

# Phospholipase C $\delta$ 1 induces E-cadherin expression and suppresses malignancy in colorectal cancer cells

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Edited by Sue Goo Rhee, Yonsei University College of Medicine, Seoul, Republic of Korea, and accepted by the Editorial Board August 7, 2014 (received for review March 24, 2014)

Colorectal cancer (CRC) is one of the most common causes of cancer-related deaths worldwide, and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations in CRC predict the ineffectiveness of EGF receptor-targeted therapy. Previous transcriptional microarray analysis suggests the association between phospholipase C $\delta$ 1 (PLC $\delta$ 1) expression and *KRAS* mutation status in CRC. However, both the roles and the regulatory mechanisms of PLC $\delta$ 1 in CRC are not known. Here, we found that the expression of PLC $\delta$ 1, one of the most basal PLCs, is down-regulated in CRC specimens compared with normal colon epithelium by immunohistochemistry. Furthermore, we examined the roles of PLC $\delta$ 1 in CRC cell lines that harbor an activating *KRAS* mutation. Ectopic expression of PLC $\delta$ 1 in CRC cells induced the expression of E-cadherin, whereas knockdown of PLC $\delta$ 1 repressed the expression of E-cadherin. Moreover, the overexpression of PLC $\delta$ 1 suppressed the expression of several mesenchymal genes and reduced cell motility, invasiveness, and *in vivo* tumorigenicity of SW620 CRC cells. We also showed that PLC $\delta$ 1 expression is repressed by the *KRAS*/mitogen-activated protein kinase (MEK) pathway. Furthermore, PLC $\delta$ 1 suppressed the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 through E-cadherin induction in CRC cells, suggesting the presence of a negative regulatory loop between *KRAS*/MEK/ERK signaling and PLC $\delta$ 1. These data indicate that PLC $\delta$ 1 has tumor-suppressive functions in CRC through E-cadherin induction and *KRAS*/MEK/ERK signal attenuation.

phospholipase C delta 1 | epithelial-to-mesenchymal transition | tumor suppressor

Colorectal cancer (CRC) is one of the most common cancers and causes of cancer-related deaths worldwide. Although CRC patients with unresectable tumors and metastasis have been treated with chemotherapy, recent advances in molecular research about CRC have resulted in the development of molecular targeted therapies, such as cetuximab and panitumumab. Chemotherapy combined with these targeted therapies improves the prognosis of CRC patients with unresectable tumors to some extent (1, 2). However, some CRC patients with activating Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations are unable to benefit from such drugs, because both cetuximab and panitumumab are epidermal growth factor (EGF) receptor-targeting agents and mutant *KRAS* constitutively activates the downstream signaling of EGF receptor (3). A more vigorous study using *KRAS*-mutant CRC is needed to identify novel molecular targets for drugs that will improve the prognosis of CRC patients with unresectable tumors, especially those with *KRAS* mutations.

Mutations in *KRAS* are found in about 40% of CRC patients. Constitutively active *KRAS* mutations lead to the hyperactivation of mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) [mitogen-activated protein kinase (MAPK)] signaling and/or phosphatidylinositol-3 kinase (PI3K) pathways (4). Activation of MEK/ERK (MAPK) signaling results in increased phosphorylation of ERK1/2, which in turn, phosphorylates several proteins related to cell cycle progression and cell motility. The PI3K pathway also promotes aberrant cell growth and survival by phosphorylation of v-akt murine thymoma

viral oncogene homolog (AKT) (4). In addition to the aberrant effects of *KRAS* mutations on cell proliferation and survival, *KRAS* mutation involvement in the epithelial-to-mesenchymal transition (EMT) has been indicated in several cancer cell types (5, 6). EMT confers cells with stem-like properties, including invasiveness, with a loss of epithelial characteristics, such as E-cadherin expression, and a gain of mesenchymal characteristics. In pancreatic cancer cells, knockdown of mutant *KRAS* causes a significant decrease in cell motility, invasiveness, proliferation, and metastasis in association with increased E-cadherin expression and decreased ERK1/2 phosphorylation, suggesting the oncogenic *KRAS* roles through ERK phosphorylation and E-cadherin suppression (6).

