C₆-ceramide nanoliposome suppresses tumor metastasis by eliciting PI3K and PKCζ tumor-suppressive activities and regulating integrin affinity modulation

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Nanoliposomal formulation of C₆-ceramide, a proapoptotic sphingolipid metabolite, presents an effective way to treat malignant tumor. Here, we provide evidence that acute treatment (30 min) of melanoma and breast cancer cells with nanoliposomal C₆-ceramide (NaL-C₆) may suppress cell migration without inducing cell death. By employing a novel flow migration assay, we demonstrated that NaL-C₆ decreased tumor extravasation under shear conditions. Compared with ghost nanoliposome, NaL-C₆ triggered phosphorylation of PI3K and PKCζ and dephosphorylation of PKCα. Concomitantly, activated PKCζ translocated into cell membrane. siRNA knockdown or pharmacological inhibition of PKCζ or PI3K rescued NaL-C₆-mediated suppression of tumor migration. By inducing dephosphorylation of paxillin, PKCζ was responsible for NaL-C₆-mediated stress fiber depolymerization and focal adhesion disassembly in the metastatic tumor cells. PKCζ and PI3K regulated cell shear-resistant adhesion in a way that required integrin αvβ3 affinity modulation. In conclusion, we identified a novel role of acute nanoliposomal ceramide treatment in reducing integrin affinity and inhibiting melanoma metastasis by conferring PI3K and PKCζ tumor-suppressive activities.

Ceramide is a sphingolipid-derived second messenger in cell membrane in response to inflammation and stress¹. It is an integral part of cellular differentiation, proliferation and apoptosis pathways. Studies indicated that endogenous ceramide metabolisms were downregulated in several cancers. Unlike long-chain ceramides, short-chain ceramides could induce cell death, which is useful for therapeutic applications in cancer. Nanoliposomal formulation improved bioavailability and solubilization of hexanoyl-D-erythro-sphingosine(C₆-ceramide)². Exogenous delivery of C₆ resulted in accumulation of ceramide in structured membrane microdomains which contained caveolin-1-enriched lipid rafts³. High doses (>20 μM) of nanoliposomal C₆-ceramide (NaL- C₆) with long-term incubation (8 ~ 24 hr) inhibited in vitro and in vivo growth of breast cancer, pancreatic cancer, chronic lymphocytic leukemia, hepatocellular carcinoma and melanoma³⁴⁷. Of note, nanoliposome-formulated ceramide significantly decreased breast carcinoma, MDA-MB-231 cell proliferation as compared with nonliposomal ceramide². However, within circulation, tumor extravasation occurs very rapidly, especially in face of hydrodynamic force⁸⁹. It is unknown whether nanoliposomal C₆-ceramide play roles in inhibiting tumor migration and metastasis upon this short encounter with tumor cells in blood stream.

The protein kinase C (PKC) family consists of at least 11 members being classified into three groups: classical, novel, and atypical PKCs, depending on their requirement for regulation by calcium and diacylglycerol (DAG)¹⁰. PKCζ, highly expressed in breast cancer cells, belongs to the atypical group, and is independent of calcium and DAG for its activities. Upon activation, PKCζ can translocate from cytosol to cell membrane¹¹. PKCζ is directly or indirectly regulated by several lipids including ceramides and phosphatidylinositol 3,4,5-triphosphate (PIP3)¹². Studies suggested that breast carcinoma cell invasiveness and metastasis were dependent on PKCζ activation¹³. Adhesion to and extravasation through the endothelial lining of blood vessels are prerequisite for establishment of tumor metastasis. Under hydrodynamic conditions, tumor cells undergo multistep adhesive interactions with vascular endothelium. This involves sialylated molecule-mediated initial tethering and integrin-mediated firm adhesion of tumor cells⁹¹⁴¹⁵. Nevertheless, some tumor cell lines, like melanoma, does not express selectin-
ligand sialyl-Lewis^x at sufficient levels to mediate tethering and rolling of tumor cells^6. Therefore, they hijack polymorphonuclear neutrophils (PMNs) or fibrin to bridge them into close proximity to the endothelial cells, thereby facilitating their subsequent migration through endothelial cells^17–21. Like melanoma cells, metastatic breast cancer cell, MDA-MB-231 is negative for sialofucosylated selectin ligands and integrin β1 and β2 integrins, like lymphocyte function-associated antigen-1 (LFA-1), Mac-1 and very late antigen-4 (VLA-4). Therefore, they were deficient in binding to endothelial intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Integrin αvβ3 was found to be expressed on MDA-MB-231 by other groups^22,23 and ourselves. Integrin αvβ3 plays important roles in breast cancer metastasis^24,25. Integrin αvβ3 can form bonds with fibrinogen which served as connecting ligands facilitating melanoma and breast cancer adhesion to endothelium in flow^26,27,28.

To evaluate the effect of short-term nanoliposomal ceramide treatment on breast cancer and melanoma migration, we studied MDA-MB-231 and Lu1205 cell static and flow migration potencies in response to 30 min NaL-C6 treatment. NaL-C6 attenuated tumor migration in a dose-dependent manner. By using mutant constructs, pharmacological inhibitors and short interference RNA (siRNA) knockdown, we discovered that NaL-C6-mediated PKCζ and PI3K phosphorylation and PKCζ dephosphorylation were responsible for reduced cell migration. As the activation of PKC isoforms and PI3K were conventionally conceived of augmenting malignancy of tumors, we uncovered a novel role of PKCζ and PI3K as tumor suppressors. The strategies of activating PKCζ might potentiate the therapeutic effect of nanoliposomal ceramide to treat tumor metastasis.

**Results**

**Acute treatment with C6 nanoliposome suppressed tumor migration.** In previous studies, it was demonstrated that C6 nanoliposomes at high dosage range and long exposure duration mediated cancer apoptosis and growth arrest^1,2,7. But it remains elusive whether acute treatment of cancer cells with C6 nanoliposomes at low dosage range had any impacts on cell phenotypes. We measured MDA-MB-231 and Lu1205 apoptosis after being treated with a variety of doses of NaL-C6 for 30 min and 12 hr^29,30. Upon 30 min 20 μM NaL-C6 incubation, only 4% MDA-MB-231 and 1% Lu1205 cells underwent apoptosis (Fig. 1a). In contrast, 20 μM NaL-C6 with a long 12 hr of exposure resulted in 35% MDA-MB-231 and 20% Lu1205 apoptosis.

