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Key Roles for the Lipid Signaling Enzyme Phospholipase D1 in the Tumor Microenvironment During Tumor Angiogenesis and Metastasis

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Abstract

Angiogenesis inhibitors confer only short-term benefits on tumor growth. We report that ablation of the lipid signaling enzyme phospholipase D1 (PLD1) in the tumor environment compromises neovascularization and growth of tumors. PLD1 deficiency suppressed activation of AKT and mitogen-activated protein kinase signaling pathways by vascular endothelial growth factor (VEGF) in vascular endothelial cells, resulting in decreased integrin-dependent cell adhesion to and migration on extracellular matrixes and reduced tumor angiogenesis in a xenograft model. In addition, mice lacking PLD1 incurred significantly fewer lung metastases. Bone marrow transplantation and binding studies identified a platelet-derived mechanism involving decreased tumor cell:platelet interaction due to impaired activation of platelet $\alpha_{IIb}\beta_3$ integrin, which decreased seeding of tumor cells into the lung parenchyma. Treatment with a small molecule inhibitor of PLD1 phenocopied PLD1 deficiency, efficiently suppressing both tumor growth and metastasis in mice. These findings reveal that PLD1 in the tumor environment promotes tumor

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growth and metastasis, and taken together with prior reports of PLD1 roles in tumor cell intrinsic adaptations to stress, suggest potential utility for PLD1 inhibitors as cancer therapeutics.

Introduction

Anti-angiogenic strategies have been approved for therapeutic use to restrict tumor growth but provide only modest benefit, yielding short-term tumor shrinkage but not improvements in long-term survival (1). Tumors thusly targeted in mice undergo “evasive resistance” characterized by accelerated metastasis (2, 3). This outcome suggests that therapeutics that target both tumor adaptation and multiple components of the tumor microenvironment that facilitate tumor growth and metastasis are required to achieve more substantial delays in tumor progression.

The signaling enzyme phospholipase D (PLD), which generates the lipid second messenger phosphatidic acid (PA) (4), is increased in abundance or activity (5–9) or mutated (10, 11) in various human cancers, and its activity in the cancer cells has been linked with proliferative signaling (12–14), evasion of growth suppressors (15, 16), resistance to cell death (17), and increased characteristics of invasiveness and metastasis (18–21). The classic PLD isoforms PLD1 and PLD2 have been linked in a cell-intrinsic manner to the pro-metastatic phenotype, although they likely have different functions in this context (22).

The development of PLD-deficient mice (23–25) and small molecule PLD inhibitors (26, 27) has made it possible to examine the role of PLD in the tumor microenvironment. Mice lacking PLD1 or PLD2 are viable and overtly normal. *Pld1*^{−/−} mice exhibit diminished $\alpha_{IIb}\beta_3$ -dependent platelet activation, making them refractory to major pathological hemostasis events such as strokes and pulmonary embolisms (23), and have defects in macroautophagy (24), which could potentially impact on cancer cell-intrinsic metabolic pathways (28). *Pld2*^{−/−} mice are protected against the synaptotoxic and memory-impairing actions of β -amyloid in a transgenic model of Alzheimer’s disease (25).

Here, we show that tumor progression requires PLD1 but not PLD2 activity in the tumor microenvironment both for primary tumor growth and independently for subsequent metastasis, suggesting that, in combination with previous reports identifying roles for intrinsic activity of both isoforms in tumor cells, small molecule PLD inhibitors could have substantial utility through inhibiting tumor progression at multiple successive stages.

Results

Tumor growth is impaired in mice lacking PLD1

Potential roles for PLD isoforms in the tumor microenvironment on tumor development were explored by subcutaneously implanting B16F10 mouse melanoma cells into wild-type, *Pld1*^{−/−} (Fig. S1A–D), and *Pld2*^{−/−} (25) mice. Growth of B16F10 tumors was attenuated in *Pld1*^{−/−} mice (Fig. 1A) but not in *Pld2*^{−/−} mice (Fig. S2A); tumors in *Pld1*^{−/−} mice were 49.0±10.1% of the size of those in wild-type mice. Similar results were obtained subsequent to implantation of mouse Lewis lung carcinoma (LLC) cells (Fig. 1B) whereas LLC tumor growth in wild-type and *Pld2*^{−/−} mice was comparable (Fig. S2B); tumor growth in *Pld1*^{+/−} mice was also comparable to that observed in wild-type mice (Fig. S2C).

Tumors initially expand using locally diffusible nutrients and are not readily detected by the immune system. Subsequently, increased nutrient demand requires the tumor to become vascularized, increasing the exposure of the tumor to the immune system. Many reasons can underlie stunted tumor growth, including immune destruction and inadequate nutrient supply. Using anti-F4/80 antibody staining, the extent of macrophage infiltration in tumors

implanted in wild-type and *Pld1*^{-/-} mice was comparable (Fig. S3), suggesting similar extents of immune surveillance and response to the growing tumors. In contrast, however, histological analysis (Fig. 1C) and immunofluorescent staining for CD31, an endothelial cell marker (Fig. 1D), revealed an almost complete absence of small blood vessels (microvessels) in the tumors growing in *Pld1*^{-/-} mice. In contrast, microvessel frequency was comparable in tumors grown in wild-type and *Pld2*^{-/-} mice (Fig. S4).