Several pieces of evidence have indicated roles of some phospholipase C (PLC) enzymes in CRC progression. PLC is a signaling molecule that hydrolyzes phosphatidylinositol-4,5-bisphosphate to generate inositol-1,4,5-trisphosphate and 1,2-diacylglycerol, which increase the intracellular Ca<sup>2+</sup> level and activate protein kinase C (PKC) signaling pathways. These pathways are involved in many biological processes, including tissue differentiation and tumorigenesis (7). Recent meta-analysis of CRC reveals that some PLC isozymes are deregulated in CRC, and low expression levels of PLC $\delta$ 1 and PLC $\epsilon$ 1 genes are associated with *KRAS* mutation status (8). PLC $\epsilon$ 1 is a ras effector with dual roles in CRC tumor progression (9–11). However, little is known about the function of PLC $\delta$ 1 in CRC. Moreover, the relationship between PLC $\delta$ 1 and *KRAS* has not been elucidated.

## Significance

We found that expression of phospholipase C $\delta$ 1 (PLC $\delta$ 1) is down-regulated in colorectal cancer (CRC) cells compared with normal colon epithelium. Ectopic expression of PLC $\delta$ 1 in CRC cells induced expression of E-cadherin, a tumor-suppressive cell-cell adhesion molecule, whereas knockdown of PLC $\delta$ 1 repressed E-cadherin expression. Moreover, PLC $\delta$ 1 overexpression reduced the malignant phenotypes of CRC. We also identified that PLC $\delta$ 1 expression is repressed by the Kirsten rat sarcoma viral oncogene homolog (*KRAS*)/mitogen-activated protein kinase kinase (MEK) pathway, which is constitutively activated in many of CRC cells. Furthermore, PLC $\delta$ 1 expression suppressed the phosphorylation of ERK1/2, which is an MEK target, by E-cadherin induction. These data suggest that PLC $\delta$ 1 has tumor-suppressive functions in CRC through E-cadherin induction and *KRAS*/MEK/ERK signal attenuation. These findings could provide a valuable strategy for CRC treatment.

Author contributions: R.S. and K.F. designed research; R.S., T.H., R.B., T.N., and Y.M. performed research; R.S. and T.H. analyzed data; and R.S. and K.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. S.G.R. is a Guest Editor invited by the Editorial Board.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1405374111/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1405374111/-DCSupplemental).

Here, we have elucidated the roles of PLC $\delta$ 1 in *KRAS*-mutant CRC cell lines. We found that PLC $\delta$ 1 regulates expression of E-cadherin, suppression of EMT, cell motility, invasiveness, and tumorigenicity. Furthermore, *KRAS*/MEK signaling repressed PLC $\delta$ 1 expression, whereas PLC $\delta$ 1 suppressed the ERK1/2 phosphorylation by E-cadherin. These data indicate that PLC $\delta$ 1 has tumor-suppressive functions in CRC through E-cadherin induction and *KRAS*/MEK/ERK signal attenuation and provide a valuable perspective on therapeutic approaches, which are also applicable to *KRAS*-mutated CRC, by regulating PLC $\delta$ 1-mediated signals.

## Results

**PLC $\delta$ 1 Is Down-Regulated in Colon Adenocarcinoma.** Because the involvement of PLC $\delta$ 1 in CRC remains virtually unknown, we first examined if PLC $\delta$ 1 protein expression is down-regulated in colon carcinoma compared with normal colon epithelium. We performed immunohistochemistry with human colon carcinoma tissue arrays, which contain 36 matched normal and adenocarcinoma tissues. PLC $\delta$ 1 expression was observed strongly in normal colon surface epithelium and moderately in crypt cells as well as in the lamina propria (Fig. 1*A*). PLC $\delta$ 1 expression was significantly diminished in CRC cells, but it was maintained in stromal cells (Fig. 1 and Fig. S1*A*). No clinicopathological features, such as sex, age, differentiation, or stage, were found to be associated with PLC $\delta$ 1 levels observed in adenocarcinoma tissues (Fig. S1*B*).

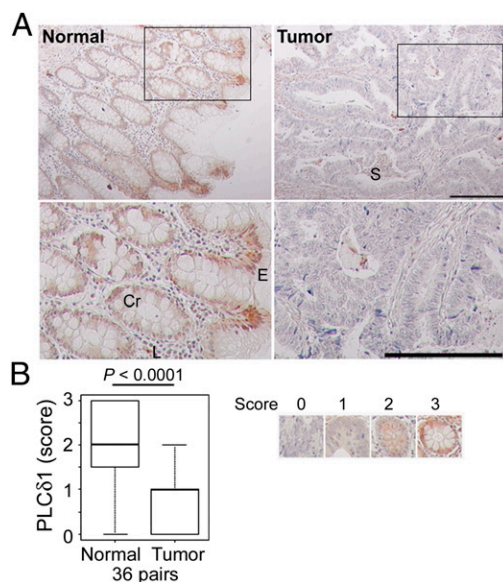
**PLC $\delta$ 1 Induces the Expression of E-Cadherin.** To elucidate the role of PLC $\delta$ 1 in CRC, we investigated PLC $\delta$ 1 expression in the CRC cell lines SW620, SW480, and DLD-1. The SW620 cell line was established from the metastatic site of a CRC patient, whereas the SW480 cell line was established from the primary tumor of the same patient. The DLD-1 cell line was established from CRC tissues from a different patient. Previously, SW620 was shown to have very low E-cadherin expression and acquired expression of Vimentin as well as EMT-promoting transcription factors, such