Next, we assessed 4-hr transwell migration of MDA-MB-231 and Lu1205 cells which received nonliposomal (NoL- C6) or liposomal C6 treatment for 30 min. Liposomal C6 were more effective to suppress MDA-MB-231 and Lu1205 cell migration than freely administrated C6 (Fig. 1b). This disparity of nonliposomal and liposomal C6 effects may be caused by the difference in the resultant accumulation of C6 within 30 min timescale^29,30. Collagen IV has been reported to function as chemotactant for melanoma migration and enhance breast cancer motility^29,31. In the absence of collagen IV, few MDA-MB-231 or Lu1205 cells migrated to the opposite side of the membrane (28 ± 5 RFU and 21 ± 3 RFU), while 100 mg/ml collagen IV in the bottom well strikingly augmented ghost nanoliposome-treated MDA-MB-231 or Lu1205 cell migration (379 ± 47 RFU and 278 ± 8 RFU) (Fig. 1c). NaL-C6 suppressed MDA-MB-231 cell migration in a dose-dependent manner. 5 ~ 20 μM NaL-C6 significantly attenuated MDA-MB-231 or Lu1205 transmigration as compared with ghost nanoliposome (p < 0.05).

Flow-regulated cancer migration plays important roles in tumor metastasis^32,33. To evaluate the effect of nanoliposomal ceramide treatment on cancer migration under hydrodynamic conditions, we utilized a flow migration device which consists of a modified 48-well Boyden chamber and a flow loop^34,35,36. To facilitate successful tumor extravasation in flow, a stable adhesion mediated by integrin is required. However, a screening of surface expressions of adhesive molecules revealed that neither MDA-MB-231 nor Lu1205 expressed integrins LFA-1 and Mac-1, the counter-receptors for endothelial ICAM-1 (Table 1)^37,38,39^40. These two cell lines express integrin αvβ3, which is a receptor for plasma protein, fibrinogen. Previous studies suggested that fibrinogen serving as a linker for cell-cell adhesion supported integrin αvβ3-dependent adhesion of melanoma cells to endothelium under flow conditions^35,40,41. Therefore, in the current flow migration settings, the transendothelial migratory properties of cancer cells were analyzed in the presence of fibrinogen at physiological concentration of 1.5 mg/ml. As expected, fibrinogen promoted the transmigration of substantial amounts of MDA-MB-231 cells at shear stresses of 2 and 4 dyn/cm^2 (512 ± 30 and 235 ± 39 cells/0.48 mm^2 filter) (Fig. 1d). At both shear stresses, NaL-C6 dramatically attenuated MDA-MD-231 and Lu1205 migration compared with ghost nanoliposome in a dose-dependent manner (p < 0.05) (Fig. 1d). At high shear stress (4 dyn/cm^2), 20 μM NaL-C6 for 30 min exposure resulted in 8.7 and 14.1 fold reduction of MDA-MB-231 and Lu1205 migration. The data indicated that acute treatment with NaL-C6 (30 min) suppressed shear-dependent tumor migration.

**Nanoliposomal C6 triggered phosphorylation of PKCζ and PI3K and translocation of PKCζ into cell membrane.** Ceramide is sphingolipid metabolite and accumulates in cell membrane lipid bilayer upon elevated sphingomyelinase activity or de novo synthesis^1,2,7,32,33. To assess the effect of acute NaL-C6 treatment on PKCζ isoform activation in MDA-MB-231 and Lu1205 cells, we determined the phosphorylation states of three PKC isoforms, PKCζ, PKCβ: and PI3K, which were previously shown to control cancer metastasis^1,2,7,33,34. Phosphorylation of PKCζ threonine 410 residue and PKCβ threonine 638 residue in activation loop domain and serine 729 in hydrophobic motifs of PKCζ contribute to activation as well as stability of enzymes^1,2,7,33. In MDA-MB-231 and Lu1205 cells, PKCζ and PKCβ were constitutively phosphorylated at residues Thr638 and Ser729, respectively, while PKCζ was marginally phosphorylated at Thr410 (Fig. 2a–c). NaL-C6 treatment reduced PKCζ phosphorylation but enhanced PKCζ phosphorylation with a maximum effect at 20 μM in both cell lines (Fig. 2a and c). In contrast, PKCβ phosphorylation level was not changed by NaL-C6 treatment (Fig. 2b).

Previous studies suggested that phosphorylated PKCζ isoforms translocate to cell membrane to regulate cell behaviors^32,35. Therefore, we evaluated the subcellular distribution of three PKC isoforms in response to nanoliposome treatment. Compared with ghost nanoliposome, NaL-C6 reduced the amounts of phosphorylated and total PKCζ in membrane fraction of tumor cells (Fig. 2d). Total PKCζ translocated into cytosolic fraction. In addition, phosphorylated membrane PKCζ was decreased with increasing doses of NaL-C6 treatment. The distributions of both phosphorylated and total PKCζ were not affected by NaL-C6 treatment (Fig. 2e). As a positive control, 12-O-tetradecanoylphorbol-13-acetate (TPA) induced a membrane translocation of PKCζ and PKCζ. On the other hand, acute NaL-C6 treatment resulted in translocation of total PKCζ from cytosol to cell membrane (Fig. 2f). Concomitantly, phosphorylation levels of PKCζ was increased in both cytosol and cell membrane. 20 μM NaL-C6 treatment resulted in a peak PKCζ phosphorylation in the cell membrane.