Tumor angiogenesis is decreased in PLD1-deficient mice

There is cross-talk between tumors and the tumor microenvironment at many stages of tumor growth and progression. Many reasons could indirectly underlie poor vascularization of the tumors, including changes in the tumor behavior such as decreased secretion of vascular growth factors in the altered genetic environment. To assess whether tumor response was a factor in the phenomenon, we subcutaneously implanted matrigel plugs embedded with vascular endothelial growth factor (VEGF). The matrigel plugs in wild-type mice became vascularized in a VEGF-dependent manner as evidenced visually and through quantitation of hemoglobin (Fig. 2A), by histological staining (Fig. 2B, upper panels), and by anti-CD31 staining to directly visualize vascular endothelial cells (Fig. 2B). In contrast, VEGF plugs in *Pld1*^{-/-} mice exhibited almost no vascularization (Fig. 2A, B).

These findings suggested an intrinsic defect in the behavior of vascular endothelial cells in *Pld1*^{-/-} mice, which was assessed with two assays of vascular endothelial cell competence. In the first assay, rings of aortic tissue were embedded in matrigel containing VEGF. Endothelial cells emigrated from aortic rings from wild-type mice and attempted to organize into microvessels (Fig. 2C). In contrast, endothelial cell emigration and microvessel formation from aortic rings prepared from *Pld1*^{-/-} mice was attenuated.

A major phenotypic characteristic of endothelial cells is the ability to assemble into tubular-like structures when grown on matrigel, forming the basis for the second assay (29). Microvascular endothelial cells isolated from wild-type pulmonary tissue formed a clearly defined and well-connected network of precapillary cords when plated on matrigel (Fig. 2D). In contrast, although *Pld1*^{-/-} lung microvascular endothelial cells proliferated indistinguishably from wild-type cells during the 3 days of culture prior to being seeded on matrigel (Fig. S5), they were subsequently incompetent at forming precapillary cords (Fig. 2D).

Taken together, these findings demonstrate an intrinsic defect in the ability of *Pld1*^{-/-} vascular endothelial cells to migrate and self-organize in model systems involving physiological substrates and growth factor stimulation.

PLD1 is required for integrin-dependent endothelial cell adhesion and angiogenic signaling

Cord and tube formation and angiogenesis critically depend on regulated adhesion to extracellular matrix proteins. We next explored whether *Pld1*^{-/-} endothelial cells were impaired in integrin-dependent or independent cell adhesion. Adhesion to polylysine- and BSA-coated substrates was comparable for wild-type and *Pld1*^{-/-} endothelial cells; however, PLD1 deficiency significantly reduced adhesion of endothelial cells to the matrix proteins fibronectin, vitronectin and collagen (Fig. 3A), which are the major ligands for the signaling-activated $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins, respectively. These results indicate that the signaling pathways facilitated by PLD1 selectively affect integrin-dependent endothelial cell adhesion.

To gain insight into the underlying molecular mechanisms, we examined angiogenic signaling pathways in lung endothelial cells. VEGF receptor activation stimulates divergent

effector pathways through phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 (30), p38 (31), and Akt (32) which direct changes in cell survival, proliferation, and migration. The latter two effector arms mediate integrin activation, enhanced endothelial cell adhesion to extracellular matrix proteins, and migration that takes place during neovascularization. Serum growth factor-stimulated PLD activity has been reported to be required for mTORC2-mediated phosphorylation of AKT at Ser⁴⁷³, but only weakly for PDK1-mediated phosphorylation of Ser³⁰⁸ (33). Amounts of total ERK1/2, p38, and AKT were comparable in wild-type and *Pld1*^{-/-} endothelial cells (Fig. 3B). PLD1 deficiency reduced basal phosphorylation of Ser⁴⁷³ in Akt and prevented VEGF-induced phosphorylation of Akt at Ser⁴⁷³ (Fig. 3C, D). *Pld1*^{-/-} endothelial cells similarly exhibited a 50% reduction in basal phosphorylation of ERK1/2 but otherwise exhibited similar kinetics to wild-type cells for VEGF-induced phosphorylation of ERK1/2. Finally, whereas basal phosphorylation of p38 was comparable between wild-type and *Pld1*^{-/-} endothelial cells, VEGF-induced phosphorylation of p38 was blunted in the absence of PLD1 (Fig. 3C, D). Taken together, these data suggest defects in VEGF receptor effector pathways through Akt and p38 that could account for the effects of PLD1 deficiency on endothelial cell adhesion and migration, and suggest that the effects of PLD1 deficiency on the receptor signaling pathway are relatively proximal to VEGF receptor activation.

