Akt was not activated by IGF1, whereas RSK was normally activated, indicating that PtdIns(3,4,5)P_3 binding to PDK1 is required for Akt, but not RSK activation.

However, the cause of death in PDK1$^{-/-}$ mice appears to be a lack of functional circulation. Because the inability to form a functional circulatory system might result from the inability of ECs to migrate, our initial approach in understanding the role of PDK1 in cell migration was to study the vessel formation in embryoid bodies (EBs) derived from ES cells lacking PDK1 and the motility of ECs differentiated from them. Moreover, to gain further insights into the role of PDK1, we modulated PDK1 activity in human ECs using retroviral vectors expressing PDK1 mutants. Our results suggest that both PDK1 and Akt are involved in EC motility.

Results

Impairment of vessel formation in PDK1$^{-/-}$ EBs

The starting point for our investigation was an examination of the role of PDK1 in blood vessel formation. We used a well-established model of early vascular plexus formation—the ES differentiation into EBs (Risau et al., 1988; Vittet et al., 1996).

PDK1$^{-/-}$ mice die at E9.5 displaying multiple abnormalities, including the lack of a circulatory system (Lawlor et al., 2002). ES cells, in which both copies of the PDK1 gene have been disrupted, are viable and proliferate at the same rate as PDK1$^{+/+}$ (Williams et al., 2000). We generated EBs from PDK1$^{+/+}$ and PDK1$^{-/-}$ ES cells. ES cells were cultured for 5 d in suspension to form EBs, and then plated on tissue culture dishes. Gross examination of EBs revealed differences in size and morphology starting from day 3 of differentiation on a gelatin-coated dish (Fig. 1 A). EBs from ES PDK1$^{-/-}$ cells exhibited reduced cell size and spreading, which were probably caused by defective cell motility and adhesion, although we could not exclude proliferation defects.

In PDK1$^{+/+}$ EBs, CD31-positive ECs aggregated in dense clusters that started to form a vascular-like network after 3 d (Fig. 1 B). On day 7, the PDK1$^{+/+}$ ECs organized into tubular structures that became more evident, numerous, and branched after 10 d (Fig. 1 B).

When PDK1$^{-/-}$ EBs were analyzed 3 d after plating, only CD31-positive cell clusters were found without any signs of vessel formation (Fig. 1 B). After 7 d, an immature network of ECs began to form in some areas of the EBs (Fig. 1 B). However, this network was unable to differentiate in vessel-like structures, and after 10 d it regressed, appearing as clusters of ECs with few branches (Fig. 1 B).

PDK1 is required for EC migration

To exclude that the observed differences might be a consequence of a reduced number of ECs, we determined whether lack of PDK1 modified the number of CD31- and Flk1-positive cells in EBs. After 3 d in culture, anti-CD31 staining confirmed the presence of ECs in both PDK1$^{+/+}$ and PDK1$^{-/-}$ EBs (Fig. 1 C). The percentage of CD31-positive cells decreased after 7 and 10 d in culture, with no considerable differences between PDK1$^{+/+}$ and PDK1$^{-/-}$ EBs (Fig. 1 C). Similar results were obtained by staining with anti-Flk1, suggesting that PDK1 is not essential for EC differentiation from ES cells (Fig. 1 D).

Directional migration is a key event in angiogenic remodelling during vascular morphogenesis (Poole and Coffin, 1991). The inability of PDK1$^{-/-}$ EBs to form a vascular network, even though ES PDK1$^{-/-}$ cells can differentiate into ECs, suggests that this phenotype could result from defective cell migration. To verify this hypothesis, we performed migration assay with cells isolated from PDK1$^{+/+}$ and PDK1$^{-/-}$ EBs and stimulated with VEGF-A. CD31-positive ECs from PDK1$^{-/-}$ EBs weakly migrated in response to VEGF-A gradient compared with those

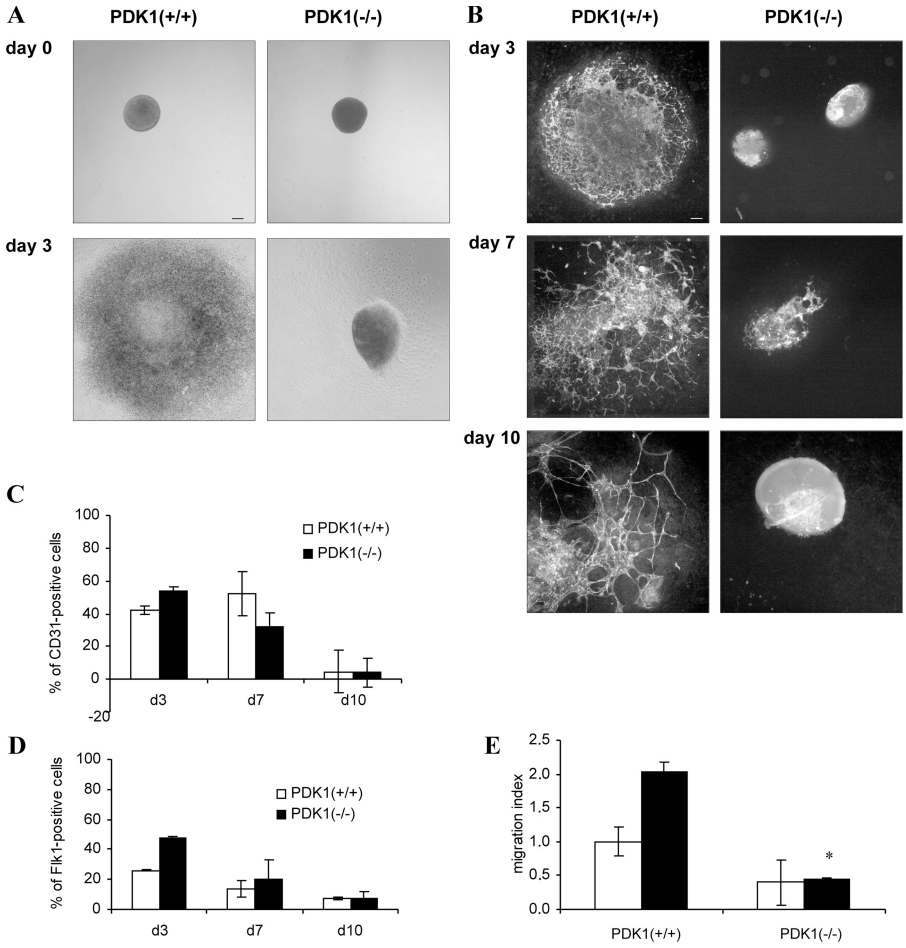

Figure 1. **Knock-out of PDK1 affects early vascular development in EBs and EB-derived EC migration.** (A) Representative phase-contrast images of EBs derived from PDK1$^{+/+}$ and PDK1$^{-/-}$ ES cells at day 0 and 3 of differentiation. (B) EBs PDK1$^{+/+}$ and PDK1$^{-/-}$ at 3, 7, and 10 d of differentiation were fixed and analyzed by indirect immunofluorescence with rat α-CD31 antibody; antigen–antibody complexes were detected with Cy2-conjugated donkey α-rat IgG. (C and D) FACS analysis of cells derived from EB PDK1$^{+/+}$ and PDK1$^{-/-}$ at different differentiation stage (3, 7, and 10 d) to evaluate percentage of CD31- and Flk1-positive cells, respectively; data were plotted as the mean ± the SD of the percentage of positive cells from three independent experiments. (E) ES cells PDK1$^{+/+}$ and PDK1$^{-/-}$ were differentiated into EBs for 3 d; they were then disaggregated, and cells were used in a chemotaxis assay, in the presence of a gradient of fibronectin (20 μg/ml) in combination (black bars) or not (white bars) with 30 ng/ml VEGF-A. Migration index is calculated assigning a value of 1 to the number of PDK1$^{+/+}$ CD31-positive cells that migrated toward fibronectin; data were plotted as the mean ± the SD of five independent experiments. Statistical significance (*, $P < 0.01$) is shown for VEGF-A–stimulated EC-PDK$^{-/-}$ compared with EC-PDK1$^{+/+}$. Images are representative of five independent experiments. Bars, 100 μm.

from PDK1$^{+/+}$ (Fig. 1 E). We also observed that, in the absence of VEGF-A, ECs derived from PDK1$^{+/+}$ EBs randomly migrate, whereas ECs from PDK1$^{-/-}$ EBs appear completely incapable of migration (Fig. 1 E).