Since the phosphorylation of PKCζ is usually associated with PI3K activation, we next determined the phosphorylation states of PI3K in MDA-MB-231 and Lu1205 cells^32,35. 30 min NaL-C6 exposure elevated the phosphorylation levels of PI3K in a dose-dependent manner (Fig. 2g). A peaked level of PI3K phosphorylation was observed as a consequence of 20 μM NaL-C6 treatment. Thus, the activity of PI3K paralleled that of PKCζ in response to NaL-C6 treatment (Fig. 2a).
Figure 1 | C_{6}-ceramide nanoliposomes suppressed MDA-MB-231 and Lu1205 migration in a dose-dependent manner. (a) MDA-MB-231 or Lu1205 cells did not undergo apoptosis after receiving acute treatment (30 min) of 5 μM, 10 μM or 20 μM ceramide nanoliposome. 12-hr treatment with ceramide nanoliposomes induced tumor cell death. % of cells undergoing apoptosis was analyzed with Annexin-v/7-ADD staining and flow cytometry. Values were mean ± SEM. n = three replicates. **p < 0.01 compared with ghost control. (b-c) Liposomal C_{6} delivery augmented the anti-migration activity of C_{6}-ceramide. MDA-MB-231 or Lu1205 cells received 1 μM, 5 μM, 10 μM or 20 μM liposomal (NaL-C_{6}) or nonliposomal C_{6}-ceramide (NoL-C_{6}) (b) or ghost nanoliposome (c) for 30 min before being used for transwell migration assays. The amounts of migrated cells were determined by calcein AM staining after 4-hr onset of experiment and were expressed as RFU. 100 mg/ml collagen IV was used as chemoattractant in the bottom well. In no collagen IV group, DMEM + 0.1% BSA was added into chemoattractant wells instead. Results were expressed as mean ± SEM. n = three replicates. *p < 0.05, **p < 0.01 compared with control for each cell type.(d) NaL-C_{6} attenuated MDA-MB-231 or Lu1205 cell transendothelial migration in a dose-dependent manner under flow conditions. Tumor cells were incubated with 1 μM, 5 μM, 10 μM or 20 μM liposomal NaL-C_{6} or ghost nanoliposome for 30 min before being introduced into flow migration chamber together with 1.5 mg/ml fibrinogen. The flow migration assay was carried out for 4 hr at shear stress of 2 or 4 dyn/cm². The migrated cancer cells were stained and counted at the bottom of filter. Results were expressed as mean ± SEM. n = three replicates. *p < 0.05, **p < 0.01 compared with ghost at the same shear stress.
PKCε was essential for nanoliposomal Cε-regulated tumor migration. Since NaL-Cε induced PKCε phosphorylation and accumulation of phosphorylated PKCε in cell membrane, we hypothesized that PKCε might play a role in NaL-Cε-induced suppression of cancer migration. To verify this, we transfected MDA-MB-231 cells with full length PKCε (PKCε FL) construct and dominantly negative PKCε(FKPKCε DN) construct which is kinase-defective and contains a point mutation in its kinase domain to assess PKCε function in cell migration. A 95% transfection efficiency was achieved at the time of functional assays as measured by fluorescently staining HA (constructs were tagged with hemagglutinin (HA)) (Fig. 3a). In the presence of ghost nanoliposomes, PKCε FL and PKCε DN overexpression had no effect on cell static transwell migration (Fig. 3b). In sharp contrast, PKCε FL potentiated suppressive effect of 20 μM nanoliposomal ceramide on MDA-MB-231 transmigration, while PKCε DN rescued MDA-MB-231 transmigration suppressed by NaL-Cε (p < 0.05).

Motility is a required process for invasion of tumor cells through the surrounding stroma. To determine whether PKCε activation was required for regulation of cell motility, wound healing assays were conducted with mutant construct-transfected MDA-MB-231 cells. In consistent with transwell migration assays, PKCε FL or PKCε DN overexpression had no effect on wound healing capability of ghost nanoliposome-treated cells (Fig. 3c). 20 μM NaL-Cε treatment for 30 min increased the size of initial wounded area. This suggests that NaL-Cε treatment resulted in actomyosin-mediated contraction of cells. PKCε FL overexpression further reduced the wound healing capacities of nanoliposomal ceramide-treated cells (p < 0.01). Conversely, PKCε DN-transfected cells briskly migrated into the wound area, reaching 60% sealing at 12 hr, after wound scratch (Fig. 3c). Upon TPA stimulation, the wound healing rates of cells receiving vector, PKCε FL and PKCε DN constructs were comparable.

Next, we analyzed the dynamics of actin cytoskeleton and focal adhesion, which are required to maintain cell shapes and promote cell migration. Ghost-treated cells exhibited thick stress fibers which traversed the cell body (Fig. 4a). In addition, in vector, PKCε FL and PKCε DN-transfected cells, the appearance of actin cytoskeleton had no obvious difference. In contrast, in NaL-Cε-treated cells, filamentous actin assembled around cell periphery, with only a few thin stress fibers located within cell body. The actin morphology in cells transfected with PKCε FL was comparable to that in cells transfected with vector. However, PKCε DN overexpression restored the morphology of actin stress fibers displayed by ghost-treated cells. The stress fibers

| Table 1 | Flow cytometry analysis of adhesion molecule expression on MDA-MB-231 (1st row) and Lu1205 (2nd row) |
|-------------------|---------------------------------------------------|
| Control IgG (Control IgM) | ICAM-1 | VLA-4 | sialyl-Leα | sialyl-Leα | αβ3 | LFA-1 | Mac-1 | CD44H |
| 7.3 ± 0.5 (11.3 ± 1.5) | 105.1 ± 4.7* | 7.8 ± 2.3 | 157.5 ± 3.6* | 131.0 ± 2.8* | 77.5 ± 1.5* | 9.2 ± 2.3 | 8.5 ± 3.6 | 129 ± 5.1* |
| 3.9 ± 0.3 (9.5 ± 2.7) | 167 ± 12.8* | 3.7 ± 1.0 | 6.5 ± 2.5 | 5.3 ± 1.6 | 55 ± 1.2* | 3.2 ± 1.1 | 3.8 ± 1.5 | 178 ± 17.2* |

Values are geometric mean fluorescence intensities ± SEM of three experiments using different batches of cells each. The parentheses indicate that the antibody used to test the expressions of adhesives molecules is IgM antibody.

*p < 0.05 compared with respective control cases.

Figure 2 | Nanoliposomal Cε-ceramide induced phosphorylation of PKCε and PI3K as well as dephosphorylation of PKCa in a dose-dependent manner. (a–c) PKCε was phosphorylated in response to acute NaL-Cε-ceramide treatment. 1 μM, 5 μM, 10 μM or 20 μM NaL-Cε or ghost nanoliposomes were incubated with 1 × 10^6 MDA-MB-231 or Lu1205 cells for 30 min. Then, the cells were subject to western blotting analysis of (a) PKCa, (b)PKCε, and (b)PKCε phosphorylation levels. Total PKCa, PKCε, and PKCε were used as loading controls. Data represent three replicates. Densitometric analysis of phosphorylated PKCa isoforms with respect to total PKCa for each treatment was shown under blots. *p < 0.05, **p < 0.01 compared with control. (d–f) PKCε was translocated into MDA-MB-231 cell membrane in response to acute NaL-Cε treatment. After being incubated with various concentrations of NaL-Cε or ghost nanoliposomes, MDA-MB-231 cells were lysed and fractionated to cytosol and membrane components. Phosphorylated and total PKCa, PKCε, or PKCε in cytosol and membrane fractions were probed with western blotting. Data represent three replicates. (g) PI3K was phosphorylated in response to acute NaL-Cε treatment. MDA-MB-231 or Lu1205 cells were incubated with nanoliposomes in the same way as (a–c). Then, the cells were subject to western blotting analysis of phosphorylated and total PI3K. Data represent three replicates. Densitometric analysis of phosphorylated PI3K with respect to total PI3K for each treatment was shown under blots. *p < 0.05, **p < 0.01 compared with ghost control.
became more robust and organized. This implied that NaL-C6 regulate the dynamics of actin cytoskeleton via PKCf.