The PLD inhibitor FIPI suppresses tumor growth

We next used FIPI, a small molecule inhibitor of PLD (27), to determine if acute inhibition of PLD1 in wild-type animals prevented *in situ* tumor vascularization and growth. FIPI has a half-life of 5.5 hours *in vivo* and moderate bioavailability (18%) (34). Vehicle or the inhibitor were injected intraperitoneally twice daily to provide continuous full inhibition of PLD, starting the day prior to implantation of the tumor cells. Mice tolerated FIPI administration well, showing no visible signs of distress and maintaining their weight similarly to vehicle-treated mice over the 11 days of treatment (Fig. S7). In wild-type mice implanted subcutaneously with tumors, FIPI treatment phenocopied the results observed in *Pld1*^{-/-} mice, namely, decreased growth of tumors (Fig. 4A; 63% reduction). This finding is unlikely to be explained by decreased proliferation of the tumor cells *per se* in the FIPI-treated animals, because FIPI did not alter rates of proliferation of the tumor cells in culture (Fig. 4B). Histological and quantitative analysis of the tumors revealed a 79% decrease in microvessels in the tumors that formed in FIPI-treated mice (Fig. 4C). Taken together, these findings identify the tumor microenvironment as being a key site at which PLD1 activity is required for *in situ* tumor growth as a consequence of its requirement in pathological neovascularization.

Tumor metastasis is impaired in PLD1 knockout mice

To assess the effect of PLD1 deficiency on tumor metastasis, we intravenously injected melanoma cells into the tail veins of wild-type and *Pld1*^{-/-} mice, and harvested and scored the lungs for metastases 14 days later. The frequency of metastasis was 50% lower in the *Pld1*^{-/-} mice than in wild-type mice (Fig. 5A). In contrast, *Pld2*^{-/-} mice had a frequency of metastasis that was identical to wild-type mice (105 ± 24% of wild-type). Although the number of metastases was reduced in *Pld1*^{-/-} mice, the size and overall appearance of the individual pulmonary foci were similar in wild-type and *Pld1*^{-/-} mice, suggesting that a decreased proliferation rate of the tumor cells in the *Pld1*^{-/-} mice was not the underlying cause of the decreased number of metastatic sites. This latter finding was again consistent with the observation that inhibition of PLD activity by FIPI did not alter rates of proliferation of the tumor cells in culture (Fig. 4B).

We thus hypothesized that PLD1 would primarily affect the initial establishment of the metastatic foci. Examination of the lung tissue in wild-type mice subsequent to tumor cell

injection into the tail vein revealed a substantial population of lodged tumor cells within 30 min (Fig. 5B) that were rapidly cleared from lung as reported previously (35, 36). Lodged tumor cells were also observed in *Pld1*^{-/-} lungs, but at lower numbers than in the wild-type lungs (Fig. 5B), consistent with the decreased number of macroscopic metastases found 14 days after injection (Fig. 5A). This finding demonstrated that the initial seeding of tumor cells to lung is PLD1-dependent.

Absence of PLD1 reduces the interaction between tumor cells and platelets

A factor that is important in metastatic seeding is coating of the tumor cells by platelets (37). Experimental metastasis is almost completely inhibited in mice in which platelets are depleted either genetically or with an antibody (38, 39). The platelet:tumor cell aggregates form within minutes, persist for several hours, and are critical for lodging of the tumor cells in the lung (40). We thus explored this possibility for the *Pld1*^{-/-} mice, beginning with examination of tumor cell:platelet interactions *in vitro*. Wild-type platelets in the resting state exhibited little binding to tumor cells, but once stimulated by platelet activating factors such as PAR4 activating peptide or thrombin, bound avidly to the tumor cells (Fig. 6A). In contrast, *Pld1*^{-/-} platelets activated by PAR4 activating peptide and thrombin displayed reduced binding to the tumor cells.

To test whether the reduction in metastasis observed in *Pld1*^{-/-} mice ensued from the absence of interaction of the tumor cells with platelets, rather than changes in properties of the lung endothelial cells or parenchyma, we performed wild-type or *Pld1*^{-/-} donor bone marrow transplantation into wild-type mice to restrict PLD1 deficiency to the transplanted cells. Wild-type mice reconstituted with *Pld1*^{-/-} bone marrow developed fewer pulmonary metastases than mice reconstituted with wild-type bone marrow (Fig. 6B), confirming that the requirement for PLD1 during metastasis is in bone marrow-derived cells, consistent with our hypothesis that the effects are due to platelet defects.

Tumor cell:platelet interaction is mediated by multiple types of receptors and interacting protein pairs. $\alpha_{IIb}\beta_3$ integrin is the most abundant adhesion receptor on the platelet surface and contributes to tumor metastasis by mediating the interactions of platelets with tumor cells (41). We have previously shown that activation of integrin $\alpha_{IIb}\beta_3$ in *Pld1*^{-/-} platelets is impaired (23), which may explain the reduction in tumor metastasis in *Pld1*^{-/-} mice. To test this hypothesis, we eliminated $\alpha_{IIb}\beta_3$ functionality in platelets using Fab fragments of the JON/A monoclonal antibody (42), which blocks the ligand binding site on the integrin (Fig. S8A). As anticipated, wild-type platelets treated with JON/A Fab fragments prior to stimulation with PAR4 activating peptide or thrombin exhibited significantly reduced interaction with tumor cells (Fig. 6C). In contrast, JON/A Fab fragment treatment did not further reduce the extent of interaction between activated *Pld1*^{-/-} platelets and tumor cells, suggesting that the impaired interaction between *Pld1*^{-/-} platelets and tumor cells in the *in vitro* model setting can be attributed to the deficiency in activation of $\alpha_{IIb}\beta_3$ integrin.