The PH domain of PDK1 is essential for vascular development and EC migration

Taking advantage of previously made ES knock-in cells in which either the PH domain (PH_{KI}/PH_{KI}) or PIF pocket (155E/155E) was disrupted, we studied the vascular phenotype of EBs derived from these ES cells (Collins et al., 2003; McManus et al., 2004). EBs generated from PDK1$^{155E/155E}$ ES cells display normal vasculature development, similar to that of PDK1$^{+/+}$ EBs (Fig. 2 A). In contrast, PDK1$^{PHKI/PHKI}$ EBs failed to develop well-defined, cordlike structures, lacking the elaborate organization displayed by PDK1$^{+/+}$ (Fig. 2 A). However, compared with PDK1$^{-/-}$ EBs, the defective vascular structures of PDK1$^{PHKI/PHKI}$ EBs appeared less severe and did not regress after 10 d (compare Fig. 1 B and Fig. 2 A). The quantification of the total length of vessel-like structures at day 7 of differentiation pointed out the impaired vascular development of PDK1$^{-/-}$ and PDK1$^{PHKI/PHKI}$ EBs compared with PDK1$^{+/+}$ and PDK1$^{155E/155E}$ EBs (Fig. 2 B).

Migration of CD31-positive ECs derived from these EBs was analyzed. As shown in Fig. 2 C, ECs derived from PDK1$^{155E/155E}$ EBs migrated in response to VEGF-A as efficiently as wild-type cells, whereas ECs from PDK1$^{PHKI/PHKI}$ EBs displayed migration defects similar to those of PDK1$^{-/-}$ cells. These results indicate that PDK1 regulates vascular formation and EC migration in a PH domain–dependent way.

VEGF-induced EC migration is increased by PDK1 overexpression

To further assess the role of PDK1 in cell migration, we studied the chemotactic response of human ECs where PDK1 was overexpressed by retroviral transduction (EC-PDK1). Contrary to ECs transduced with vector alone (EC-vector), either EC-PDK1 or ECs expressing a membrane-tagged form of PDK1 (EC-PDK1caax) migrated more efficiently in a gradient of VEGF-A (Fig. 3 A). Few cells migrated in the absence of chemoattractant, and the overexpression of PDK1 did not increase the number of migrating cells; in some batches of EC-PDK1caax, we observed a relatively higher number of migrating cells in basal conditions compared with EC-vector, but not a statistically significant amount. To distinguish between chemotaxis toward VEGF-A gradient and random motility induced by VEGF-A, we added the same concentration of VEGF-A in both the upper and lower compartments of the Boyden chamber. The results clearly showed that PDK1 and PDK1caax were not able to significantly enhance random migration (Fig. 3 A).

This result was supported by time-lapse videomicroscopy experiments, in which ECs were homogenously stimulated with

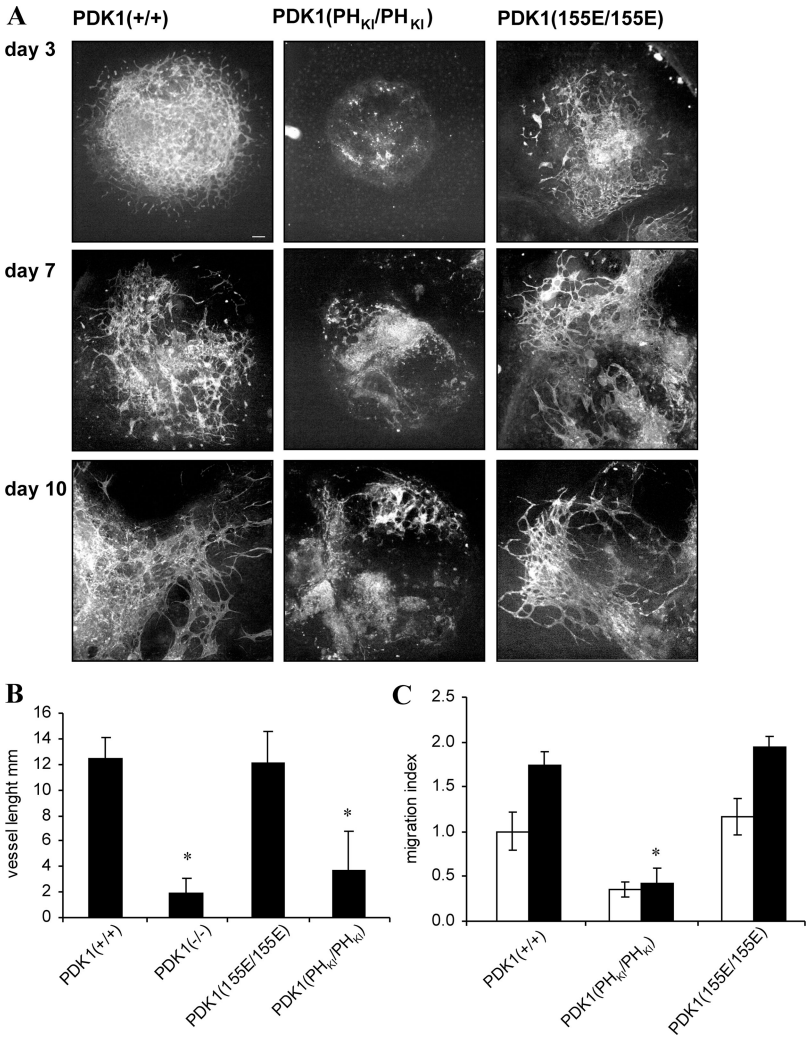

Figure 2. **The PH domain of PDK1 is essential for vessel formation in EBs and for EB-derived EC migration.** (A) EB PDK1$^{+/+}$, PDK1$^{PHKI/PHKI}$, and PDK1$^{155E/155E}$ at 3, 7, and 10 d of differentiation were fixed and analyzed by indirect immunofluorescence with rat α-CD31 antibody; antigen–antibody complexes were detected with Cy2-conjugated donkey α-rat IgG. Images are representative of five independent experiments. Bar, 100 μm. (B) 10 EB PDK1$^{+/+}$, PDK1$^{-/-}$, PDK1$^{PHKI/PHKI}$, and PDK1$^{155E/155E}$ at 7 d of differentiation from 5 different experiments were analyzed with imaging software to measure the total length of vessel-like structures. Data were plotted as the mean ± the SD. Statistical significance (*, $P < 0.01$) is shown for PDK$^{-/-}$ and PDK1$^{PHKI/PHKI}$ EBs compared with PDK1$^{+/+}$ EBs. (C) ES cells PDK1$^{+/+}$, PDK1$^{PHKI/PHKI}$ and PDK1$^{155E/155E}$ were differentiated into EBs for 3 d; they were then disaggregated, and cells were used in a chemotaxis assay, in the presence of a gradient of 20 μg/ml fibronectin in combination with 30 ng/ml VEGF-A (black bars) or not (white bars). Migration index is calculated assigning a value of 1 to the number of PDK1$^{+/+}$ CD31-positive cells that migrated toward fibronectin; data were plotted as the mean ± the SD of five independent experiments; statistical significance (*, $P < 0.01$) is shown for VEGF-A–stimulated EC-PDK1$^{PHKI/PHKI}$ compared with EC-PDK1$^{+/+}$.

VEGF-A. Fig. 3 B shows that neither PDK1 nor PDK1caax were able to increase random motility compared with EC-vector.

We considered whether the PDK1-enhanced migration might reflect increased activation of PDK1 by VEGF-A. The regulatory mechanisms controlling PDK1 activity are poorly understood. PDK1 has been reported to be constitutively active in resting cells and autophosphorylated at S241 (Casamayor et al., 1999). According to these previous data, we observed that S241 was basally phosphorylated in ECs, and that stimulation with VEGF-A did not modify the phosphorylation level (Fig. 3 C, top). The high level of S241 phosphorylation of EC-PDK1 compared with EC-vector depends on the high expression level of exogenous proteins, as demonstrated by the amount of myc-tagged protein (Fig. 3 C, bottom). We then examined the phosphorylation of Akt, which is the main substrate of PDK1. Upon stimulation with VEGF-A, T308 of Akt was very poorly phosphorylated in EC-vector (Fig. 3 D, first row, and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200607053/DC1). In contrast, we found that in EC-PDK1 and EC-PDK1caax the level of phosphorylation was already detectable without stimulation and increased upon VEGF-A stimulation (Fig. 3 D, first row, and Fig. S1 A). Interestingly, S473 of Akt, which is not a substrate of PDK1, showed similar levels of phosphorylation in unstimulated EC-vector, EC-PDK1, and EC-PDK1caax; in the presence of VEGF-A, the level of phosphorylation increased in all different cell types at the same rate (Fig. 3 D, second row, and Fig. S1 B). To evaluate the kinase activity of Akt we looked at the phosphorylation of two substrates: GSK3β and FKHR/AFX. As shown in Fig. 3 D, the phosphorylation of GSK3β and FKHR/AFX in PDK1 and PDK1caax ECs increased in either basal condition and upon VEGF-A stimulation, and it partially correlated with the phosphorylation of T308, but not with that of S473 of Akt. Collectively, these results indicate that Akt phosphorylation level at T308 correlates with the EC's ability to migrate, whereas the role of S473 seems to be marginal.