By staining paxillin, a focal adhesion marker, we showed that in ghost-treated cells, vector, PKCf FL and PKCf DN overexpression did not lead to the difference in focal adhesion distributions (Fig. 4a). They all displayed bright punctate focal adhesions which were colocalized with the end of thick stress fibers. When cells were treated with NaL-C6, focal adhesion stainings became dim. Small focal adhesions were visible at cell periphery and they almost disengaged with thin stress fibers. PKCf FL transfection did not change the appearance of focal adhesions. On the contrary, PKCf DN overexpression reverted the loss of focal adhesion complexes and thick stress fibers induced by NaL-C6 treatment. Focal adhesions moved from the cell periphery to cell body where they were associated with thick stress fibers. Quantitative analysis of the average focal adhesion size and number in a cell revealed that in NaL-C6-treated cells, PKCf FL decreased but PKCf DN increased the size and number of focal adhesions (Fig. 4b–c).

Figure 3 | NaL-C6-mediated inhibition of MDA-MB-231 migration was dependent on functional activity of PKCf. (a) Empty vector, PKCf FL, or PKCf DN were transfected into MDA-MB-231 cells. The transfection efficiency of these constructs was detected with anti-HA and anti-Alexa 350 staining. >95% cells received target genes. The migration of empty vector, PKCf FL, or PKCf DN transfected cells in response to NaL-C6 treatment was assessed by transwell migration assay (b) and wound healing assay (c). (b) For transwell migration assays, plasmid-transfected MDA-MB-231 cells were treated with ghost or 20 μM NaL-C6 for 30 min before being loaded into transwell inserts. Results were expressed as mean ± SEM. n = three replicates.*p < 0.05 compared with empty vector control. (c) PKCf DN rescued suppressive effect of NaL-C6 on cell wound healing capacity. For wound healing assays, confluent cell monolayers in 6 well plates were scratched and the area (in number of pixels) that cells migrated into the wound over a period of 12 hr was measured. Plasmid-transfected MDA-MB-231 cells were treated with ghost liposome, 20 μM NaL-C6, or 200 nM TPA for 30 min before assay. Results were expressed as mean ± SEM. n = three replicates.**p < 0.01,†p < 0.01 compared with NaL-C6 + vec.
Figure 4 | NaL-C₆ reduced stress fiber formation and inhibited focal adhesion disassembly through PKCζ activation. (a) Vector, PKCζ FL or PKCζ DN-transfected MDA-MB-231 cells were treated with ghost or 20 μM NaL-C₆ for 30 min. Cells were stained with rhodamine-phalloidin and paxillin antibody. The right panel shows magnified views of the boxed area in the merged images. Bar = 10 μm. (Green = paxillin, red = F-actin). To verify the presence of PKCζ in given individual cells, the cells were further subject to anti-HA and anti-Alexa 350 staining. The cells shown in the images were all overexpressing target constructs. (b–c) Quantification of the average number and size (μm²) of paxillin-containing focal adhesions in vector, PKCζ FL or PKCζ DN-transfected cells treated with 20 μM C₆-ceramide or ghost nanoliposomes for 30 min using ImageJ software. 12 cells were analyzed per condition in each experiment. Results were expressed as mean ± SEM. *p < 0.05 compared with vector control.
Disassembly of focal adhesion complexes is usually accompanied by the dephosphorylation of paxillin molecules. To investigate whether NaL-C6 treatment and PKCζ disturbance may regulate the phosphorylation states of paxillin, MDA-MB-231 cells were stained with anti-paxillin antibody and 4G10, an antibody against phosphotyrosine on proteins. In ghost-treated cells, 4G10 staining appeared as large dots and colocalized with paxillin in cell body, implying that paxillin were tyrosine phosphorylated (Fig. 5a). Upon NaL-C6 treatment, paxillin and 4G10 dots became smaller and localized at cell periphery (Fig. 5b). The line-scanned fluorescence intensity profiles of paxillin were not correlated with those of 4G10, suggesting that paxillin was dephosphorylated. While PKCζ FL transfection did not alter the phosphorylation states of paxillin, PKCζ DN overexpression resulted in the presence of larger 4G10-stained dots in cell body where they were colocalized with focal adhesions. The line-scanned fluorescence intensity profiles of paxillin were in phase with those of 4G10 (Fig. 5c–d). This data implied that NaL-C6 regulated focal adhesion disassembly and paxillin dephosphorylation in a PKCζ-dependent manner.

At a shear stress of 4 dyn/cm², NaL-C6 administration abrogated cell migration ability with only a small number of cells migrating through the human umbilical vascular endothelial cell (HUVEC) monolayer (Fig. 6a middle vs left). PKCζ DN eliminated the inhibitory effect of NaL-C6 on cell migration (Fig. 6a right vs middle). At shear stresses of 2 and 4 dyn/cm², PKCζ DN overexpression rescued NaL-C6-suppressed cell extravasation (p < 0.01). To further determine the role of PKCζ in regulating cell migration, we analyzed PKCζ phosphorylation levels in cells remaining in circulation and undergoing transmigration after 4-hr flow migration assays. As shown in Fig. 6c, nanoliposomal Ca²⁺-ceramide upregulated PKCζ phosphorylation levels in flowing cells in a dose-dependent manner. In contrast, PKCζ phosphorylation in migrated cells was unresponsive to up to 20 μM NaL-C6 treatment. These results implied that PKCζ participated in NaL-C6-regulated cell migration in flow.

**PI3K inhibition rescued tumor migration suppressed by acute Ca²⁺ nanoliposome treatment.** To determine the role of PI3K in NaL-C6-induced suppression of cancer migration, we assessed transwell migration of MDA-MB-231 cells which received PI3K inhibitors, wortmannin and LY294002. Wortmannin and LY294002 significantly attenuated the transmigration of NaL-C6-treated cells (Fig. 7a). Nevertheless, NaL-C6-treated cells receiving wortmannin or LY294002 migrated more vigorously than those receiving DMSO. Wortmannin and LY294002 increased the amounts of migrating cells by 29% and 64%, respectively. In addition, wortmannin and LY294002 promoted the sealing of the wound for NaL-C6-treated cells (Fig. 7b). Of note, wortmannin which is a more potent inhibitor for PI3K had a larger impact on cell motility than LY294002. Wortmannin exposure completely reverted the cell contraction phenotype induced by NaL-C6 treatment and promoted cell longitudinal migration. Wortmannin or LY294002 treatment significantly increased NaL-C6-suppressed MDA-MB-231 migration at shear stresses of 2 and 4 dyn/cm² (Fig. 7c). At a shear stress of 4 dyn/cm², PI3K phosphorylation levels were boosted by NaL-C6 treatment in flowing cells but not migrated cells (Fig. 7d). This suggested that PI3K was critical for ceramide nanoliposome-regulated cell migration.