Finally, to determine whether the impaired activation of $\alpha_{IIb}\beta_3$ integrin explained the decreased metastasis in *Pld1*^{-/-} mice, we blocked $\alpha_{IIb}\beta_3$ using the JON/A Fab antibody fragments in wild-type and *Pld1*^{-/-} mice before injecting them with B16F10 tumor cells. In wild-type mice, blockade of $\alpha_{IIb}\beta_3$ led to a 50% reduction in metastasis (Fig. 6D, S8B), indicating a partial but non-redundant role for this integrin as previously reported (41). However, in contrast to our findings with the *in vitro* model system (Fig. 6C), blockade of the $\alpha_{IIb}\beta_3$ -mediated platelet:tumor cell interaction in *Pld1*^{-/-} mice almost completely eliminated pulmonary metastases (Fig. 6D, S8B). These findings reveal that additional platelet-dependent mechanisms underlie the decreased frequency of tumor metastasis in *Pld1*^{-/-} mice.

The PLD inhibitor FIPI inhibits tumor metastasis

We examined whether use of FIPI to acutely ablate PLD activity inhibited tumor metastasis. FIPI efficiently inhibited PLD activity in platelets (Fig. 7A) with a dose responsiveness similar to our previous report (27) and almost fully eliminated binding of activated platelets to tumor cells in the *in vitro* setting (Fig. 7B). Because our findings suggest that PLD1 is required in the early steps of metastasis, namely during seeding in the pulmonary vascular bed and extravasation into the parenchyma, we employed FIPI to fully inhibit PLD activity before injecting B16F10 cells and for the next 20 hours. The quantification of pulmonary metastasis two weeks later revealed that blockade of PLD1 in the early stage of metastasis led to a 65% decrease in the frequency of metastatic foci (Fig. 7C), comparable or greater to that observed in *Pld1*^{-/-} mice (Fig. 5A).

Taken together, these experiments demonstrate an additional, related role for PLD1 in cancer progression, involving supporting the metastasis of cancer cells to distal sites.

Discussion

Roles for the PLD isoforms in cancer cells in the context of proliferation, survival under stressed conditions, migration, and invasion have been studied. This report provides evidence that PLD1 activity in the tumor microenvironment is critical for tumor growth and metastasis, and describes the use of small molecule PLD inhibitors to suppress tumor progression.

We report here that the decreased tumor growth is accompanied by diminished tumor vascularization, which provides a basis for the decreased growth. Nonetheless, it should be noted that immune responses can play important supportive or suppressive roles in tumor growth. We did not observe differences in macrophage density in the tumors or in the surrounding regions, but it remains possible that immune responses could differ in *Pld1*^{-/-} mice and affect the rate of tumor growth.

Several studies have previously raised the possibility of roles for PLD1 in vascularization. In zebrafish, use of antisense morpholino oligonucleotides to knock down PLD1 was reported to block developmental angiogenesis of intersegmental vessels, resulting in little to no blood circulation and pericardial edema (43). However, this phenotype was rescued in a non-cell autonomous manner through transplantation of non-vascular notochord (mesodermal) tissue, suggesting that the angiogenesis failure was secondary to disrupted signaling from an independent tissue. In contrast, *Pld1*^{-/-} mice did not exhibit this developmental phenotype, and we show using several approaches that deficiency of PLD1 in mice resulted in primary defects in the vascular endothelial cells. Taken together, the phenotype we report here for *Pld1*^{-/-} mice would appear to be unrelated to the one described for PLD1 suppression in zebrafish.

Using an RNAi approach, a role for PLD1 in hypoxia and VEGF-mediated pathologic retinal angiogenesis has also been proposed, through a Src-dependent PLD1-PKC γ -cPLA₂ activation pathway (44). Here, by using knockout mice instead of RNAi, we established a role of PLD1 in tumor angiogenesis through separate signaling pathways. VEGFR2 is the major mediator of VEGF-induced responses in endothelial cells and its signaling pathways are relatively well characterized. The Tyr¹¹⁷⁵ autophosphorylation site docks phospholipase C- γ (30), which indirectly mediates activation of the mitogen-activated protein kinase pathway, and the adaptor protein Shb, which activates phosphatidylinositol 3-kinase resulting in stimulation of Akt (32). Phosphorylation of Tyr¹²¹⁴ in VEGFR2 activates Cdc42 and p38 (31). Our data reveal that in the experimental setting we examined, absence of PLD1 blocks the VEGFR2-PI3K-Akt signaling pathway (potentially through mTORC2) and

blunts the VEGFR2-p38 pathway. Because these effector arms mediate VEGF-induced adhesion and migration of endothelial cells, the decreased signaling we observed suggests a molecular mechanism underlying the decreased angiogenesis in *Pld1*^{-/-} mice. However, PLD1 has been linked to many signaling pathways (4), and there may be additional effectors altered in function in the absence of PLD1 in the setting of VEGF stimulation of vascular endothelial cells.