It was important to be sure that the increased migration did not result from effects on proliferation or survival. A high level of Akt activity was reported to confer EC survival in the absence of attachment (Marte and Downward, 1997). Moreover, in some cell lines, PDK1 regulates cell proliferation and survival, although ES PDK1$^{-/-}$ cells do not display any growth defects (Flynn et al., 2000; Cho et al., 2001). To determine the effects of PDK1 on these biological processes, we studied the growth and the survival rate of ECs expressing the wild-type and the membrane-tagged PDK1 mutant. PDK1 expression caused slight increase of VEGF-A–induced cell proliferation

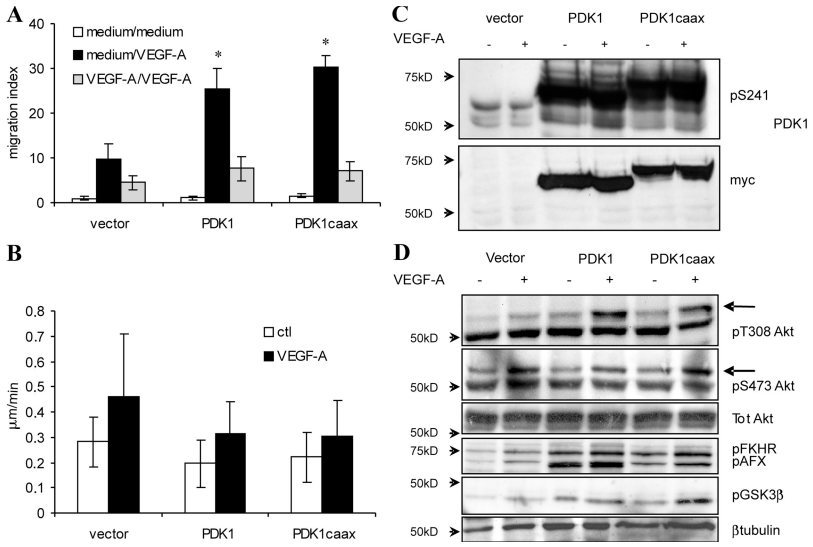

Figure 3. **Overexpression of PDK1 enhances VEGF-induced EC migration.** (A) ECs infected with retroviruses carrying wild-type PDK1 (PDK1), membrane-targeted PDK1 (PDK1caax), and not carrying anything (vector) were used in chemotaxis assays in the presence of 10 ng/ml VEGF-A, added in the lower compartment of the chamber (medium/VEGF-A) or in both the upper and lower ones (VEGF-A/VEGF-A). Migration index is calculated assigning a value of 1 to the number of vector cells migrated in absence of stimulus; data were plotted as the mean ± the SD of six independent experiments. Statistical significance (*, $P < 0.01$) is shown for VEGF-A–stimulated EC-PDK1 and PDK1caax compared with vector ECs. (B) ECs infected with the indicated retroviruses were plated on gelatin, with or without 100 ng/ml VEGF-A. Time-lapse videomicroscopy was performed, and images were recorded every 10 min for 6 h. Velocity values shown are means of 60 cells from three different experiments. Data were plotted as the mean ± the SD. (C and D) Cells were serum deprived for 2 h, and then stimulated or not stimulated with VEGF-A for 10 min; 50 µg of each lysate was immunoblotted with the indicated antibody. Western blots shown are representative of five experiments performed with similar results.

only after 96 h of stimulation (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200607053/DC1). As for proliferation, cell viability in the absence of serum and growth factors was not dramatically increased by PDK1 and PDK1caax expression (Fig. S2 B).

The promigratory effect of PDK1 requires its PH domain and catalytic activity and is PI3K dependent

PDK1 contains a C-terminal PH domain, a centrally located catalytic domain, and a 50-aa N-terminal region that binds a Ral-GEF (Tian et al., 2002). Additionally, recent studies indicate that the ability of PDK1 to phosphorylate S6K, SGK, and RSK is dependent on a docking site, the PIF pocket, which is located on the small lobe of the PDK1 kinase domain (Biondi et al., 2001). To determine which region was required for PDK1-induced migration, PDK1 mutants of these regions of the protein were produced and tested for their ability to modulate VEGF-A–induced EC migration (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200607053/DC1). ECs expressing PDK1 kinase-dead (PDK1-KD), PDK1 lacking the PH domain (PDK1-ΔPH) or the Ral-interacting domain (PDK1-Δ50), and PDK1 with a mutation on the PIF pocket (PDK1-L155E) were assayed for migration properties in a Boyden chamber in the absence or presence of VEGF-A as chemoattractant. The PDK1-KD mutant lost the ability to enhance EC migration (Fig. 4 A), and, in some experiments, it even exhibited a reduced motility compared with EC-vector (not depicted). As expected, the kinase-dead mutant was unable to enhance the phosphorylation of T308 of endogenous Akt (Fig. 4 B, fourth row).

ECs transduced with PDK1-Δ50 and PDK1-L155E were assayed in a similar manner and still showed an increased migration when stimulated by VEGF-A (Fig. 4 A). In contrast, PDK1-ΔPH did not function in enhancing EC migration (Fig. 4 A). When tested for the ability to phosphorylate Akt, it showed levels of T308 phosphorylation comparable with that of EC-vector (Fig. 4 B, fourth row).

As we have previously shown for PDK1 and PDK1caax, the expression of all PDK1 mutants did not change the level of S473 Akt phosphorylation in both unstimulated and VEGF-A–stimulated conditions (Fig. 4 B, fifth row).

The experiments described above suggested that, in addition to the kinase activity, the presence of the PH domain is also required for the promigratory effect of PDK1.

Because it has been demonstrated that the PH domain of PDK1 binds with high affinity to PtdIns(3,4,5)P_3 and this interaction enhances its ability to activate Akt (Alessi et al., 1997), we investigated the potential involvement of PI3K, the enzyme generating Ptdins(3,4,5)P_3, in the mechanism of PDK1-induced migration. Treatment with the inhibitor of PI3K, LY294002, reduced both basal and VEGF-A–stimulated EC migration (Fig. 5 A). The inhibition of PI3K activity completely abolished the effect of PDK1 and PDK1caax expression on EC migration, suggesting that production of PtdIns(3,4,5)P_3 is necessary for PDK1 activation and the subsequent promigratory effect.

As PtdIns(3,4,5)P_3 is required for both PDK1 and Akt activity, the inhibitory effect of LY294002 was not attributable exclusively to one of them. To clarify this point, we treated ECs expressing Akt-myr, which is a membrane-targeting mutant form of Akt that is PtdIns(3,4,5)P_3 independent, with LY294002, and we observed the phosphorylation level of T308 of Akt. In unstimulated conditions, T308 is highly phosphorylated, whereas a little increase was observed after VEGF-A stimulation (Fig. 5 B). Unexpectedly, the inhibition of PI3K activity with LY294002 did not decrease the phosphorylation level of T308 of Akt, but exclusively inhibited the phosphorylation increase stimulated by VEGF-A (Fig. 5 B). These results (Fig. 5, A and B) clearly show that during VEGF-A–stimulated migration, PI3K activation is required for both PDK1 and Akt activity.

To assess the role of Akt in the PDK1-induced migration, we performed experiments with ECs infected with lentiviral vectors carrying shRNA sequences designed to silence the expression of Akt. The best performing sequence among those

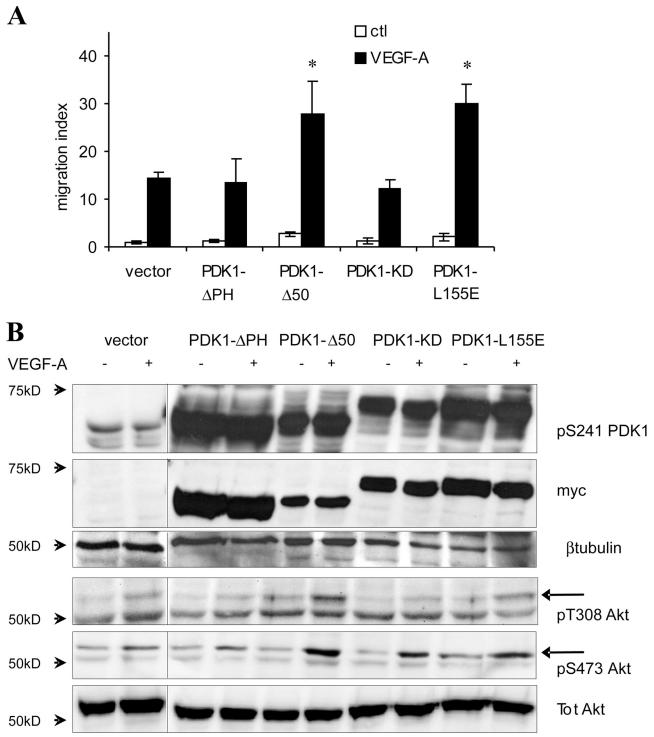

Figure 4. **The promigratory effect of PDK1 requires its PH domain and its catalytic activity.** (A) ECs were infected with retroviruses carrying PDK1 with a deleted PH domain (ΔPH), PDK1 with of the first 50 amino acids deleted (Δ50), PDK1 kinase-dead (KD), PDK1 mutated in the PIF pocket (L155E), and retroviruses not carrying anything (vector); these cells were used in chemotaxis assays in presence of a gradient of VEGF-A (10 ng/ml). Migration index is calculated assigning a value of 1 to the number of vector cells migrated in the absence of stimulus; data were plotted as the mean ± the SD of six independent experiments. Statistical significance (*, $P < 0.01$) is shown for VEGF-A–stimulated EC-PDK1-Δ50 and PDK1-L155E compared with vector ECs. (B) Cells were serum deprived for 2 h, and then stimulated or not stimulated with VEGF-A for 10 min; 50 μg of each lysate was immunoblotted with the indicated antibody. Western blots shown are representative of five experiments performed with similar results.

tested caused a decrease in Akt expression of ∼70% (Fig. 5 C). The reduction of Akt expression paralleled the reduced ability to migrate in response to VEGF-A in both EC-vector and EC-PDK1 (Fig. 5 C).

