# RhoA mediates the expression of acidic extracellular pH-induced matrix metalloproteinase-9 mRNA through phospholipase D1 in mouse metastatic B16-BL6 melanoma cells

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**Abstract.** Solid tumors are characterized by acidic extracellular pH ($pH_e$). The present study examined the contribution of small GTP-binding proteins to phospholipase D (PLD) activation of acidic $pH_e$-induced matrix metalloproteinase-9 (MMP-9) production. Acidic $pH_e$-induced MMP-9 production was reduced by C3 exoenzyme, which inhibits the Rho family of GTPases; cytochalasin D, which inhibits actin reorganization; and simvastatin, which inhibits geranylgeranylation of Rho. Small interfering RNA (siRNA) against *RhoA*, but not against *Rac1* or *Cdc42*, significantly inhibited acidic $pH_e$ induction of MMP-9. Pull-down assays showed that acidic $pH_e$ increased the activated form of RhoA. Forced expression of constitutively active RhoA induced MMP-9 production, even at neutral $pH_e$. *RhoA* siRNA also reduced acidic $pH_e$ induced PLD activity. Specific inhibition of PLD1 and *Pld1* gene knockout significantly reduced acidic $pH_e$-induced MMP-9 expression. In contrast, PLD2 inhibition or knockout had no effect on MMP-9 expression. These findings suggested that RhoA-PLD1 signaling is involved in acidic $pH_e$ induction of MMP-9.

## Introduction

Tumor cells generate energy through aerobic glycolysis rather than through oxidative phosphorylation in mitochondria. Thus, tumor cells are characterized by acidic extracellular pH ($pH_e$), a phenomenon known as the Warburg effect, due to lactate production through aerobic glycolysis. $H^+$ generated during glycolysis must be secreted to maintain a neutral or weakly alkaline intracellular pH. Several mechanisms are involved in $H^+$ secretion, including transporters and/or exchangers such as monocarboxylate transporters (MCTs), which act as $H^+$-lactate co-transporters, and $Na^+/H^+$ exchangers (1). Acidity is also caused by $CO_2$ generated through the pentose phosphate pathway (2). We have reported that acidic $pH_e$ induces the expression of matrix metalloproteinase-9 (MMP-9), acting for type IV collagen break down (3,4). The ability of acidic $pH_e$ to induce tumor invasion and metastasis (5-10) suggests that acidic $pH_e$ is an important microenvironment supporting tumor malignancy.

The epithelial mesenchymal transition (EMT) is a critical process for tumor invasion and metastasis. EMT can be mediated by growth factors, including transforming growth factor (TGF)-β (11). Notably, we and others have reported that acidic $pH_e$ induces EMT in several cell models (10,12). Although actin cytoskeletal reorganization is involved in EMT, disruption of this pathway was reported associated with the downregulation of MMP-9 expression (13,14). Indeed, the intracellular signaling pathway that involves acidic $pH_e$ has not been completely elucidated (15-25).

Phospholipase D (PLD) is an esterase that hydrolyzes the phospholipids of plasma membranes of mammalian cells. PLD has two major isozymes, PLD1 and PLD2 (26), which localize at perinuclear regions and plasma membranes, respectively (27). Activators of these enzymes include phosphatidylinositol 4,5-bisphosphate (PIP2), small GTPases and ADP-ribosylation factors. Although melanocytes possess negligible levels of PLD1 and PLD2, melanomas express much higher levels of PLD1 (28). In contrast, PLD2 plays a role in the progression of colorectal cancer (29). Thus, the association between these isozymes and malignant phenotypes is likely cell type specific. We have reported that acidic $pH_e$ induces *Mmp-9* mRNA expression through intracellular pathways involving $Ca^{2+}$-triggered PLD, mitogen activated protein (MAP) kinase and nuclear factor-κB (NF-κB) (15,16) and acidic sphingomyelinase and NF-κB (16). However, the mechanism underlying acidic $pH_e$-induced PLD isozyme expression remains unclear. The present study was designed to assess the role of the RhoA-PLD1 axis in acidic $pH_e$-induced MMP-9 production.