Although angiogenesis has become an attractive target for drug therapy due to its key role in tumor growth, anti-angiogenic therapy has been reported to promote accelerated tumor metastasis. In some mouse cancer models, the tumors show initial sensitivity to VEGFR2 blockade, but become more aggressive, invasive and metastatic after several weeks of treatment (2, 3). The small molecule approach we demonstrate here may be attractive in that it targets an additional step in the tumor progression, namely metastatic seeding, in addition to tumor cell-intrinsic roles associated with metastasis (22, 45).

The mechanism through which PLD1 facilitates tumor metastasis is incompletely understood. One possibility is that *Pld1*^{-/-} platelets, which have impaired activation of $\alpha_{IIb}\beta_3$ integrin at intermediate amounts of platelet stimulation (23), provide a weaker physical shield for the tumor cells or are needed as bridges to lodge at sites of metastasis. Although few *Pld1*^{-/-} platelets interacted with tumor cells in an *in vitro* model system due to defective activation of $\alpha_{IIb}\beta_3$ integrin, we still observed a lowered frequency of metastasis in the *Pld1*^{-/-} mice after blocking $\alpha_{IIb}\beta_3$ using monovalent JON/A antibody. This indicates that there was at least partial activation of $\alpha_{IIb}\beta_3$ integrin in the *Pld1*^{-/-} platelets *in vivo* and revealed the involvement of additional or other mechanisms *in vivo* that underlie platelet- and PLD1-dependent tumor metastasis. Intriguingly, platelets activate signaling pathways in tumor cells that facilitate the prometastatic phenotype by locally releasing transforming growth factor (TGF) β_1 (37). PLD1 may not only facilitate $\alpha_{IIb}\beta_3$ -mediated contact between platelets and the tumor cells, but also play roles in the release of TGF β_1 (46).

It is widely accepted that cancer patients have a venous thromboembolic event risk that represents a leading cause of death in hospitalized patients with cancer (47, 48) and that anticoagulation improves long-term survival in this population (49, 50). Moreover, increased risk of venous thromboembolism is an emerging complication of many angiogenesis inhibitors such as bevacizumab (51). We have reported that PLD1 plays a critical role in platelet activation and stable thrombus formation in the setting of high shear forces - in the absence of PLD1, thrombi are unstable under conditions of rapid flow (23). As a result, mice lacking PLD1 are protected in pathological conditions that require this stability, such as is seen in models of pulmonary embolism, stroke, and aortic thrombosis. These findings raise the possibility that use of a small molecule PLD1 inhibitor might avoid a serious side effect associated with anti-angiogenic therapy and lower the frequency of thrombosis in cancer patients.

Finally, although we found roles for PLD1 but not PLD2 in the tumor microenvironment for tumor growth and metastasis, suggesting that the optimal therapeutic might be one that was selective for PLD1, numerous reports on cell-intrinsic roles for PLD2 in transformation, increased invasiveness and migration have been reported (22, 45). Hence, a small molecule dual inhibitor of PLD1 and PLD2 such as FIPI may ultimately provide the most utility.

Materials and Methods

Animals

Pld1^{-/-} mice were generated in the C57BL/6 x 129Sv background and backcrossed to C57BL/6 mice for more than 10 generations. Sex-matched 6- to 8-week-old wild-type and

Pld1^{-/-} mice were used for the experiments, which were performed using protocols approved by the SBU Institutional Animal Care and Use Committee (IACUC).

Cell culture

Mouse melanoma cells (B16F10), mouse Lewis lung carcinoma cells (LLC) and human breast cancer cells (MDA-MB-231) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Primary lung endothelial cells were cultured in DMEM containing 20% FBS, 100 µg/ml heparin (Sigma), 100 µg/ml endothelial cell growth stimulant (Biomedical Technologies), nonessential amino acids and sodium pyruvate (Invitrogen).

Immunohistochemistry

5 µm paraffin sections from fixed tumor tissues were dewaxed in xylene and dehydrated through alcohols. After blocking with 5% goat serum, sections were stained with goat anti-CD31 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4°C and then washed before incubation in Alex-fluor-546-conjugated secondary antibody for 1 hour. After washing, sections were mounted with vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Slides were imaged using a Leica TCS SP5 laser scanning confocal microscope.

Western blotting

Endothelial cells serum-starved for 4 hours and in some experiments then stimulated with 50 ng/ml VEGF were lysed using RIPA buffer and the protein concentration of the samples determined using the Bradford method. Equal amounts of protein were loaded onto SDS-PAGE gels and transferred to nitrocellulose membranes. Western blots were performed using primary antibodies against phospho-Akt (Ser473), phospho-extracellular signal-regulated kinase 1/2 (Erk1/2), phospho-p38 (Cell signaling Technology, Beverly, MA) and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The blots were developed using secondary antibodies conjugated with Alexa Fluor 680 or IRDye 800 (Rockland Immunochemicals, Gilbertsville, PA). Fluorescent signals were detected with an Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NE).