PDK1 localizes at the leading edge of migrating ECs

PDK1 has been shown to move to the plasma membrane in response to platelet-derived growth factor and insulin (Anderson et al., 1998; Egawa et al., 2002). However, other studies have not supported these reports (Currie et al., 1999). To determine the subcellular localization of PDK1 in ECs responding to VEGF-A, we stained EC with anti-myc and -PDK1 Abs. In unstimulated cells, PDK1 was localized in the cytoplasm and perinuclear region (Fig. 6 A). Once the cells were stimulated with VEGF-A, a PDK1 fraction translocated to the plasma membrane (Fig. 6 B). As expected, EC-PDK1caax showed a strongly marked plasma membrane localization in both stimulated and unstimulated cells (Fig. 6, D and C). Deletion of the PH domain of PDK1 resulted in a loss of its ability to localize to the plasma membrane upon VEGF-A stimulation (Fig. 6, F and G, for quantification).

The association of PDK1 with the plasma membrane in stimulated cells and its involvement in the process of directional cell migration prompted us to test whether PDK1 localized at the leading edge of migrating cells. After wounding a confluent monolayer of EC-PDK1, we detected PDK1 in the lamellipodia at the leading edge of migrating cells in the direction of the wound (Fig. 7 A). In contrast, PDK1-ΔPH failed to move to the leading edge, whereas PDK1caax was localized all around the plasma membrane (Fig. 7, B and C).

To test whether PDK1 phosphorylated Akt in the lamellipodia at the leading edge, we stained a wounded monolayer of PDK1-transfected murine embryonic fibroblasts (MEFs) with anti-PDK1 and anti-pT308Akt antibodies. The level of phosphorylation on T308 of Akt increased at the wound edge and colocalized with PDK1 on large regions of the plasma membrane (Fig. 7, D–F). In this experiment, MEFs were used instead of ECs to avoid the interference of GFP of infected ECs. The MEF behavior was similar to that of ECs in migration assays (unpublished data) and in translocation of PDK1 to plasma membrane in response to PDGF (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200607053/DC1).

Spatial distribution of PDK1 and Akt regulates chemotaxis

These findings suggested that both PDK1 and Akt move to the leading lamellipodia, where Akt is phosphorylated by PDK1. It has been demonstrated in different cell types that motility and chemotaxis rely on the activation of PI3K, and chemotactic factors elicit intracellular PtdIns(3,4,5)P_3 gradients in the plasma membrane. Therefore, the localization of PDK1 at the leading edge could be a consequence of an intracellular gradient of PtdIns(3,4,5)P_3, mediated by the binding of PtdIns(3,4,5)P_3 to the PH domain of PDK1. To further investigate the importance of PDK1 localization at the leading edge in cell motility, we compared behaviors of ECs expressing membrane-targeted mutants of PDK1 (PDK1caax), Akt (Akt-myr), and PI3KCA (p110caax) in chemotaxis experiments. The expression of the membrane-targeted catalytic subunit of PI3K did not increase EC migration, but rather, in some experiments, slightly inhibited chemotaxis (Fig. 8 A). Similar results were obtained by Funamoto et al. (2002), who observed chemotaxis defects after the expression of membrane-targeted PI3KA in *D. discoideum* PI3K1/2-null cells.

In contrast, when we transduced EC with membrane-targeted Akt, a strong increase in migrating cells both in the absence and presence of VEGF-A was observed (Fig. 8 A). This increase in motility is characterized by the presence of multiple pseudopodia (unpublished data).

These findings raise the question of whether restricted activation of PDK1 and Akt to the leading edge is critical for the chemotaxis process or not. We considered the possibility that PDK1 and Akt interact with each other in a manner that depends on PtdIns(3,4,5)P_3. In this model, the correct membrane localization of both occurs in response to lipid. To address this issue, we transduced ECs with PDK1caax and Akt-myr together, expecting that PDK1–Akt complex might form in higher concentrations, but that neither component could be localized properly.

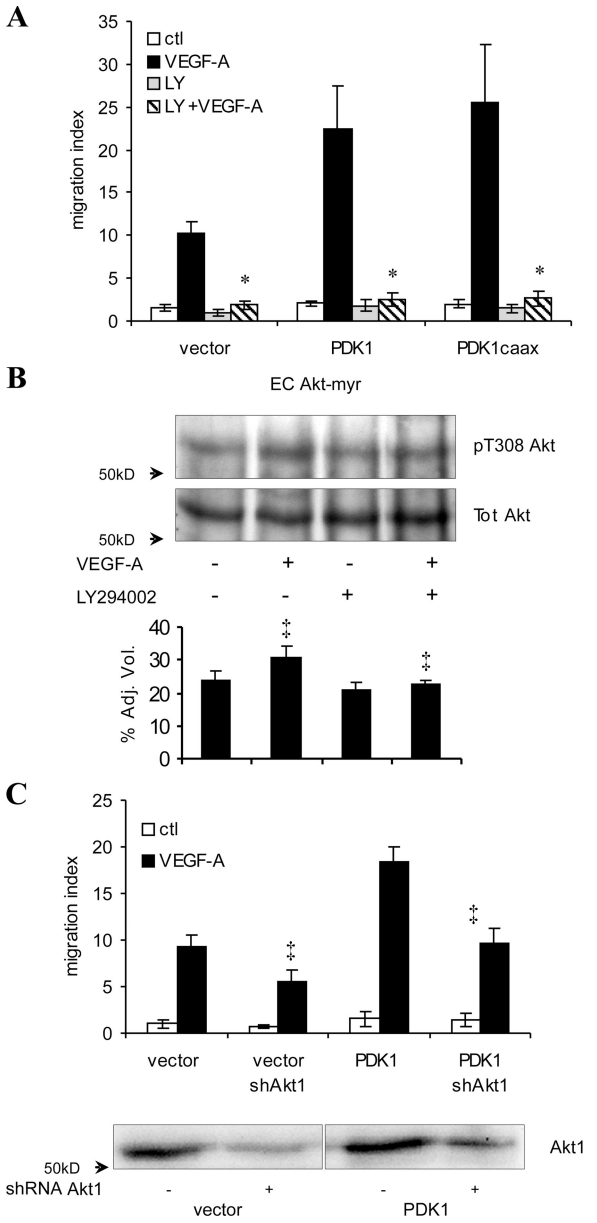

Figure 5. **The promigratory effect of PDK1 is PI3K-dependent and requires Akt.** (A) ECs infected with retroviruses carrying wild-type PDK1, membrane-targeted PDK1, and not carrying anything (vector) were used in chemotaxis assays in the presence of 10 ng/ml VEGF-A; 50 μM of the PI3K inhibitor LY294002 was added to the lower chamber, in combination or not with VEGF-A, and to the cells in the upper chamber. Migration index is calculated assigning a value of 1 to the number of vector cells migrated in the absence of stimulus; data were plotted as the mean ± the SD of six independent experiments; statistical significance (*, $P < 0.01$) is shown for LY294002-treated+ VEGF-A–stimulated cells compared with VEGF-A–stimulated cells. (B) EC-Akt-myr were serum deprived for 2 h, pretreated or not with 50 μm LY294002 for 45 min, and then stimulated or not with 30 ng/ml VEGF-A for 10 min; cells were lysed, and overexpressed Akt-myr was immunoprecipitated with α-HA antibody; immunocomplexes were separated by SDS-PAGE and immunoblotted with indicated antibody. Three independent experiments were acquired with the molecular imager ChemiDoc XRS, and densitometric analysis was performed with Quantity One software. Data were plotted as the mean ± the SD of the percentage of adjusted volume. Statistical significance is shown for VEGF-A–stimulated cells compared with control (‡, $P < 0.05$) and for LY294002-treated+ VEGF-A–stimulated cells compared with VEGF-A-stimulated (‡, $P < 0.05$). Western blot shown is representative of three experiments performed with similar results. (C) Vector ECs and EC-PDK1 were infected with lentiviruses carrying shRNA for Akt1 or with vector; after 72 h, the cells were used in chemotaxis assays in the presence of a gradient of VEGF-A (10 ng/ml). Migration index is calculated assigning a value of 1 to the number of vector cells migrated in absence of stimulus; data were plotted as mean ± SD of three independent experiments. Statistical significance (‡, $P < 0.05$) is shown for VEGF-A–stimulated shAkt1 ECs compared with respective controls. 50 μg of lysate of each cell type was immunoblotted with α-Akt1 mAb. Western blot shown is representative of three experiments performed with similar results.