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## Materials and methods

*Reagents*. Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, and High Capacity RNA-to-cDNA kits were purchased from Life Technologies (Grand Island, NY, USA). Simvastatin and C3 exoenzyme from Clostridium botulinum were from Merck Millipore (Darmstadt, Germany). Rhodamine-phalloidin and RhoA Pull-down Activation Assay Biochem kits were from Cytoskeleton, Inc. (Denver, CO, USA). Isogen total RNA extraction kits were purchased from Nippon Gene (Tokyo, Japan), and SYBR Premix Ex *Taq* II and Xfect Transfection reagent were from Takara Bio (Tokyo, Japan). VU0359595 and CAY10594 were from Cayman Chemical (Ann Arbor, MI, USA). Fetal bovine serum (FBS) was from HyClone Laboratories Inc. (South Logan, UT, USA). The blocking reagent N102 was from NOF Corp. (Tokyo, Japan). Immobilon-P PVDF membranes and chemiluminescence reagent were from Merck Millipore (Billerica, MA, USA). Avidin-conjugated horseradish peroxidase (HRP) was from Bio-Rad Laboratories (Hercules, CA, USA). Dual reporter assay kits were from Toyo Ink (Tokyo, Japan).

*Vectors and transfection*. Constitutively active RhoA vector (pRK5mycL63RhoA) and its control vector (pRK5myc) were the kind gifts of Dr Alan Hall, Memorial Sloan Kettering Cancer Center (New York, NY, USA). The luciferase reporter gene constructs driven by the 5'-flanking region of human MMP-9 (-670) were from Dr Douglas D. Boyd, MD Anderson Cancer Center (Houston, TX, USA). pSpCas9(BB)-2A-GFP (PX458) was obtained from Addgene (Cambridge, MA, USA), and the cytomegalovirus-driven *Renilla* luciferase reporter vector (pRL-CMV) was from Promega (Madison, WI, USA). Vectors were transfected with Xfect Transfection reagent according to the manufacturer's protocol.

*Cell and cell culture*. B16-BL6 cells were kindly provided by Dr Kaoru Miyazaki, Yokohama City University (Yokohama, Japan). The basal culture medium consisted of a 1:l mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12) supplemented with 4 mM phosphoric acid, 15 mM HEPES, 1.8 g/l $NaHCO_3$, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin sulfate, adjusted to the desired pH with HCl or NaOH. For serial culture, basal medium at pH 7.4 was supplemented with 10% fetal bovine serum (FBS). All cells were cultured at 37˚C in a 5% $CO_2$ incubator.

*Immunofluorescence microscopy*. Cells were incubated in basal medium (pH 7.4) containing 10% FBS for 1 day on glass coverslips in 6-well plates. The cells were fixed with 4.0% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, and permeabilized in PBS containing 0.2% Triton X-100. After washing with PBS, the cells were incubated with 20% N102 blocking reagent in PBS. For F-actin staining, the cells were incubated with rhodamine-phalloidin (1 U/ml) for 30 min at room temperature, followed by three washes with PBS.

*Zymography*. MMP-9 activity was assessed by zymography, essentially as previously described (3,4,10,15,16,30). Briefly, conditioned media were collected from the cultures and centrifuged to remove cellular debris. The supernatants were mixed with 2.5 volumes of acetone and centrifuged. The precipitated proteins were dissolved in non-reducing sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The samples were electrophoresed on 7.5% polyacrylamide gels containing 0.1% gelatin. The gels were washed with 2.5% Triton X-100 containing 50 mM Tris-HC1 (pH 7.5) and 0.1 M NaCl at room temperature for 2 h, and incubated in 50 mM Tris-HC1 (pH 7.5) containing 10 mM $CaCl_2$ at 37˚C for 20 h, and stained with Coomassie brilliant blue R-250.