PI3K-regulated PKCζ phosphorylation and PKCα dephosphorylation were crucial for NaL-C6-suppressed cancer migration. To further verify the role of PI3K and PKCζ in regulating cell migration, MDA-MB-231 and Lu1205 cells were transfected with PI3K or PKCζ siRNA. PI3K and PKCζ siRNA effectively knocked down target gene expressions (Fig. 8a). PI3K knockdown reduced the phosphorylation level of PKCζ and elevated phosphorylation level of PKCα in both NaL-C6-treated MDA-MB-231 and Lu1205 cells. This may suggest that PI3K was activated upstream of PKCζ and PKCα in response to acute NaL-C6 treatment. PI3K or PKCζ knockdown rescued MDA-MB-231 and Lu1205 transwell migration, wound healing and flow migration potencies (Fig. 8b–d). At 4 dyn/cm², PI3K and PKCζ knockdown increased MDA-MB-231 cell migration by 4.7- and 4.3-fold, respectively. PKCα is known to regulate myosine light phosphorylation and actin contraction, thereby promoting cell migration. Since PKCα phosphorylation was downregulated in response to NaL-C6 treatment, we hypothesized that PKCα deactivation was also required for suppression of cell migration. To test the hypothesis, we transfected cells with constitutively active PKCζ (PKCζ CAT). PKCζ CAT overexpression enhanced MDA-MB-231 and Lu1205 transwell migration and flow migration capacities (Fig. 8e–f).

**Integrin αβ3 affinity modulation was involved in PKCζ and PI3K-dependent suppression of migration by NaL-C6.** Cell adhesion to endothelium and development of shear-resistant bonds were critical for tumor extravasation in flow. To assess whether the suppression of the transmigration in flow by NaL-C6 was caused by disruption of cell firm adhesion, we employed cell detachment assay. To conduct this assay, fibrinogen was coated as a substrate in petri dish prior to parallel plate chamber assembly. MDA-MB-231 cells treated with NaL-C6 for 30 min were allowed to settle onto the coated fibrinogen before step-load shears were exerted. 20 μM NaL-C6 exposure reduced the number of bound cells with increasing shear rate (0 ~ 1600 sec⁻¹) (Fig. 9a). In contrast to PKCζDN and PKCζ CAT, PKCζ CAT transfection rescued the suppression of cell adhesion by NaL-C6 at each shear rate (Fig. 9a).

Since MDA-MB-231 and Lu1205 cells express integrin αβ3, which can form bonds with fibrinogen and promote tumor firm adhesion in flow, we next investigated the effect of integrin αβ3 siRNA knockdown on cell shear-resistant adhesion. Compared with scrambled siRNA control, integrin αβ3 siRNA knockdown significantly reduced cell adhesion (NaL-C6+ vec+ scr vs NaL-C6+ vec+ siR) (Fig. 9b). In the cells which were depleted of integrin αβ3 with siRNA, PKCζ DN transfection failed to restore cell adhesion ability suppressed by NaL-C6 (NaL-C6+ vec+ scr vs NaL-C6+ vec+ PKCζ DN+ siR). PI3K inhibition by wortmannin considerably increased the amounts of bound cells compared with DMSO (Fig. 9c). Integrin αβ3 knockdown abrogated the effect of wortmannin on cell adhesion.

Integrin affinity is regulated by divalent cations. Addition of Mn²⁺ or removal of Ca²⁺ results in increased ligand-binding affinity and adhesiveness of αβ3 integrin17,28. WOW-1 antibody which specifically recognizes αβ3 activation-epitope was used to probe integrin binding affinity modulated by ions28. In the presence of Mn²⁺, WOW-1 binding increased by 2.5-fold, while in the presence of Ca²⁺, WOW-1 binding decreased by 3-fold (Fig. 9d). Mn²⁺ restored MDA-MB-231 shear-resistant adhesion suppression by NaL-C6. On the other hand, Ca²⁺ addition significantly reduced the adhesion of PKCζ DN-transfected and NaL-C6-treated cells. These results implied that affinity modulation of integrin αβ3 was required for PI3K and PKCζ-dependent cell adhesion weakening mediated by acute NaL-C6 treatment.

**Discussion**

Cancer metastasis is highly coordinated, multistep process, involving tumor undergoing epithelial-mesenchymal transition, traveling in blood stream, lodging onto vascular endothelium and extravasation4. By using wound healing, transwell migration and flow migration assays, we revealed that short-term C6 nanoliposome treatment suppressed melanoma and breast cancer migration without inducing cell apoptosis. In addition, we found that NaL-C6 initiates a very distinct signaling pathway to suppress cancer extravasation under...
Figure 5 | C6-ceramide nanoliposome-induced focal adhesion disassembly was regulated by dephosphorylation of paxillin through PKCζ activation.