These cells showed a reduced VEGF-A–induced migration compared with EC-PDK1caax or EC-Akt-myr alone (Fig. 8 A). However, ECs expressing Akt-myr transduced with wild-type PDK1 did not change their ability to migrate in comparison with nontransduced cells, demonstrating that the inhibitory effect was not caused by the double infection (Fig. 8 A).

Finally, we analyzed by immunofluorescence the localization of PDK1 and pT308Akt in a wounded monolayer of double-infected EC. We detected both PDK1 and pT308Akt on the lamellipodia at the leading edge of migrating EC-PDK1 (Fig. 8, B and C, respectively). In ECs carrying both PDK1 and Akt-myr, a gradient of phosphorylated Akt along the cells was still present (Fig. 8 E), in accordance with the localization of PDK1 at the leading edge (Fig. 8 D). On the other hand, when PDK1 was forced to the whole plasma membrane by infecting the cells with PDK1caax, phosphorylated Akt was mainly localized at the front of migrating cells (Fig. 8 G). When both PDK1 and Akt were constitutively linked to the membrane, the staining of pT308Akt was evident along the entire surface of the cells (Fig. 8 I).

These data, together with chemotaxis results (Fig. 8 A), suggest that proper membrane localization of both PDK1 and Akt are required to correctly instruct the chemotaxis process.

Discussion

Cells with altered PI3K or PTEN activity can usually migrate, but exhibit a significantly reduced ability to move directionally toward a chemoattractant gradient (Comer and Parent, 2002). However, the mechanism behind how PtdIns(3,4,5)P_3 accumulation is followed by the formation of leading edge is still obscure. Although it has been described as a positive feedback loop between PtdIns(3,4,5)P_3 and Rac GTPase, resulting in enhanced formation of membrane protrusions at the leading edge, relatively little is known about how PI3K downstream effectors regulate cell migration in response to external stimuli (Merlot and Firtel, 2003; Affolter and Weijer, 2005). Moreover, there is not yet a consensus about the importance of localized PI3K signaling during migration of mesenchymal cells (e.g., fibroblasts and EC) that do not adopt an amoeboid movement, and especially not when tyrosine kinase receptor signaling is involved (Affolter and Weijer, 2005; Schneider and Haugh, 2005).

In this study, we found that PDK1, a PtdIns(3,4,5)P_3-binding protein, plays an important role in the regulation of cell migration stimulated by VEGF-A, a ligand of the tyrosine kinase VEGF receptor 1 and 2. We observed that cells lacking PDK1 exhibited a reduced motility, and completely lost their ability to migrate in response to a chemoattractant in vitro. The role of PDK1 in cell migration is controlled by its PH domain because knock-in cells with a mutated PH domain, and therefore unable

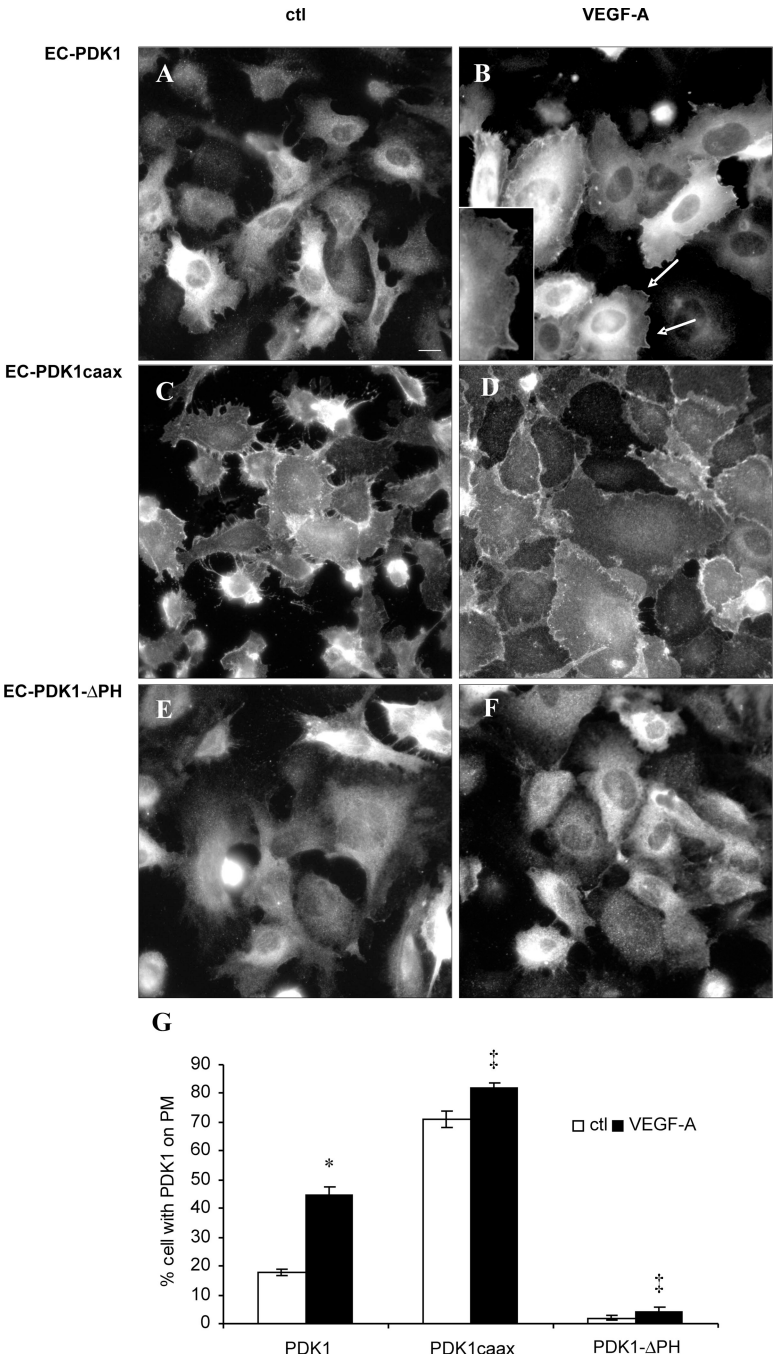

Figure 6. **PDK1 translocates to the membrane after VEGF-A stimulation.** ECs infected with the indicated retroviruses were seeded on gelatin-coated glass coverslips; they were then serum deprived and stimulated (B, D, and F) or not (A, C, and E) with 50 ng/ml VEGF-A. Cells were then fixed and analyzed by indirect immunofluorescence with mAb α-PDK1 (A–D) or mAb α-myc (E and F); antigen–antibody complexes were detected with Alexa Fluor 488–conjugated donkey α-mouse IgG. Bar, 10 μm. Images shown are representative of >50% of observed cells. (G) A total of 200 cells from three independent experiments were analyzed to calculate the percentage of cells showing PDK1 staining on plasma membrane. Data were plotted as the mean ± the SD. Statistical significance is shown for VEGF-A–stimulated EC-PDK1 compared with control cells (*, $P < 0.05$) and VEGF-A–stimulated EC-PDK1caax and PDK1-ΔPH compared with VEGF-A–stimulated EC-PDK1 (‡, $P < 0.05$).

to bind PtdIns(3,4,5)P_3, migrate at the same reduced rate as knock-out cells. Thus, it is plausible that PDK1 may follow the internal gradient of PtdIns(3,4,5)P_3 generated by VEGF-A–dependent PI3K activation, and translocates to the plasma membrane in the direction of the stimulation. We can, indeed, observe that in response to VEGF-A, PDK1 moves to the plasma membrane at the leading edge of migrating cells and phosphorylates Akt on T308.