*Pull-down assays*. Active RhoA was measured using RhoA Pull-down Activation Assay Biochem kits. Because the GTP-bound form of active RhoA specifically binds rhotekin peptide, consisting of the Rho binding region (amino acids 7-89), fused to glutathione S-transferase (GST-rhotekin), this complex was purified using glutathione affinity beads and active RhoA level analyzed by immunoblotting with anti-RhoA antibody as previously described (15). Briefly, the complexes were separated by 10% SDS-PAGE and transferred onto Immobilon-P PVDF membranes. The membranes were blocked with TBS-T (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) containing 20% blocking reagent N102, treated with anti-RhoA antibody, and incubated with biotin-conjugated secondary antibody and avidin-conjugated horseradish peroxidase. Signals were detected with enhanced chemiluminescence reagents. As a positive control, total RhoA level was analyzed in cell lysates.

*Mmp-9 promoter assay*. MMP-9 promoter activity was measured using luciferase reporter gene assays with an MMP-9 promoter construct, as previously described (15). Transfection efficiency was monitored by co-transfection with *Renilla* luciferase reporter vector (pRL-CMV), with reporter activity measured using a dual reporter assay kit, according to the manufacturer's protocol.

*Gene knockout of PLD isozymes*. Cells with knockout of either the PLD1 or PLD2 were generated using the CRISPR-Cas9 system (31). Single guide RNA (sgRNA) sequences targeting PLD1 and PLD2 were 5'-gtggaatatgacgcatctccagg-3' and 5'-ttg aggtccaggtcggaaaaagg-3', respectively. Vectors were transfected with Xfect Transfection reagent. Clones showing lack of target gene expression were selected by the colony formation method.

*PLD activity*. PLD activity was indirectly measured using PLD assay kits (BioVision, Milpitas, CA, USA), according to the manufacturer's protocol. Because PLD cleaves phosphatidylcholine to choline and phophatide, choline was oxidized with choline oxidase, yielding $H_2O_2$, which was measured using a $H_2O_2$-sensitive probe at 570 nm.

*Reverse transcription quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was purified with Isogen and reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription kit. Sequences corresponding to *Pld1*, *Pld2*, *MMP-9* and *Actb* (β-actin) mRNAs were amplified using specific primers and SYBR Premix Ex *Taq* II in a Thermal Cycler Dice real-time system (TP-870; Takara Bio). The

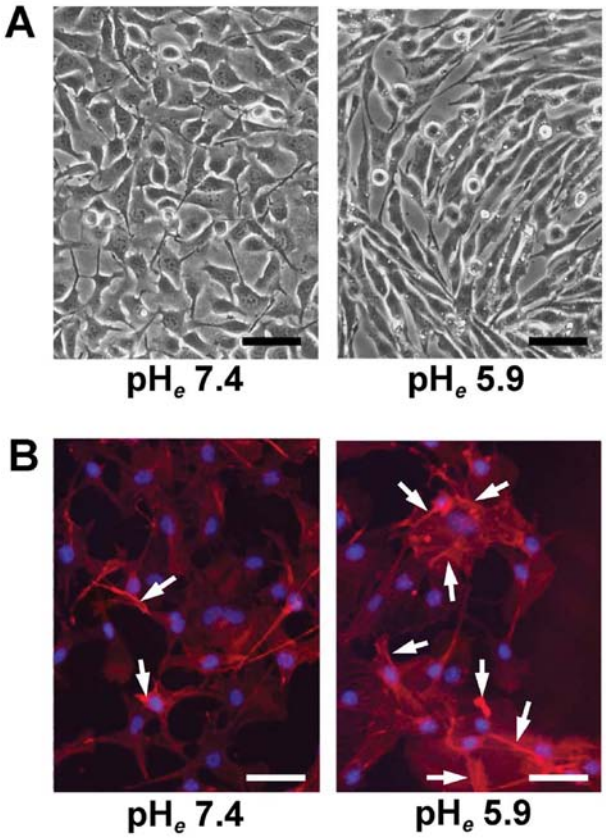


Figure 1. Morphological changes in BL6 cells in acidic medium. Serum-starved cells were cultured in physiological pH and acidic medium at 37˚C. (A) Phase contrast microscopy of cells incubated for 48 h in acidic medium. (B) Immunofluorescence assay of cells incubated in acidic medium for 24 h, fixed with 4.0% paraformaldehyde and stained for F-actin with rhodamine-phalloidin (red). Most acidic pH$_e$-induced actin stress fibers were associated with the plasma membrane (white arrows). Scale bar, 50 $\mu$m.