Platelet isolation

Following cardiac puncture, blood was drawn into acid citrate dextrose (ACD, 1:7 ratio, v/v). PRP (platelet-rich plasma, PRP) was obtained after centrifugation at 100 x *g* for 15 min at room temperature and diluted in modified Tyrode's-HEPES buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 5 mM glucose, and 1 mM MgCl₂, pH 7.3).

Bone marrow transplantation

6-week-old wild-type recipients were gamma-irradiated with a lethal dose of 1000 rad and injected intravenously with 5 × 10⁶ donor bone marrow cells. Experimental pulmonary metastasis assays were then performed 10 weeks post-transplantation, by which time the bone marrow was fully reconstituted.

Measurement of PLD activity

PLD activity was determined by measuring the accumulation of [³H] phosphatidylbutanol ([³H] Ptd-But) as described previously (27, 52). Washed platelets were labeled with [³H] palmitic acid (10 µCi/ml) at 37 °C for 1 hour and incubated with different concentration of FIPI or DMSO for 30 min. The platelets were pre-incubated with 0.4% 1-butanol for 3 min and then incubated with thrombin (0.5 U/ml) in the presence of CaCl₂. Reactions were

stopped by the addition of ice-cold chloroform/methanol (1:2) and incubation on ice for 20 min. The lipids were extracted by addition of ice-cold chloroform and water (1:1), collection of the organic phase, and separation by thin-layer chromatography. [³H] Ptd-But bands were identified through co-migration with standards and quantified by scintillation.

Tumor implantation

B16F10 mouse melanoma cells or mouse Lewis Lung Carcinoma cells (LLC) cells (1×10^6) were suspended in 100 μ l serum-free DMEM and subcutaneously injected into the dorsal flank of recipient mice. Tumors were harvested 10–12 days after injection, measured for volume, weighed and fixed with 4% paraformaldehyde (PFA) in PBS.

***In vivo* Matrigel plug assay**

Mice received subcutaneous injections of 500 μ l growth factor-reduced matrigel (BD Biosciences, Bedford, Massachusetts) mixed with 60 ng/ml VEGF and 100 U/ml heparin. On day 7, matrigel plugs were removed, fixed in 4% PFA and embedded in paraffin. Sections were stained with hematoxylin and eosin or immunostained using anti-CD31 antibodies. For quantification, matrigel plugs were harvested and digested by Drabkin reagent (Sigma, St. Louis, MO). Hemoglobin amounts were quantified according to the manufacturer's instructions.

Aortic ring assay

Thoracic aortas were surgically dissected and the mouse aortic ring assay performed as described previously (53). Aortic rings were placed between two layers of growth factor-reduced matrigel supplemented with 50 ng/ml VEGF and overlaid with MCDB-131 endothelial cell growth medium (Invitrogen, Grand Island, NY) containing 2.5% FBS. Aortic rings were cultured at 37°C for 7 days with changes of media every other day. Images were acquired using an inverted microscope and the number of microvessels sprouting from each aortic ring counted in a double-blind fashion.

Primary lung endothelial cell isolation

Lungs from 7-day-old mice were minced and digested with 1 mg/ml collagenase type I (Roche, Indianapolis, IN) in HBSS for 1 hour. After passing through a 70 μ m pore size cell strainer (BD Biosciences, Bedford, Massachusetts), endothelial cells were purified by immunosorting using CD31-conjugated magnetic beads and cultured in gelatin-coated flasks in DMEM+20%FCS+100U/100 mg/ml penicillin and streptomycin/2mM L-glutamine/heparin (100ug/ml)/endothelial cell growth stimulant ECGS (100ug/ml)/nonessential amino acid/sodium pyruvate, removing non-adherent cells after 24 hours. When the cells reached confluency, a second round of immunosorting using CD102-conjugated magnetic beads was performed and the cells cultured again as above. Primary endothelial cells at passages 2 to 4 were used for experiments after their purity was confirmed to be greater than 90% by anti-CD102 immunostaining and FACS analysis.

Endothelial tube formation assay

Growth factor-reduced matrigel was added (100 μ l) to 96-well plates and allowed to polymerize at 37°C for 1 hour. Primary lung endothelial cells (3×10^4) were seeded on the matrigel in DMEM containing 50 ng/ml VEGF and incubated at 37°C for 12 hours. Images of the cells were taken using an inverted microscope and the number of cords in 4 representative pictures counted.

Cell adhesion assay

96-well plates were coated overnight at 4°C with 1 µg/ml fibronectin, vitronectin, collagen, poly-L-lysine or 2% BSA and blocked with 2% BSA at 37°C for 1 hour. Primary lung endothelial cells (5×10^4) were then seeded and incubated at 37°C for 1 hour. Non-adherent cells were removed by gently washing with pre-warmed PBS and adherent cells fixed and stained with 0.1% crystal violet. After washing, the crystal violet dye retained in the wells was dissolved in 10% acetic acid and the absorbance of each well at 595 nm measured using a multiwell plate reader (Bio-rad, Hercules, CA).