The essential role of PDK1 in cell migration is supported by the findings that PDK1 knock-out EBs exhibit evident developmental defects that can be ascribed to defective EC motility. The vascular phenotype displayed by PDK1$^{-/-}$ EBs is obvious and cannot be accredited to lack of EC differentiation. In PDK1$^{-/-}$ EBs, the number of differentiated EC is similar to that of PDK1$^{+/+}$ EBs, but the ECs are not able to form capillary-like structures. Given that cell migration is a critical event in the angiogenic remodelling during vascular morphogenesis (Poole and Coffin, 1991), we suggest that the vascular phenotype observed in the PDK1$^{-/-}$ EBs could be caused by reduced cell motility. This hypothesis is supported by the evidence that PDK1$^{-/-}$ mouse embryos die at E9.5, displaying multiple abnormalities, including lack of somites, dorsal root ganglia, forebrain, and a circulatory system (Lawlor et al., 2002). The lack of dorsal root ganglia, together with the absence of branchial arches, strongly suggests a defective migration of neural crest cells. Moreover, ES cells lacking PDK1, which failed to activate

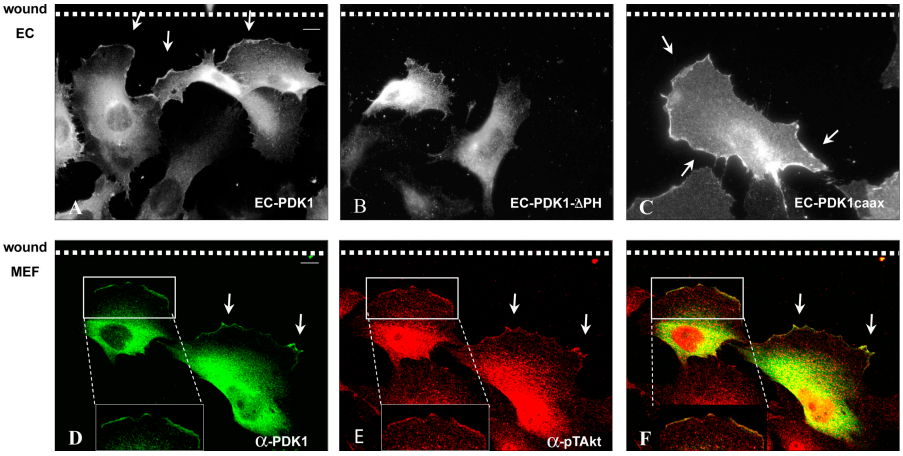

Figure 7. **PDK1 localizes at the leading edge of migrating ECs in a PH domain–dependent way and colocalizes with pT308Akt in migrating MEFs.** (A–C) ECs infected with indicated retroviruses were seeded at high density on gelatin-coated glass coverslips; monolayer cells were wounded by dragging a plastic pipette tip across the cell surface; and 50 ng/ml VEGF-A was added to the medium. After 6 h, cells were fixed and analyzed by indirect immunofluorescence with mAb α-PDK1 (A and C) or mAb α-myc (B); antigen–antibody complexes were detected with Alexa Fluor 488–conjugated donkey α-mouse IgG. (D–F) MEFs transiently transfected with PDK1 were seeded at high density on gelatin-coated glass coverslips; monolayer cells were wounded by dragging a plastic pipette tip across the cell surface; and medium supplemented with 50 ng/ml PDGF was added. After 6 h, cells were fixed and double stained with mAb α-PDK1 (green) and rabbit α-pT308Akt (red); antigen-antibody complexes were detected with Alexa Fluor 488–conjugated donkey α-mouse IgG and Alexa Fluor 555–conjugated donkey α-rabbit. Images shown are representative of >50% of observed cells. Boxed regions are enlargements of the cell's leading edge. Bars, 10 μm.

Akt and RSK, are viable, despite the reported important role of Akt and RSK in regulating survival and proliferation. Although it has been reported that PDK1 plays an important role in cell proliferation and survival in some cell lines, at least for ES cells and ECs, PDK1 is not intrinsically required for survival and proliferation (Flynn et al., 2000; Cho et al., 2001). Collectively, our results and the phenotype of PDK1 knock-out mice (Lawlor et al., 2002) indicate that PDK1 is necessary for EC migration in vitro and in vivo, and potentially regulates the migration process of other cell types and tissues.

Expression of wild-type and mutant PDK1 was used to provide insight into how migration might be regulated. The initial finding that overexpression of PDK1 promotes cell migration supports the central role of PDK1 in this process. Interestingly, overexpression of PDK1 exclusively increased cell motility in the presence of a chemoattractant, such as VEGF-A, but did not modify the basal cell motility. The ability of PDK1 to increase EC migration is kinase-dependent, as demonstrated using the kinase-dead mutant. Some PDK1 domains that are important in other functions, such as the RalGEF-interacting N terminus and the PIF pocket, are not involved in this process. The PH domain is clearly critical. Both the ECs expressing PDK1 lacking a PH domain and the EB-PDK1$^{PHKI/PHKI}$–derived cells make this point. However, inactivation of the PH domain in EBs gave rise to a vascular phenotype less severe than that caused by complete deletion of PDK1. In contrast, the motility of EC derived from EB-PDK1$^{PHKI/PHKI}$ was completely defective and comparable to that of EB-PDK1$^{-/-}$. These results suggest that other PDK1 domains may be involved in the vascular network formation, regulating different biological processes rather directional motility.

Consistent with these observations, PDK1-induced migration was blocked by PI3K inhibitor. Upon activation of VEGF receptor, activation of PI3K promotes the membrane localization of PDK1 and Akt, resulting in an increase of cell migration. Whether PtdIns(3,4,5)P_3 is also required for PDK1 activation in EC is unclear. A previous report (Stephens et al., 1998) and our experiments indicate that PDK1 is constitutively active, suggesting that the involvement of PI3K in PDK1 activation could be preferentially linked to PtdIns(3,4,5)P_3-induced conformational changes of Akt that enable PDK1 to phosphorylate this kinase (Stokoe et al., 1997). In addition to this mechanism, we demonstrated that PDK1 activity can be positively regulated by VEGF-A in cells expressing the membrane-targeted mutant of Akt, which does not require conformational changes to be phosphorylated.

The increased phosphorylation of Akt-T308 in PDK1-overexpressing cells paralleled the chemotaxis increase, whereas no phosphorylation change of S473 was observed. This suggests that T308 phosphorylation determines the activation state of Akt, whereas S473 is dispensable. However, two substrates of Akt, GSK3β and FKHR, were more phosphorylated in PDK1-overexpressing cells, but their phosphorylation level did not completely correlate with the EC ability to migrate. A possible explanation could be that high levels of T308 phosphorylation modify the substrate specificity of Akt, stimulating its activity on other substrates. A similar event has recently been described for S473. Cells lacking the kinase for S473 retained the Akt activity, but the ability to phosphorylate some substrates was dramatically reduced (Jacinto et al., 2006).

The evidence that PDK1 overexpression does not modify the basal cell motility suggests that this enzyme is mainly involved in the directional movement. Knowledge on chemotaxis mechanisms is essentially derived from studies on leukocytes and *D. discoideum* amoeba, which are able to move rapidly toward a variety of chemoattractants. These studies have demonstrated that PH domain–containing proteins specific for PtdIns(3,4,5)P_3 accumulate at the leading edge of migrating cells and that PI3K and PTEN associate with the membrane at the front and back, respectively, of chemotaxing cells (Funamoto et al., 2002; Iijima and Devreotes, 2002). As with the aforementioned cell type, motility of cells with nonamoeboid movement, such as fibroblasts and EC, rely on the activation of PI3K, and PDGF gradients elicit intracellular PtdIns(3,4,5)P_3 gradients in the plasma membrane (Haugh et al., 2000; Shiojima and Walsh, 2002). However, there are indications that, in these

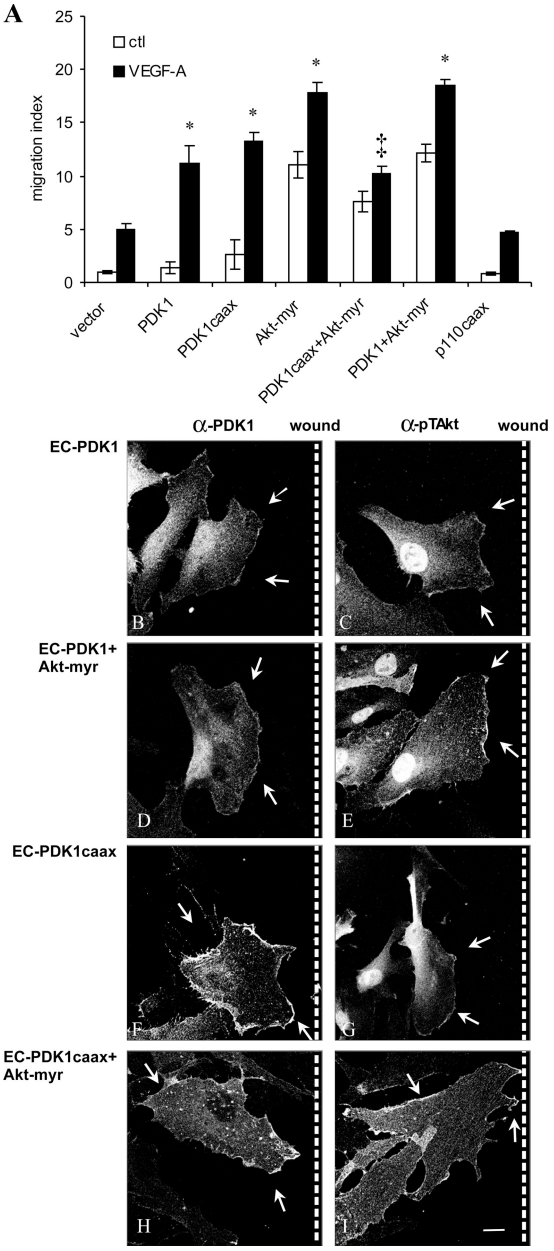

Figure 8. **Spatial distribution of PDK1 and Akt regulates chemotaxis.** (A) ECs transduced with retroviruses carrying membrane-targeted forms of PDK1 (PDK1caax), Akt (Akt-myr), PI3K-catalytic subunit p110 (p110caax), both PDK1caax and Akt-myr, both PDK1 and Akt-myr, and vector were used in chemotaxis assays in the presence of a gradient of VEGF-A (10 ng/ml). Migration index is calculated assigning a value of 1 to the number of vector cells migrated in the absence of stimulus. Data were plotted as the mean ± the SD of three independent experiments. Statistical significance is shown for VEGF-A–stimulated EC-PDK1, PDK1caax, Akt-myr, PDK1+Akt-myr (*, $P < 0.05$) compared with EC-vector and for VEGF-A–stimulated PDK1caax+Akt-myr (‡, $P < 0.05$) compared with EC-PDK1+Akt-myr. (B–I) ECs infected with indicated retroviruses were seeded at high density on gelatin-coated glass coverslips; monolayer cells were wounded by dragging a plastic pipette tip across the cell surface; and 50 ng/ml VEGF-A was added to the medium. After 6 h, cells were fixed and analyzed by indirect immunofluorescence with mAb α-PDK1 (B, D, F, and H) or rabbit α-pT308Akt (C, E, G, and I); antigen–antibody complexes were detected with Alexa Fluor 405–conjugated donkey α-mouse or α-rabbit IgG. Images shown are representative of >50% of observed cells. Bars,10 μm.