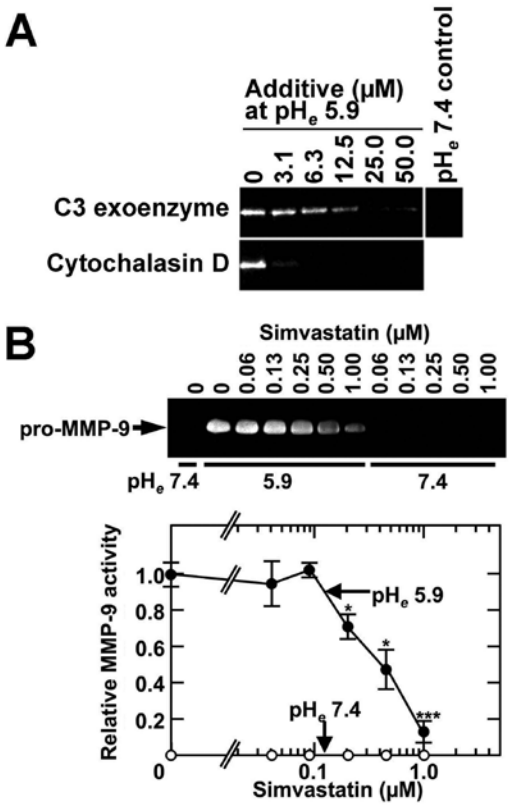


Figure 2. Effects of C3 exoenzyme (A), cytochalasin D (A), and simvastatin (B) on acidic pH$_e$-induced MMP-9 expression. Serum-starved cells were pre-incubated with C3 exoenzyme, simvastatin or cytochalasin D at indicated concentration for 1 h and incubated in acidic medium in the presence of the respective inhibitors for 24 h. MMP-9 activity in the culture medium was analyzed by zymography. Data are expressed as means ± SE (n=3). $^{*}$P<0.05; $^{***}$P<0.001.

specific primer sequences were 5'-acacaccggtgtgcggatct-3' (upstream) and 5'-cgtggtctgggtgtctcatc-3' (downstream) for *Pld1* (134 bp); 5'-catcagcatgacagctatgcc-3' (upstream) and 5'-ttcttccgccttcctcttga-3' (downstream) for *Pld2* (219 bp); 5'-gccctggaactcacacgaca-3' (upstream) and 5'-ttggaaactcacacgccagaa-3' (downstream) for *Mmp-9* (85 bp); and 5'-catccgtaaagacctctatgccaac-3' (upstream) and 5'-atggagccaccgatccaca-3' (downstream) for *β-actin* (85 bp). The levels of expression of *Pld1*, *Pld2* and *Mmp-9* mRNAs were normalized to those of β-actin mRNA in the same samples.

*Statistical analysis*. Statistical significance was determined by the Student's t-test. P-values <0.05 were considered to indicate a statistically significant result.

## Results

*Reduction of Rho inhibits acidic pH$_e$-induced MMP-9 production*. We have reported that acidic pH$_e$-induced morphological changes in B16-F10 melanoma (3) and Lewis lung carcinoma (10) cell lines, with these cells showing a fibroblastic morphology. Similarly, an acidic pH$_e$-induced morphological changes, independently of cell density, in B16-BL6 cells (Fig. 1A), and enhanced the accumulation of F-actin aggregates, most of which were associated with the plasma membrane (Fig. 1B). Because small G-proteins such as RhoA play important roles in actin reorganization (32), we tested whether cytochalasin D and C3 exoenzyme, which specifically inhibit actin polymerization and Rho, respectively, reduced acidic pH$_e$-induced MMP-9 production, finding that both of these agents inhibited MMP-9 induction (Fig. 2A). Because geranylgeranylation is essential for Rho activation and statins can inactivate Rho by reducing geranylgeranylation (32), we tested the effects of simvastatin on these cultures, finding that simvastatin inhibited acidic pH$_e$-induced MMP-9 production in a dose-dependent manner (Fig. 2B). To assess the contribution of RhoA to the acidic pH$_e$ signaling pathway responsible for the induction of MMP-9, we treated cells with *RhoA* siRNA. We found that *RhoA* siRNA significantly inhibited MMP-9 induction, whereas *Rac1* siRNA and *Cdc42* siRNA did not (Fig. 3A). Pull-down assays showed that acidic pH$_e$ increased the level of active RhoA (Fig. 3B). Furthermore, transduction of a constitutively active RhoA expression vector into B16-BL6 cells resulted in the induction of MMP-9, even at physiological pH$_e$ (Fig. 3C). Taken together, these findings suggested that RhoA is a molecule that transduces acidic signaling to induce expression of MMP-9.