Pharmacological inhibition of PLD using FIPI

For in culture experiments, 750 nM FIPI was added to the culture medium from a 7.5 mM stock in DMSO 30 minutes prior to the experiment. For the primary tumor experiments, mice received daily sets of i.p. injection of 1 mg/kg body weight FIPI (5-Fluoro-2-indolyl des-chlorohalopemide, Sigma) in 4% DMSO/96% saline in the morning and 3 mg/kg FIPI eight hours later. FIPI is 18% bioavailable (34); at 1 mg/kg (2.4 µM), an equally distributed effective T_0 concentration would be 426 nM. This should provide 11 hours of full inhibition, based on 100 nM FIPI being required for full inhibition (27) and an *in vivo* $T_{1/2}$ of 5.5 hours (34). A dose of 3 mg/kg should provide 20 hours of full inhibition. A dose of 1 mg/kg followed by a dose of 3 mg/kg 8 hours later should provide 22 hours of full inhibition subsequent to the second dose. A dose of 3 mg/kg followed by a dose of 3 mg/kg 12 hours later should provide 21 hours of full inhibition subsequent to the second dose.

Experimental pulmonary metastasis

10^5 B16F10 melanoma cells suspended in 100 µl PBS were injected into the tail veins of 6- to 8-week-old mice. To block the activity of $\alpha_{IIb}\beta_3$ integrin, mice were injected retro-orbitally with Fab fragments of the antibody JON/A (100 µg) (42) or vehicle control 2 hours before the B16F10 cell injection. Two weeks later, the mice were killed and the lungs fixed in Bouin's solution. The number of metastatic nodules present across the surface of the five lobes of the lung was counted under a dissecting microscope.

Adherence of Platelets to Tumor Cells

Washed platelets were fluorescently labeled using the Vybrant CFDA SE cell tracer kit and activated by PAR4 or thrombin. B16F10 cells were grown to 50% confluency in 6-well plates and washed twice with HBSS. 3×10^6 fluorescence-labeled platelets were incubated with B16F10 cells for 20 min at room temperature with shaking. Wells were washed with HBSS and fixed with 4% PFA for 15 min. Images were taken using fluorescence microscopy and the mean fluorescent intensity quantified using NIH ImageJ software. To block the activity of $\alpha_{IIb}\beta_3$ integrin, platelets were incubated with JON/A Fab fragments (5 µg/ml) for 10 min before adding them to the B16F10 cells.

Short-term metastasis experiments

2×10^6 B16F10 cells were fluorescently labeled using the Vybrant CFDA SE cell tracer kit (Invitrogen, Grand Island, NY) and injected into mice through the tail vein. Mice were killed 30 min, 2 or 5 hours after injection. To prevent collapse, lungs were inflated with Optimal Cutting Temperature (OCT) embedding medium through the trachea before harvest, embedded in Tissue Freezing Medium (Electron Microscopy Sciences), and frozen at -80°C. Cryosections were prepared and imaged using fluorescence microscopy. The mean fluorescence intensity of each section was quantified using NIH ImageJ software.

Statistical Analysis

The two-tailed independent Student *t*-test was used to compare the continuous variables between two groups. ANOVA with Bonferroni correction was used for comparison of multiple groups. All data were derived from independent experiments; cells and mice were not pooled across experiments. Statistical significance was set at 0.05 for all tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Tumor growth and angiogenesis are suppressed in *Pld1*^{-/-} mice

Mouse B16F10 melanoma and Lewis lung carcinoma cells were implanted s.c. in wild-type and *Pld1*^{-/-} mice and the resulting *in situ* tumors removed 10 days later for analysis. Representative B16F10 melanoma (A) and Lewis lung carcinoma (B) tumors recovered from the wild-type and *Pld1*^{-/-} mice are shown. Average weights and sizes of the tumors $\pm$ s.d. are presented in the bar graph (n=6 mice per group). C, B16F10 tumor sections stained by hematoxylin and eosin. Microvessels are clearly visible in tumors from wild-type mice (arrows). Inset, magnification of boxed region. D, B16F10 tumor sections stained using DAPI to indicate nuclei, and anti-CD31 to visualize endothelial vessels which were quantitated as presented in the bar graph. C, D: n = 4 fields of cells per group. *, $P < 0.05$; **, $P < 0.01$ by Student's *t*-test. Scale bars: 1 cm (A, B); 50 μ m (C, D). MVD, microvessel density.

Fig. 2. Neoangiogenesis is impaired in *Pld1*^{-/-} mice

A, Hemoglobin contents of control- and VEGF-containing matrigel plugs implanted in wild-type and *Pld1*^{-/-} mice for 10 days ($n = 4$ mice for each group, $\pm$ s.d.). Inset shows representative plugs recovered from the mice. B, Representative pictures of matrigel plug sections from wild-type and *Pld1*^{-/-} mice stained with H&E (top) and anti-CD31 antibody (bottom). C, Wild-type and *Pld1*^{-/-} mouse aortic rings were embedded in growth factor-containing matrigel and cultured at 37°C for 7 days. The number of microvessel sprouts from each aortic ring was counted ($n = 3$ mice for each genotype); representative images shown. D, Primary lung endothelial cells were seeded on matrigel and incubated with VEGF. Results are expressed as the mean number of cords $\pm$ s.d.; $n = 6$ independent sets of cells, representative images shown. *, $P < 0.05$; **, $P < 0.01$ by Student's t -test. Scale bars: 50 μm (B); 20 μm (C).