cells, the PtdIns(3,4,5)P_3-mediated spatial gradient–sensing mechanism differs (Arrieumerlou and Meyer, 2005; Schneider and Haugh, 2005).

Our observations that PDK1 overexpression regulates EC chemotaxis, together with PDK1 localization at the leading edge of migrating cells, demonstrate that the PI3K signaling pathway regulates EC directional motility in accordance with the previously proposed models in leukocytes and *D. discoideum*. Moreover, the expression of membrane-targeted PI3K in ECs did not increase, rather than decrease, the chemotaxis. Similar results have been described by Funamoto et al. (2002) in *D. discoideum* cells, in which they observed a chemotaxis deficiency in PI3K1/2$^{-/-}$ cells expressing membrane-targeted PI3K.

However, these models, which are focused on PI3K local accumulation, are not able to explain why EC transduced with PDK1 membrane-targeted mutant exhibit a great increase in chemotaxing cells compared with wild-type EC.

We suggest a model in which the PH domain of PDK1 and Akt contributes to their localization; as long as either PDK1 or Akt is localized in a lipid-specific way, a signaling gradient results (Fig. 9). When one of them is overexpressed and forced to the plasma membrane, the result is the local increase of Akt activation and chemotaxis. When both are overexpressed and forced to the membrane, the gradient of PtdIns(3,4,5)P_3 is no longer needed for localization and activation that would result in directional migration (Fig. 9).

The enrichment of PDK1 and Akt at the leading edge contributes to a local increase of Akt phosphorylation at T308 that could be responsible for the stimulation of directional migration. This model is confirmed by immunofluorescence staining of Akt pT308 showing that only when both PDK1 and Akt are membrane-anchored is Akt local activation lost. In spite of this, the high level of phosphorylation of Akt-myr observed in unstimulated condition or in presence of PI3K inhibitor is difficult to explain. In these conditions, PDK1 mainly localizes to the cytosolic and perinuclear regions; thus, it cannot efficiently phosphorylate the T308 of Akt-myr. A possible explanation is that the presence of the Myr tag on Akt leads to effects that are not solely related to its constitutive membrane association.

In conclusion, we demonstrate that PDK1 is required for migration of ECs in vivo and in vitro. Moreover, the increase of chemotaxing cells, obtained in PDK1-overexpressing ECs, indicates that one of the mechanisms by which PDK1 controls the motility is the regulation of the directional migration. We also suggest that PDK1-mediated Akt activation at the leading edge is responsible for this effect.

To our knowledge, this is the first genetic evidence that the PI3K signaling pathway controls the motility response mediated by tyrosine kinase receptors and the directional motility process in ECs.

Materials and methods

Cell culture

Human ECs were isolated from umbilical cord vein, characterized, and grown as previously described (Bussolino et al., 1992). MEF and Phoenix cells were grown in DME (Cambrex) supplemented with 10% FCS, 2 mM

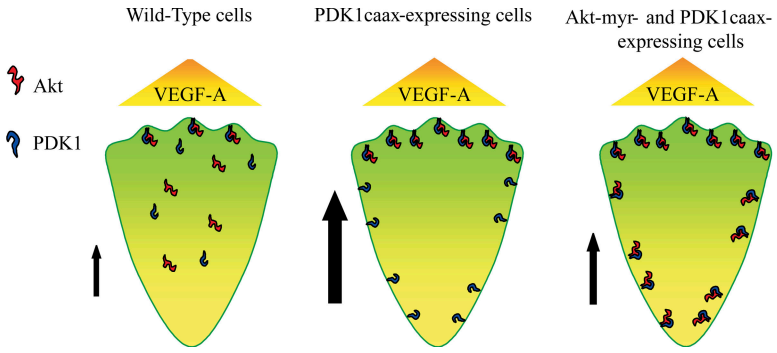

Figure 9. **Proposed model of PDK1 action on directional cell migration.** Stimulation of wild-type ECs with VEGF-A induces the local activation of PI3K and the formation of a gradient of PtdIns(3,4,5)P_3 (indicated with green) from the front to the rear of the cell that allows the polarized localization of PDK1 and Akt on the leading edge and directional migration; overexpression of membrane-anchored PDK1 strongly increases local level of Akt activation and, consequently, chemotaxis; in contrast, when ECs overexpress both PDK1caax and Akt-myr, activation of Akt is uniformly distributed along the membrane and ECs migrate less efficiently. The arrow sizes are indicative of the migration index.

L-Glutamine (Cambrex), and antibiotics. MEFs were transiently transfected with Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. All ES cells (PDK1$^{+/+}$, PDK1$^{-/-}$, PH_{KI}/PH_{KI}, and 155E/155E) were provided by D.R. Alessi (University of Dundee, Dundee, UK) and maintained in an undifferentiated state by culture on a feeder layer of MEFs pretreated with 10 μg/ml mitomycin C (Sigma-Aldrich), in high-glucose DME (Invitrogen), 15% FBS (HyClone), 0.1 mM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), L-Glutamine, antibiotics, and 1,000 U/ml LIF (CHEMICON International, Inc.).

Retroviral vector construct and EC infection

The cDNAs of wild-type and mutant PDK1 (with the exception of L155E) and the cDNA of the membrane-targeted catalytic subunit of PI3K (p110caax) were previously described (Tian et al., 2002). The L155E mutant of PDK1 was made by site-directed mutagenesis. The cDNA of membrane-targeted Akt1 (Akt1-myr) was provided by W. Sessa (Yale University, New Haven, CT; Morales-Ruiz et al., 2000). All cDNAs (with the exception of HA-tagged Akt1-myr) were myc-tagged by polymerase chain reaction and subcloned under the control of LTR promoter into EcoRI–EcoRI sites of the retroviral vector Pinco, which also contains GFP cDNA as tracking marker (Primo et al., 2005). cDNAs of myc-tagged PDK1 and PDK1caax were also subcloned into EcoRI–EcoRI sites of Pinco modified with dsRed cDNA instead of GFP for double-infection experiments. The amphotropic cell line Phoenix was transfected with retroviral vectors, and the retroviral supernatants obtained were collected, filtered (0.45 μm; Millipore), and supplemented with 4 μg/ml of polybrene (Sigma-Aldrich). Medium of ECs were replaced with the appropriate retroviral supernatants, and cells were incubated at 37°C with 5% CO_2 for 5 h. 72 h after infection, cells were analyzed for GFP expression by microscopy and for specific transgene expression by Western blot.

shRNA sequences and lentiviral preparation

Short hairpin RNAs (shRNA) against human Akt1 (4 sequences) were designed according to the TRC shRNA guidelines (Moffat et al., 2006), and subcloned into the MluI–ClaI sites of the pLVTHM vector (Wiznerowicz and Trono, 2003), which was provided by D. Trono (University of Geneva, Geneva, Switzerland). Efficacy of the constructs was tested through transduction into Sup-M2-TS cells and Western blot analysis of total cell lysates with α-Akt1–specific antibody (Cell Signaling Technology) after 4 d. The sequence for the sense oligonucleotides for the most effective knockdown constructs is: 5′-GGACTACCTGCACTCGGAGAA-3′ (based on positions 1,207–1,238 of human AKT1). Self-inactivating retroviral and lentiviral particles were produced as previously described (Piva et al., 2006). Aliquots of virus, plus 8 μg/ml of polybrene, were used to infect exponentially growing cells (10^5/ml). Fresh medium was supplemented at 24 h after the infection. The infectivity was determined (after 72 h) by FACS analysis of GFP-positive cells.