*PLD1 is a downstream effector of acidic pH$_e$ signaling in MMP-9 induction*. We previously reported that PLD is important for transducing acidic pH$_e$ signaling (15,16). Moreover,

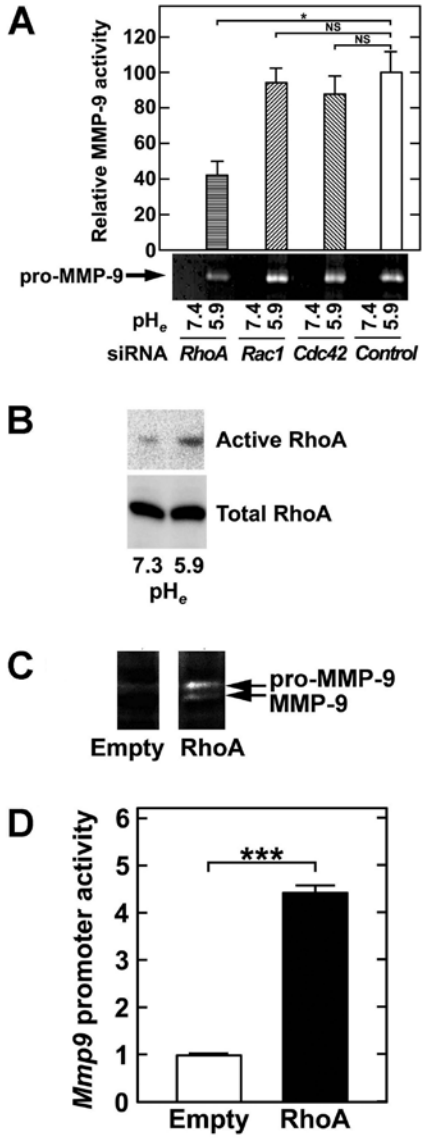


Figure 3. RhoA is a transducer of acidic $pH_e$ signaling that induces MMP-9 production. (A) *RhoA* siRNA but not *Rac1* or *Cdc42* siRNA inhibited acidic $pH_e$-induced MMP-9 expression. The siRNAs were transfected into cells under serum-free conditions. After 24 h, the cells were stimulated by acidic medium for another 24 h, and MMP-9 secretion into the culture medium was analyzed by zymography. The intensity of gelatinolytic bands was measured and plotted relative to control at $pH_e$ 5.9. (B) Assessment of acidic $pH_e$ activated RhoA by pull-down assays using GST-Rhotekin. Introduction of a constitutively active RhoA expression vector (pRK5mycL63RhoA) into the cells induced MMP-9 activity that was detected by zymography. The control consisted of the empty vector pRK5myc (C), with promoter activity determined by dual promoter assays (D). Data were expressed as mean ± SE (n=3). $^{*}$P<0.05; $^{***}$P<0.001; NS, not significant.

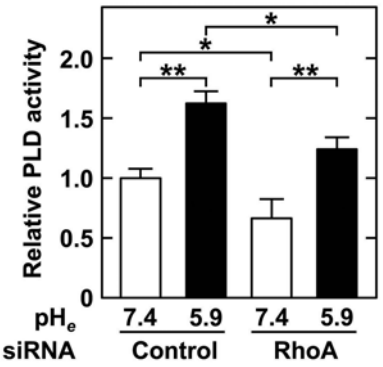


Figure 4. *RhoA* siRNA reduces PLD activity. Cells transfected with *RhoA* siRNA were cultured in neutral and acidic medium for 24 h. The cells were lysed and PLD activity in the supernatants determined. Data were expressed as mean ± SE (n=3). $^{*}$P<0.05; $^{**}$P<0.01.