(a–d) MDA-MB-231 cells were transfected with vector(a–b), PKCζ FL(c), or PKCζ DN(d). Then, they were treated with ghost (a) or 20 μM ceramide nanoliposomes (b–d) before being co-stained with anti-paxillin antibody (green) and 4G10 (phospho-tyrosine specific antibody) (red). The profiles in the panel below show the fluorescence intensity patterns of focal adhesions from line scans in the merged images, which were analyzed by Image J. Bar = 5 μm. 12 cells were analyzed per condition in each experiment.
Figure 6 | NaL-C₆ suppressed tumor extravasation under flow conditions in a way that required PKCζ activation. (a) PKCζ DN partially restored the migratory potency of MDA-MB-231 cells which was suppressed by NaL-C₆ as shown by images of transmigrated cells at the bottom of the filter. After 4-hr flow migration experiments at a shear stress of 4 dyn/cm², chambers were disassembled and filters were stained. Images were taken from 10× objective. Ghost + vector: cells were transfected with empty vector and treated with ghost nanoliposomes; NaL-C₆ + vector: cells were transfected with empty vector and treated with C₆-ceramide nanoliposomes; NaL-C₆ + PKCζ DN: cells were transfected with PKCζ DN constructs and treated with C₆-ceramide nanoliposomes. (b) PKCζ DN rescued NaL-C₆-suppressed MDA-MB-231 migration at a shear stress of 2 or 4 dyn/cm². Vector or PKCζ DN-transfected MDA-MB-231 cells were treated with ghost or 20 μM C₆-ceramide nanoliposomes for 30 min. Then, cells were introduced into flow migration chamber together with 1.5 mg/ml fibrinogen. The number of cells migrated after 4 hr assay was measured. Results were expressed as mean ± SEM. n = three replicates. *p < 0.05 compared with NaL-C₆ + vector. (c) The lack of migratory potency of MDA-MB-231 cells was correlated with phosphorylation of PKCζ. MDA-MB-231 cells were treated with various doses of C₆-ceramide nanoliposomes or ghost nanoliposomes for 30 min prior to flow migration experiments. After flow migration at 4 dyn/cm² for 4 hr, flowing cells were collected from the circulation loop and migrated cells were trypsinized. Flowing and migrated cells were subjected to western blotting analysis of phospho-PKCζ and total PKCζ. Data represent three replicates.
both static and hydrodynamic conditions (Fig. 10). Pharmacological inhibition of PI3K, transient expression of dominantly negative PKCζ construct, and siRNA knockdown of PI3K or PKCζ, suppressed cell adhesion and migration. PI3K is activated by NaL-C6 to mediate phosphorylation of PKCζ and dephosphorylation of PKCα. PKCζ plays double roles in regulating cell migration. On one hand, it induces cytoskeletal architecture disruption, paxillin dephosphorylation and focal adhesion disassembly; on the other
Figure 8 | PI3K was activated upstream of PKCα and PKCα to initiate a signaling cascade to suppress MDA-MB-231 or Lu1205 migration. (a) siRNA targeting PI3K reduced PKCα phosphorylation and increased PKCα phosphorylation. MDA-MB-231 or Lu1205 cells were transfected with scrambled siRNA, PI3K siRNA or PKCα siRNA before being treated with 20 μM C6 nanoliposome for 30 min. Subsequently, the cells were subject to Western blotting analysis of phosphorylated and total PI3K, PKCα and PKCα levels. PI3K, PKCα and PKCα siRNAs exhibited >90% knockdown efficiencies. Data represent three replicates. (b–c) PI3K or PKCα knockdown rescued NaL-C6-suppressed MDA-MB-231 and Lu1205 static transwell migration (b) and wounding healing potentials (c). MDA-MB-231 or Lu1205 cells were transfected with scrambled siRNA, PI3K siRNA or PKCα siRNA before being treated with 20 μM C6 nanoliposome for 30 min. Results were expressed as mean ± SEM. n = three replicates. **p < 0.01 compared with scrambled. (d) PI3K or PKCα knockdown rescued NaL-C6-suppressed MDA-MB-231 and Lu1205 migration at a shear stress of 2 or 4 dyn/cm². Scrambled, PI3K or PKCα siRNA-transfected MDA-MB-231 or Lu1205 cells were treated with ghost or 20 μM C6-ceramide nanoliposomes for 30 min. Then, cells were introduced into flow migration chamber together with 1.5 mg/ml fibrinogen. The number of cells migrated after 4-hr assay was measured. Results were expressed as mean ± SEM. n = three replicates. **p < 0.01 compared with NaL-C6 vector. (e–f) PKCαCAT restored NaL-C6-suppressed MDA-MB-231 and Lu1205 static transwell migration (e) and flow migration (f). MDA-MB-231 or Lu1205 cells were transfected with vector or PKCαCAT. The transfection efficiency was probed by western blotting with anti-HA antibody. Then, cells were treated with 20 μM C6-ceramide nanoliposomes for 30 min before being assessed for transwell (e) and flow migration abilities (f). **p < 0.01 compared with NaL-C6+vector.
Figure 9 | Loss of integrin αvβ3-mediated shear-resistant adhesion was involved in suppressive effect of NaL-C6 on tumor migration via PKCf and PI3K. (a) PKCf DN but not PKCα DN and PKCε DN conferred NaL-C6-treated cells shear-resistant adhesive capacity. Fibrinogen was coated as substrate before the parallel plate flow chamber was assembled. Transfected MDA-MB-231 cells were settled onto fibrinogen for 7 min prior to initiation of the assays. Step-load shears (0, 50, 100, 200, 400, 800, and 1600 sec⁻¹) were applied to attached cells. The percentage of cells remaining bound to fibrinogen after each shear step was determined (expressed as % cells remaining bound). Results were expressed as mean ± SEM from three independent experiments. *p < 0.05 compared with NaL-C6 vector at each shear rate. (b–c) Silencing integrin αvβ3 by siRNA compromised the ability of PKCf DN (b) or PI3K inhibitors (c) to rescue cell adhesion. Scrambled or integrin αvβ3-targeting siRNA was introduced into MDA-MB-231 cells which were transfected with empty vector or PKCf DN constructs(b) or incubated with DMSO, 500 nM wortmannin or 10 μM LY294002 (c). These cells were treated with 20 μM C6 nanoliposome for 30 min before being injected into parallel plate chamber for cell detachment assays. The percentage of cells remaining attached to fibrinogen after each shear step was determined (expressed as % cells remaining bound). Results were expressed as mean ± SEM from three independent experiments. *p < 0.05 compared with NaL-C6 vector scramble at each shear rate. vec, vector; scr, scrambled siRNA; siR, integrin αvβ3 siRNA. (b) Modulation of integrin αvβ3 affinity was required for NaL-C6-mediated and PKCf-dependent cell adhesion weakening. The ability of MDA-MB-231 cells to bind to ligand-mimetic antibody Fab WOW-1 (10 μg/ml) in the presence or absence of 1 mM CaCl2 or 250 μM MnCl2 were analyzed by flow cytometry. The mean fluorescence intensity of staining was measured from three experiments. Results were expressed as mean ± SEM. **p < 0.01 compared with normal; ††p < 0.01 compared with calcium. Untransfected or PKCf DN-transfected MDA-MB-231 cells were incubated with 20 μM C6 nanoliposome for 30 min before being subject to cell detachment assay in the presence or absence of 1 mM CaCl2 or 250 μM MnCl2. *p < 0.05 compared with C6; †p < 0.05 compared with NaL-C6+PKCf DN.
hand, it reduces the affinity of integrin $\alpha_3\beta_3$, thereby weakening cell adhesion in flow. The inhibitory activity of PKC$_a$ and PI3K in NaL-C$_6$-treated cells distinguish them from pro-mitogenic and pro-migratory activities ascribed to conventional and novel PKC isoforms and PI3K/Akt axis.