Chemotaxis assay

Chemotaxis assays with human ECs were performed in a Boyden chamber, as previously described (Primo et al., 2005). In brief, PVP-free polycarbonate filters (8 μm pore size; Neuroprobe) were coated with 1% gelatin for 2 h at 37°C. 10 ng/ml VEGF-A (R&D Systems) dissolved in serum-free medium was seeded in the lower compartment of the chamber; cells were serum starved overnight, and then suspended in serum-free medium at a concentration of 2.5×10^6 cells/ml, and 50 μl of the suspension was added to the upper compartment. For experiments with EBs, at day 3 of differentiation, they were disaggregated with PBS and 2 mM EDTA for 5 min at 37°C and trypsin for 1 min at 37°C. Cells were seeded on the upper side of a 24-well, 8-μm pore HTS FluoroBlok insert (BD BioSciences; 1×10^5 cells/well) that was coated on the lower side with 20 μg/ml fibronectin and incubated in EB medium supplemented with 2% FCS, rather than 20% FCS; the lower compartment was filled with EB medium and 2% FCS with or without 30 ng/ml VEGF-A.

After 5 h of incubation at 37°C with 5% CO_2, the upper surface of the filters was scraped with a rubber policeman, and the filters were fixed and stained with Diff-Quick (Dade Behring) or rat α-CD31, as described in Indirect immunofluorescence. Four random fields of each sample in the lower surface of the filters were counted at 10× magnification.

Motility assay

Starved ECs were plated onto gelatin-coated 24-well plates and allowed to adhere in serum-free medium for 1 h at 37°C. 100 ng/ml VEGF-A was or was not added to the medium, and ECs were observed with an inverted microscope equipped with a thermostatic and CO_2-controlled chamber (AS MDW workstation; Leica). Fluorescent video images of ECs were recorded at 10-min intervals for 6 h with a charge-coupled device camera (Orca HiRes; Hamamatsu Photonics) and analyzed using DIAS image processing software (Solltech). Speed parameter (in micrometers/minute) of 60 cells from three different experiments were calculated and plotted.

Immunoprecipitation

Confluent cells were serum deprived for 2 h, pretreated or not pretreated with 50 μm LY294002 for 45 min, and stimulated or not stimulated with 30 ng/ml VEGF-A for 10 min. Cells were transferred on ice, washed three times with cold PBS containing 1 mM Na orthovanadate, and lysed in RIPA-modified buffer containing 20 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.5% Na desossicolate, 0.1% SDS, 5 mM EDTA, and protease and phosphatase inhibitors (50 μg/ml pepstatin, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 100 μM $ZnCl_2$, 1 mM Na orthovanadate, and 10 mM NaF). After centrifugation (15 min at 10,000 *g*), supernatants were precleared by incubation for 1 h with protein G–Sepharose (GE Healthcare). Samples (700 μg of proteins) were incubated with rat α-HA (Roche) for 2 h to isolate overexpressed Akt-myr, and immune complexes were recovered on protein G–Sepharose. Beads were washed four times with lysis buffer and detected by immunoblot. Proteins were separated by SDS-PAGE electrophoresis, transferred to polyvinylidene difluoride (PVDF) membrane (Millipore), incubated with rabbit α-pT308Akt and, after stripping, rabbit α-Akt (Cell Signaling Technology), and visualized by ECL system (GE Healthcare).

Western blot analysis

For lysates, cells were serum deprived for 2 h and stimulated or not with 30 ng/ml VEGF-A for 10 min. Total proteins were extracted in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol) and quantified, and equal amounts of each sample were resolved by SDS-PAGE and transferred to PVDF membrane. After blocking with TBS/0.1% Tween 20/5% BSA, membranes were incubated with primary antibody overnight at 4°C. The following primary antibodies were used: rabbit α-pS241PDK1, α-pS473Akt, α-pT308Akt, α-Akt, α-pFKHR, α-pGSK3β (all from Cell Signaling Technology), mAbs α-myc, and α/β tubulin (both Santa Cruz Biotechnology). Immunoreactive proteins were identified with secondary antibody coupled to HRP antibody and visualized by ECL.

EBs

For in vitro differentiation of ES cells, the same ES medium was used, except that LIF was omitted and the FBS concentration was 20% (EB medium).

The hanging drop procedure was followed (Gualandris et al., 2000). ES cells grown to confluence on the feeder layer of MEFs were harvested with trypsin and centrifuged. The pellet was resuspended in ES medium, and ES plus MEF cell suspension was seeded on a tissue culture dish for 30 min at 37°C. During this time, MEFs attached, whereas ES cells remained in suspension. This step was repeated twice. The ES cell suspension was centrifuged, and the pellet was resuspended in EB medium for counting. 30-μl drops, containing 400 cells, were placed on the undersurface of the lids of Petri dishes and incubated at 37°C. After 2 d, the cell aggregates contained in the drops were collected and cultured in suspension in bacterial Petri dishes for 3 d. The aggregates were then transferred onto gelatin-coated regular culture dishes, where they spread and differentiated; this was day 0 of differentiation. For fluorescence immunostaining, at day 0 EBs were plated onto gelatin-coated glass coverslips in 24-well plates; after 3, 7, or 10 d of differentiation, they were fixed with paraformaldehyde 3.7% in PBS for 20 min at room temperature, washed three times with PBS, and stained as described in Indirect immunofluorescence. The quantification of total length of vessel-like structures stained by α-CD31 was performed with the imaging software winRHIZO Pro (Regent Instruments, Inc.), as described by Cascone et al. (2005).

FACS analysis

EBs at different differentiation stages were rinsed twice and then disaggregated with PBS 2 mM EDTA for 5 min at 37°C and trypsin for 1 min at 37°C. 2×10^5 cells were then incubated on ice with 5 μg/ml of the following primary antibody for 30 min: rat α-Flk1 (Becton Dickinson), rat α-CD31, and control rat IgG. After three washes with PBS 1% BSA, cells were incubated on ice with 2.5 μg/ml R-phycoerythrin–conjugated α-rat antibody (Southern Biotechnology Associates) for 20 min. After final washes with PBS, samples were fixed with PBS, 1% BSA, and 2% PAF and analyzed using FACScan (Becton Dickinson).

Wound healing

ECs or MEFs were seeded at high density on gelatin-coated glass coverslips; after 12 h of adhesion, monolayer cells were wounded by dragging a plastic pipette tip across the cell surface and 50 ng/ml VEGF-A or PDGF was added to serum-free medium. After 6 h, cells were washed with PBS, fixed with PAF 3.7% for 10 min at room temperature, and analyzed by indirect immunofluorescence, as described in the following section.

Indirect immunofluorescence

The protocol described was followed both for cells (ECs and MEFs) and EBs. For immunofluorescence staining, ECs were plated onto gelatin-coated glass coverslips in 24-well plates. After 12 h of adhesion in complete medium, they were serum deprived for 2 h and then stimulated or not stimulated with 50 ng/ml VEGF-A. Medium with growth factors was removed, and cells were fixed with 3.7% PAF for 10 min at room temperature. After fixation, cells or EBs were rinsed three times with PBS, and then quenched with 50 mM NH_4Cl for 20 min at room temperature, washed twice with PBS, and permeabilized with PBS 0.5% Triton X-100 for 5 min at room temperature. After two washes with PBS, coverslips were blocked with PBS 0.3% Triton X-100, 1% donkey, 1% or goat serum for 1 h at room temperature, and incubated with primary antibodies overnight at 4°C in a humidified chamber. For EC staining of EBs, rat α-CD31 (1:100; BD Biosciences) was used, and for ECs and MEFs, mAb α-PDK1 (1:80; BD Biosciences), mAb α-myc (1:40; Santa Cruz Biotechnology) and rabbit monoclonal α-pT308Akt (1:80; Cell Signaling Technology) were used. After three washes with PBS, coverslips were incubated for 1 h at 37°C in a humidified chamber with fluorescent secondary antibodies; donkey α-rat Cy2 (1:200; Jackson ImmunoResearch Laboratories), donkey α-mouse Alexa Fluor 488 or goat α-mouse Alexa Fluor 405 (1:400; Invitrogen), donkey α-rabbit Alexa Fluor 555, or goat α-rabbit Alexa Fluor 405 (1:400). Coverslips were then rinsed three times with PBS, mounted, and analyzed using an inverted fluorescence microscope (DM IRB; Leica) equipped with 63×/1.30 HCX Plan-Apochromat (Carl Zeiss MicroImaging, Inc.) glycerin-immersion and 4×/0.10 C Plan objectives or a confocal laser-scanning microscope (TCS SP2 with DM IRE2; Leica) equipped with 63×/1.40 HCX Plan-Apochromat oil-immersion objective. Confocal images are the maximum projections of a z section of ∼1.50 μm. The images were arranged and labeled using Photoshop software (Adobe).

Online supplemental material

Fig. S1 shows the quantification of Western blots presented in Fig. 3 D. Fig. S2 shows that PDK1 overexpression didn't affect the proliferation rate and survival capacity of ECs. Fig. S3 shows the PDK1 mutants used in the overexpression experiments. Fig. S4 shows the localization of PDK1 in MEF stimulated or not stimulated with PDGF. Online supplemental materials are available at http://www.jcb.org/cgi/content/full/jcb.200607053/DC1.

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