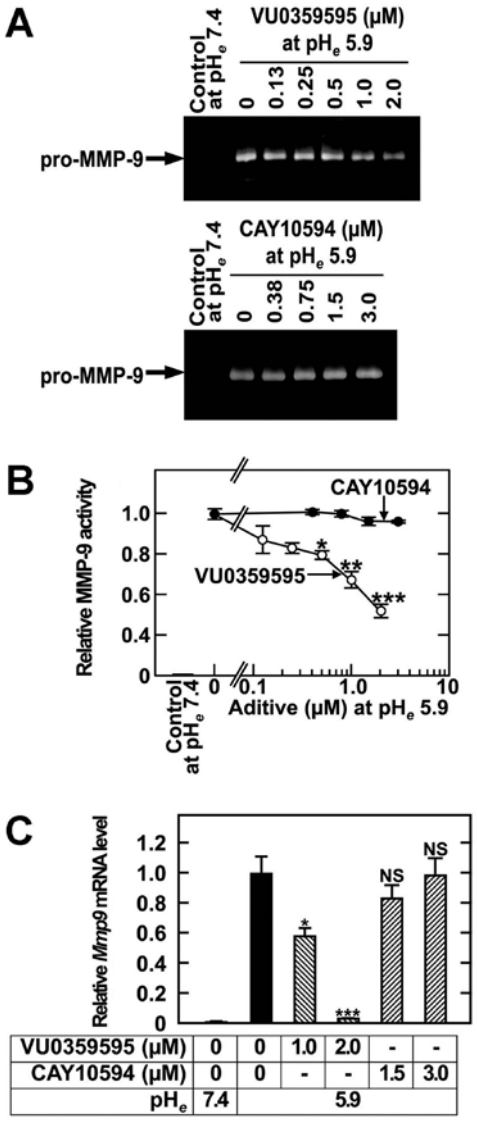


Figure 5. Attenuation of acidic $pH_e$-induced MMP-9 production by a specific inhibitor of PLD1, but not of PLD2. Serum-starved cells were pretreated with either the PLD1 inhibitor VU0359595 or the PLD2 inhibitor CAY10594 at neutral pH for 1 h then stimulated by acidic medium containing the respective inhibitor for 24 h. (A) Zymography; (B) intensity of the gelatinolytic bands in panel A. Control values ($pH_e$ 7.4) are hidden by the basal line due to the small value; (C) RT-qPCR. Data are expressed as mean ± SE. $^{*}$P<0.05; $^{**}$P<0.01; $^{***}$P<0.001; NS, not significant.

RhoA has been found to activate PLD (26). Therefore, we tested the effects of *RhoA* siRNA on PLD activity. *RhoA* siRNA reduced not only PLD activity at $pH_e$ 5.9 but also the basal PLD activity at $pH_e$ 7.4 (Fig. 4). Because PLD has two isozymes, PLD1 and PLD2 (26), we determined which one is involved in the acidic $pH_e$ induction of MMP-9. We found that the specific PLD1 inhibitor VU0359595 significantly reduced acidic $pH_e$-induced MMP-9 production, whereas the specific PLD2 inhibitor CAY10594 did not (Fig. 5A and B). This was confirmed by RT-qPCR assessments of the level of