In the current study, C$_6$-ceramide was delivered in a nanoliposomal form which presents as an effective way of reducing the hydrophobicity of the ceramide and increasing its membrane transport$^2$. Upon liposomal administration, ceramide is likely to be inserted into membrane lipid bilayer and localized to structured microdomain where it can be associated with signaling proteins and initiate intracellular signaling cascades$^3$. In the current study, we found that in contrast to nonliposomal ceramide, liposomal ceramide more effectively suppressed tumor migration. The roles of sphingolipid metabolites in regulating cell migration remain elusive. Long-chain ceramide C$_{16}$ was reported to enhance mouse embryonic stem cell migration in a dose- and time-dependent manner$^{29}$. On the other hand, sphingosine-1-phosphate which can be converted to ceramide via sphingosine-1-phosphate phosphatase and ceramide synthase inhibited chemotactic motility of breast cancer cells$^{40}$. Furthermore, C$_2$ ceramide was reported to suppress cancer invasiveness through downregulating MMP-2 expression$^{41}$. In the current study, the migration-suppressive effect of nanoliposomal C$_6$ was a consequence of activating of PKC$_a$ and PI3K. It was likely that short- and long-term ceramide treatments orchestrated different signaling pathways to mediate migratory inhibition and apoptosis. Long-term ceramide treatment initiated a pro-apoptotic pathway involving inactivation of Akt$^v$, while acute ceramide treatment recruit and activate PI3K. In the current study, we focused on the 30-min acute effect of C$_6$ nanoliposome. This is because upon adhesion to endothelium, tumor rapidly extravasates with a time period of less than 1 hr$^{49}$. The time duration for circulating tumor to interact with nanoliposome is short, especially in face of shear force. Thus, studying tumor responses to acute, rather than prolonged, NaL-C$_6$-treatment may be a more accurate reflection of physiological conditions.

In agreement with previous studies, the current study showed that NaL-C$_6$ treatment decreased membrane localization of phosphorylated PKC$\alpha$.$^5$. PKC$\alpha$ is known to regulate myosin light chain phosphorylation and actomyosin-mediated cell migration. PKC$\alpha$ activation was critical for focal adhesion formation and integrin-mediated cell migration$^{43}$. Dephosphorylation of PKC$\alpha$ by nanoliposomal ceramide may decrease myosin light chain phosphorylation, preventing cell migration. PKC$_a$ was shown to participate in cell adhesion and migration. By using inhibitors, PKC$_a$ was suggested to promote epidermal growth factor-mediated breast cancer chemotaxis$^{44}$. PKC$_a$ in neutrophils regulated chemotactant-induced actin assembly, integrin-dependent adhesion and cell migration$^{45}$. PKC$_a$ also participated in the migration of CD34+ progenitor cells$^{46}$. In the current study, PKC$_a$ suppressed tumor migration in response to NaL-C$_6$ treatment, implying that different stimuli may confer PKC different functionalities.

PI3K is a family of lipid kinases which transduce signals from various growth factors and cytokines by generating phospholipids$^{47}$. In response to stimulation, PI3K is recruited to the membrane by direct interaction of its p85 subunit with tyrosine phosphate motifs on receptors. The activated p110 catalytic subunit generates phosphatidylinositol-3,4,5-triphosphate, which serves as docking sites for several signaling proteins. PI3K was suggested to participate in integrin $\alpha_3\beta_3$-mediated melanoma migration, by inducing actin stress fiber formation and enhancing integrin $\alpha_3\beta_3$ avidity$^{48}$. Activation of PI3K by Cdc42 and Rac1 alters actin organization, leading to increased motility and invasiveness. In the current study, activation of PI3K was negatively correlated with cell migration, suggesting an inhibitory role of PI3K on the migration of nanoliposomal ceramide-treated cells.

Temporal and spatial regulation of cytoskeletal organization and focal adhesion formation plays an essential role in cell migration. It was evident that nanoliposomal ceramide administration had an impact on both cell motility and receptor-mediated adhesion. Cell motility is driven by actin-based protrusion at cell’s leading edge$^{49}$. Previous studies showed that mutations in paxillin phosphorylation sites reduced focal adhesion formation$^{50}$. It was also suggested that phosphorylation of paxillin promotes the association of unphosphorylated focal adhesion kinase with paxillin at newly growing focal contact sites, thereby promoting cell motility and migration$^{51,52}$. Paxillin association with stress fiber at adhesion sites may be critical for transmitting propulsive forces and serve as traction points over which cell moves$^{49}$. Therefore, cancer cell migration speed is a function of cell adhesion strength, as regulated by focal adhesion size and number. Highly polymerized cytoskeleton exerts tension on adhe-

Figure 10 | Proposed mechanism of C$_6$ nanoliposome-mediated suppression of tumor migration.
sion sites and allows retraction of cell, increasing the migration speed of cells. It is likely that ceramide suppresses cell motility by mediating PKCζ-dependent stress fiber disassembly and dephosphorylation of paclitaxel.

To undergo extravasation in blood vessel, cancer cells must tether and firmly adhere to endothelium. Different molecular constituents were involved in this process. Understanding the molecular mechanisms of the dynamic flow physically opposes cell attachment. Cell adhesion strengthening through α5β1 integrin activation enhances cell shear migration in flow. The affinity status of integrin α5β1 was regulated by “inside-out” signaling. Studies indicated that integrin α5β1 activation status regulated breast cancer metastasis and metastasis downstream of naoliposomal ceramide may facilitate the development of therapeutic strategies to prevent tumor metastasis.

**Methods**

**Cell culture and reagent.** DOPE, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), dioleylphosphatidylcholine (DOPC), D-erythro-hexanoyl-sphingosine (Cc-ceramide), 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000], and N-octanoyl-sphingosine-1-succinyl(methoxy(polyethylene glycol)-750) (PEG(750)-C6) were purchased from Avanti Polar Lipids (Alabaster, AL), 12-O-tetradecanoylphorbol-13-acetate (TPA), anti-PKCζ, anti-PP2, anti-PKCζ, anti-phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) and anti-PI3K antibodies were purchased from Cell Signaling Technology (Massachusetts, MA). Anti-HA, anti-paxillin, anti-phospho-PKCζ (Thr410) and anti-phospho-PKCζ (Ser729) were purchased from Santa Cruz (Dallas, TX). Mouse IgG anti-human α5β1 (anti-CDS1/61, clone 23Cr6), CD44, VLA-4 and ICAM-1 (clone BBGII-11) were purchased from R&D Systems (Minneapolis, MN). 4G10 (antiphosphotyrosine antibody) was obtained from Millipore (Billerica, MA). Rhodamine-phallodin, 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI), calcine-AM and mouse anti-human mAbs against Mac-1 (anti-CD11b) and LFA-1 (anti-CD11a) were purchased from Invitrogen (Carlsbad, CA). Mouse anti-human mAbs directed to siyl-Lea and siyl-Leb were purchased from Calbiochem (San Diego, CA). 24-well transwell devices with 0.8 mm pore size were purchased from BD Falcon (San Jose, CA, USA). To measure the binding of a ligand to the cell, antibodies Fab W20-1 (generously donated by Dr. Sanford Shattil from The Scripps Research Institute), were cultured for 30 min with 10 µg/ml Fab in 135 mM NaCl, 2.5 mM MgCl2, 3.3 mM NaH2PO4, 8.3 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, pH 7.4, with or without 250 µM MnCl2 or 1 mM CaCl2. Cells were washed and incubated with TRITC anti-mouse IgG and analyzed on FACSCalibur (BD, San Jose, CA, USA). To measure the binding of a ligand to the cell, antibodies Fab W20-1 (generously donated by Dr. Sanford Shattil from The Scripps Research Institute), were cultured for 30 min with 10 µg/ml Fab in 135 mM NaCl, 2.5 mM MgCl2, 3.3 mM NaH2PO4, 8.3 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, pH 7.4, with or without 250 µM MnCl2 or 1 mM CaCl2. Cells were washed and incubated with TRITC anti-mouse IgG and analyzed on FACSCalibur (BD, San Jose, CA, USA).