Fig. 3. Reduced integrin-dependent cell adhesion and impaired angiogenic signaling by *Pld1*^{-/-} endothelial cells

A, Primary lung endothelial cells were plated and allowed to adhere on vitronectin (Vn), fibronectin (Fn), collagen (Col), poly-L-lysine (PLL) or BSA. Adhesion of wild-type endothelial cells on the varied matrix proteins was assigned a value of 1 (n = 3 sets of cells for each group; error bars show s.d.); **, *P* < 0.01 by Student's *t*-test. B, Lysates from serum-starved endothelial cells were assessed using Western blotting for AKT, p-ERK1/2, p38, and β-actin. Representative experiment of five shown. C, Serum-starved endothelial cells were stimulated with VEGF for the indicated times and analyzed by Western blotting using antibodies against p-Akt (Ser473), p-ERK, p-p38 and β-actin. Representative experiment of three shown. Densitometric readings were obtained using ImageJ software and the signals normalized to the loading controls. Fold changes over unstimulated wild-type were calculated and averaged as shown in (D) ± s.d. VEGFR2 abundance was comparable in wild-type and *Pld1*^{-/-} endothelial cells (Fig. S6).

Fig. 4. FIPI blocks tumor growth and angiogenesis

A, Growth curves of vehicle control- and FIPI-treated B16F10 tumors. Tumor volumes were measured on alternate days starting on day 4, and the mice sacrificed on day 10 to determine tumor weights ($n = 6$ mice per group, $\pm$ s.d.). B, FIPI has no effect on tumor cell proliferation. B16F10 and MDA-MB-231 cells were incubated with increasing concentrations of FIPI for 3 days and the number of viable cells determined (full inhibition of PLD is achieved at 100 nM, (27)). Results of 3 experiments in triplicate are expressed as the mean inhibitory rate $\pm$ s.d. C, Microvessel density in control- and FIPI-treated B16F10 tumors was assessed by anti-CD31 immunostaining and quantitating 4 randomly chosen fields. **, $P < 0.01$ by Student's t -test. Scale bar: 50 μ m.

Fig. 5. PLD1 deficiency diminishes the metastatic potential of circulating tumor cells

A, Wild-type and *Pld1*^{-/-} mice (n=12 mice) were injected with B16F10 cells and the number of metastatic foci on the lung surface counted under a dissecting microscope ($\pm$ s.d., **, $P < 0.01$ by Student's *t*-test). Representative metastatic lungs are shown for comparison. B, Wild-type and *Pld1*^{-/-} mice were injected with fluorescently-labeled B16F10 cells and analyzed at successive time points after injection. Representative lung sections are shown; mean fluorescence intensities of random fields from the sections were quantified using NIH ImageJ software (3 images per mouse, 3 mice per group; $\pm$ s.d. shown; *, $P < 0.05$ by Student's *t*-test). Scale bars: 50 μ m (B); 15 μ m (inset in B).

Fig. 6. The interaction between tumor cells and platelets is impaired in *Pld1*^{-/-} mice

A, Fluorescently-labeled control and PAR4 peptide- or thrombin-activated wild-type and *Pld1*^{-/-} platelets were assayed for stable interaction with B16F10 tumor cells ($n = 4$ independent sets of platelets; $\pm$ s.d. shown). B, Lethally-irradiated wild-type mice that had received transplants of bone marrow from wild-type and *Pld1*^{-/-} mice were intravenously injected with B16F10 cells. Metastases were assayed as in Fig. 5 ($n = 3$ mice per group). C, Platelets were incubated with JON/A Fab fragments before the *in vitro* B16F10 tumor cell interaction assay was performed ($n = 4$ independent experiments). D, Wild-type and *Pld1*^{-/-} mice were injected with JON/A Fab fragments before injection of B16F10 cells. Lungs were removed 14 days after tumor cell injection and surface metastases quantitated ($n=12$ mice per group). **, $P < 0.01$; ***, $P < 0.001$ by Student's *t*-test (A, B) or ANOVA with Bonferroni correction (C, D); n.s., not significant.

Fig. 7. FIPI inhibits tumor metastasis

A, Inhibition of PLD activity in platelets by FIPI. Platelets isolated from wild-type mice were treated with different concentration of FIPI or DMSO. Activities are shown in comparison to basal activity (n = 3 independent experiments, $\pm$ s.d.). B, FIPI was injected i.p. into the mice before platelet isolation. The platelets were fluorescently labeled and assayed for interaction with B16F10 cells as above (n = 4 independent experiments). C, Wild-type mice injected with FIPI before i.v. injection of B16F10 tumor cells were analyzed for pulmonary metastases (n = 12 mice). *, $P < 0.05$; **, $P < 0.01$ by Student's *t*-test.