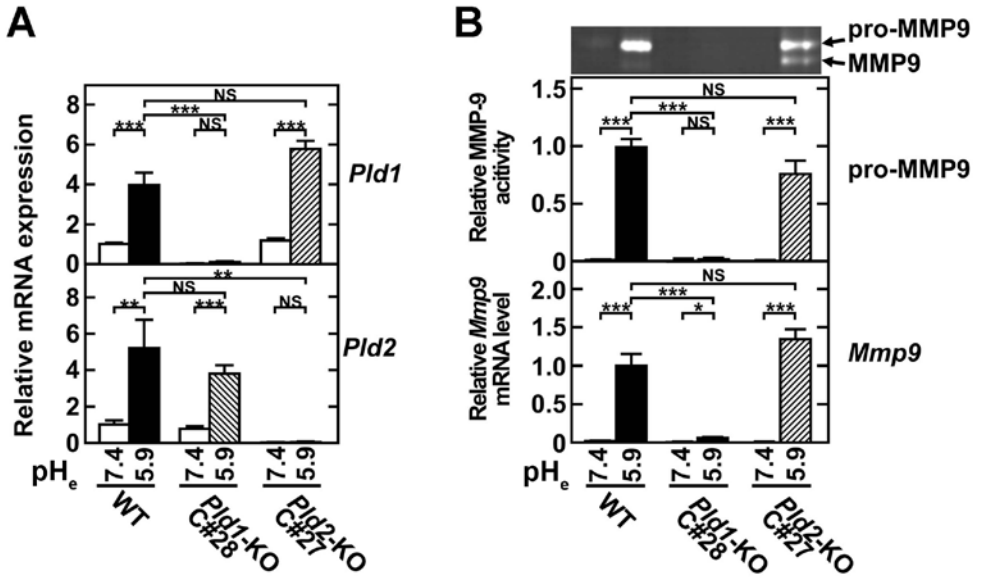


Figure 6. *Pld1* gene knock-out cells do not respond to acidic $pH_e$. The CRISPR/Cas9 system was used to knock out the *Pld1* or *Pld2* gene. Cell clones were isolated by colony formation. (A) Confirmation of *Pld1* and *Pld2* mRNA levels in each clone by RT-qPCR. *Pld1* knockout clone #28 (*Pld1*-KO C#28); *Pld2* knockout clone #28 (*Pld2*-KO C#27). These clones and their parental BL6 cells with wild-type (WT) *Pld* genes were serum-starved and treated with acidic medium. (B) Zymography (upper panel), intensity of gelatinolytic activity of zymograms (middle panel), and RT-qPCR for *Mmp-9* (bottom panel). Data are expressed as mean ± SE. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; NS, not significant.

*Mmp-9* mRNA expression (Fig. 5C). Furthermore, we used the CRISPR-Cas9 system to knockout either PLD isozyme (Fig. 6A), finding that PLD1 KO completely inhibited acidic $pH_e$-induced MMP-9 production whereas PLD2 KO had no effect on its expression (Fig. 6B). This result was also confirmed by RT-qPCR for *Mmp-9* mRNA (Fig. 6B). Acidic $pH_e$ also enhanced the levels of both PLD1 and PLD2 mRNAs (Fig. 6C). Taken together, these findings suggest that RhoA-PLD1 plays an important role in acidic $pH_e$ induction of MMP-9.

## Discussion

The present study expands on our previous findings, showing that acidic $pH_e$ activates PLD leading to MMP-9 induction. The present study initially focused on the mechanism of PLD activation. PLD activity has been closely associated with actin reorganization, as small GTPases such as the Rho family and Arf synergistically activate PLD (26). RhoA is a more potent PLD activator than other members of the Rho family such as Rac1 and Cdc42, with the latter two shown to rescue PLD activity when RhoA was inactivated (33). This study showed that RhoA knockdown by a specific siRNA reduced acidic $pH_e$-induced MMP-9 expression, whereas Rac1 and Cdc42 siRNAs had no effect. These findings indicate that Rac1 and Cdc42 are not involved in acidic $pH_e$ signaling and that they cannot rescue RhoA activity in the PLD-MMP-9 axis. These findings are in good agreement with results showing the importance of RhoA activation in MMP-9 expression (34,35). Our and other research groups have investigated the role of acidic $pH_e$ in the induction of EMT (10,12), showing that RhoA activation is important for actin reorganization in EMT. Cytochalasin D was found to enhance mesenchymal epithelial transition along with E-cadherin by reducing RhoA activity and reduced expression of vimentin and N-cadherin (13). Sustained Ras activity was shown to reduce Rac, which can inhibit Rho function, whereas induction of Rho can result in EMT in transformed cells. Cytoskeletal rearrangement induced by acidic $pH_e$ is therefore important for EMT through the RhoA-PLD axis.