**Flow cytometry.** To examine adhesion molecule expression, cells were incubated with saturating concentrations of primary mAbs directed against specific adhesion molecules (ICAM-1, VLA-4, siyl-Lea, α5β1, LFA-1, Mac-1 and CD44) in DPBS containing 1% BSA for 20 min at 4°C and then washed twice. After an additional 30 min incubation with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse Fab fragment (1 µg/106 cells; Jackson Immuno Research, West Grove, PA) at 4°C, the cells were resuspended in DPBS and fixed with 1% paraformaldehyde for 1 hour and analyzed by a GUAVA flow cytometry (GUAVA technologies, Burlington, CA) and FACS Calibur (BD, San Jose, CA, USA). To measure binding of a ligand to the cell, antibodies Fab W20-1 (generously donated by Dr. Sanford Shattil from The Scripps Research Institute), were cultured for 30 min with 10 µg/ml Fab in 135 mM NaCl, 2.5 mM MgCl2, 3.3 mM NaH2PO4, 8.3 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, pH 7.4, with or without 250 µM MnCl2 or 1 mM CaCl2. Cells were washed and incubated with TRITC anti-mouse IgG and analyzed on FACSCalibur (BD, San Jose, CA, USA).

**Plasmid transfection.** MDA-MB-231 or Lu2105 cells were transiently transfectioned with empty pCDNA3 expression vector, PKCζ FL, PKCζ DN (generous gift from Dr. J. Moscat), constitutively active PKCζ (PKCζ CAT), dominantly negative PKCζ (PKCζDN), or dominantly negative PKCζ (PKCζ DN) constructs. 2.5 µg plasmids were transfected into cells with 7.5 µg Mirus TransIT Transfection reagent (TransIT-T1; Mirus Bio LLC, Madison, WI) for 6.5 h, ensuring efficient transfection and successful transfection efficiency. Flow cytometry was performed with a FACSCalibur (BD, San Jose, CA, USA), as previously described with a slight modification. Briefly, DSPC, DOPE, 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000], and N-octanoyl-sphingosine-1-succinyl(methoxy(polyethylene glycol)-750) (PEG(750)-C6) were purchased from Avanti Polar Lipids (Alabaster, AL), 12-O-tetradecanoylphorbol-13-acetate (TPA), anti-PKCζ, anti-phospho-PKCζ, anti-PP2, anti-phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) and anti-PI3K antibodies were purchased from Cell Signaling Technology (Massachusetts, MA). Anti-HA, anti-paxillin, anti-phospho-PKCζ (Thr410) and anti-phospho-PKCζ (Ser729) were purchased from Santa Cruz (Dallas, TX). Mouse IgG anti-human α5β1 (anti-CDS1/61, clone 23Cr6), CD44, VLA-4 and ICAM-1 (clone BBGII-11) were purchased from R&D Systems (Minneapolis, MN). 4G10 (antiphosphotyrosine antibody) was obtained from Millipore (Billerica, MA). Rhodamine-phallodin, 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI), calcine-AM and mouse anti-human mAbs against Mac-1 (anti-CD11b) and LFA-1 (anti-CD11a) were purchased from Invitrogen (Carlsbad, CA). Mouse anti-human mAbs directed to siyl-Lea and siyl-Leb were purchased from Calbiochem (San Diego, CA). 24-well transwell devices with 0.8 mm pore size were purchased from Corning (Corning, NY). TransIT-LT 2020 was purchased from Mirus (Mirus Bio LLC, Madison, WI). Super Signal West pico chemiluminescence reagent and ImmunoPure antibody goat anti-mouse IgG horseradish peroxidase was from Thermo Scientific (Rockford, IL). Wortmannin, LY294002, recombinant fibroblasts, TNFα, a bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO).

Lu2105 and MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM/F12, supplemented with 10% fetal bovine serum (FBS). HUVECs (American Type Culture Collection) were maintained in F12K media supplemented with 50 µg/ml heparin (Mallinkrodt Baker, Inc.), 30 µg/ml endothelial growth factor (Sigma-Aldrich, Shanghai, China) and 10% FBS. Cells in passage number of 5-10 were used for experiments. DMEM/F12, F-12K, non-essential amino acids, sodium pyruvate, penicillin/streptomycin and 1-glutamine were all purchased from Gibco (Gaithersburg, MD).
Western blotting. After functional assays, tumor cells were collected and rinsed with DPBS, and lysed with RIPA lysis buffer (20 mM Tris, 5 mM MgCl₂, 1 mM PMSF, 20 mg/ml aprotonin, 10 mg/ml leupeptin, 1 mM Na₃VO₄, and 20 mM β-mercaptoethanol). The lysates were centrifuged at 12,000 g for 10 min, and the supernatant was stored at −80°C. The protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit.

Transwell migration. Transmigration was assessed by the ability of cells to migrate through a porous (8 μm) polycarbonate membrane of a transwell device (Corning, NY) to a chemotactic cuvette (collagen IV). Cells were plated into the upper chamber of the transwell insert at a density of 10⁵ cells/well, and allowed to adhere for 2 hr. The migration chambers were then treated with 20 ng/ml of recombinant human stromal-derived factor-1α (R&D Systems) for 8 hr to allow adhesive molecule levels to reach maximum. The center 12 wells of each 24-well plate were filled with soluble chemoattractant type IV collagen (100 μg/ml). Tumor cells were plated in triplicate in 24-well plates in 0.5 ml volume and the percentage of specific apoptosis is calculated using the following formula: Apoptosis (%) = (%Annexin-V-/Annexin-V/APOC positive cells in assay well – % Annexin-V-/Annexin-V/APOC positive in the control well)X100/(100 – % Annexin-V-/Annexin-V/APOC positive in the control well).

Statistical analysis. All data were obtained from at least three independent experiments and expressed by means ± SEM. Statistical significance was determined using Student’s t-test or ANOVA. Tukey’s test was used in post hoc analysis for ANOVA. A probability value of p < 0.05 or p < 0.01 was considered to be statistically significant.
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