TPA is an analog of diacylglycerol that activates protein kinase C (PKC) isoforms such as conventional PKC ($DAG^-$ and $Ca^{2+}$-dependent; α, βI, βII and γ) and novel PKC (DAG-dependent, but $Ca^{2+}$-independent; δ, ε, η and θ), contributing to the activation of AP-1 and PLD (36). We have shown that TPA did not induce MMP-9 expression in B16-BL6 cells at neutral $pH_e$ but enhanced MMP-9 expression at acidic $pH_e$, at least in part through NF-κB, but not AP-1 activation (15,16). However, bisindolylmaleimide III, a potent PKCa inhibitor, attenuated MMP-9 expression at low $pH_e$ (data not shown).

PLD has two major isozymes, PLD1 and PLD2 (26). To determine which of these isozymes contributed to acidic $pH_e$ signaling, we used isozyme specific inhibitors and gene knockout technique for each PLD. These experiments showed that PLD1 was responsible for the induction of *Mmp-9* mRNA expression at acidic $pH_e$. Human melanoma cells predominantly express PLD1 (28). Although PLD1 activity is inducible, PLD2 activity is constitutive and shows a reduced response to stimulation by small GTPases (27). TPA was found to enhance *Pld1*, but not *Pld2*, promoter activity, resulting in MMP-9 secretion through PKCβII-Ras-MAP kinase-NF-κB signaling (37). Our results also showed that PLD1, but not PLD2, was associated with acidic $pH_e$ induced MMP-9 production. We also found that increased PLD activity may be due to increases in both *Pld1* gene expression and RhoA-induced PLD1 activation, as well as to $Ca^{2+}$ influx (16), suggesting the existence of an intracellular signaling loop for gain of function of PLD1. Acidic $pH_e$ increased the expression of *Pld2* mRNA in PLD1-KO cells, but these cells did not respond to acidic medium. This finding suggests that PLD2 cannot compensate for PLD1 in the acidic $pH_e$ signaling.

We have reported that PLD activation leads to MMP-9 expression through NF-κB (15). Deletion of the NF-κB-binding-site from the *Mmp-9* promoter construct attenuated acidic $pH_e$-induced *Mmp-9* promoter activity when compared with wild-type (WT) promoter (15). In contrast, the promoter activity ratio between neutral and acidic $pH_e$ was similar in the mutant and WT constructs, suggesting that PLD-NF-κB signaling is essential for *Mmp-9* expression. In agreement, this study showed that RhoA siRNA reduced PLD activities but had no effect on the activity ratio of neutral and acidic $pH_e$. Taken together, these findings indicate that PLD1 activity is essential in the induction of *Mmp-9* mRNA, with additional signaling, such as acidic sphingomyelinase, required for further induction of *Mmp-9* gene expression by acidic $pH_e$ (16).

Generally, tumor cells become hypoxic ~100 $\mu$m from blood vessels. Because the expression of metabolic enzymes in glycolysis, such as glyceraldehyde dehydrogenase (GAPDH) and enolase, is dependent on hypoxia-inducible factor (HIF) (38), increased tumor acidity may occur in hypoxic areas, although hypoxia may not be essential for tumor acidity (2). Increases in glucose products, such as pyruvate and lactate, can result in the accumulation of HIF-induced proteins under aerobic conditions (39), indicating that glycolysis can associate the signaling of both hypoxia and acidic $pH_e$. Hypoxia has been shown to induce the EMT in gastric cancer cells through TGFβ signaling in an autocrine manner (40). TGF acts synergistically with other growth factors, including epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (41,42). Further analysis is required to determine relationships between growth factors and acidic $pH_e$ signaling

In conclusion, the present study investigated whether acidic $pH_e$, a hallmark of malignant tumors, contributes to the metastatic phenotype through the RhoA-PLD1 axis. Inhibition of isoprenylation by statins such as simvastatin is useful for preventing metastasis driven by acidic $pH_e$.

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