as Zeb1 and Snail. These reports suggest that SW620 cells have undergone EMT, whereas SW480 and DLD-1 cells have not (12). Consistent with these reports, we observed the very low expression of E-cadherin in SW620 cells, whereas robust E-cadherin expression was observed in DLD-1 and SW480 cells (Fig. 2*A*). Interestingly, we found that PLC $\delta$ 1 expression was very low in SW620 cells, whereas it was relatively high in DLD-1 cells (Fig. 2*A*). Because of these results, we investigated the role of PLC $\delta$ 1 in SW620 cells in association with the expression of E-cadherin. We found that PLC $\delta$ 1 overexpression in SW620 significantly up-regulated E-cadherin protein levels (Fig. 2*B*). Similar results were also obtained by quantitative real-time PCR (qRT-PCR), showing that PLC $\delta$ 1 induced E-cadherin expression transcriptionally (Fig. 2*C*). The E-cadherin and PLC $\delta$ 1 mRNA expression levels in PLC $\delta$ 1-overexpressing clones were in the range between levels observed in SW480 and DLD-1 cells (Fig. S2), suggesting that the levels of PLC $\delta$ 1 overexpression and restored E-cadherin are biologically relevant. Confocal microscopy showed that E-cadherin was localized at the cell–cell junctions in PLC $\delta$ 1-overexpressing cells, suggesting the functional restoration of E-cadherin by PLC $\delta$ 1 (Fig. 2*D*). This restoration of E-cadherin was attenuated by PLC $\delta$ 1-targeting siRNA (Fig. 2*E*). Cell morphology was also changed with E-cadherin expression, because individual rounded cells adapted an epithelial morphology with increased cell-to-cell tight adhesions (Fig. 2*F*).

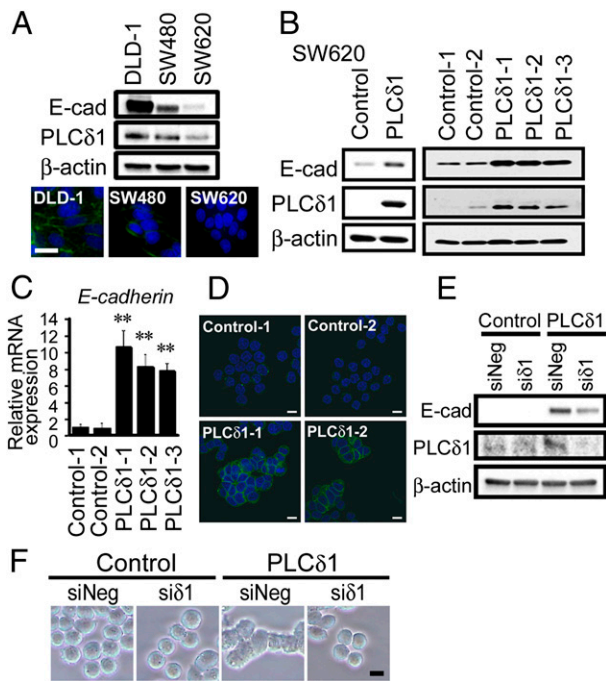
**Endogenous PLC $\delta$ 1 Contributes to E-Cadherin Expression in a Lipase-Dependent Manner.** Because PLC $\delta$ 1 overexpression induced E-cadherin expression, we next tried to evaluate the more physiological effects of PLC $\delta$ 1 on E-cadherin expression in CRC cell lines by silencing experiments. In SW620 and SW480 cells, siRNA-mediated PLC $\delta$ 1 knockdown reduced E-cadherin expression (Fig. 3*A* and Fig. S3). In DLD-1 cells, PLC $\delta$ 1 knockdown also reduced E-cadherin expression and junctional localization (Fig. 3*B*). Because functional redundancy between PLC isoforms is possible, we also examined whether another PLC $\delta$  isoform, PLC $\delta$ 3, also regulates E-cadherin expression. Although, PLC $\delta$ 3 expression was significantly down-regulated in CRC (Fig. S4) and ectopic expression of PLC $\delta$ 3 in SW620 up-regulated E-cadherin expression (Fig. S5*A*), knockdown of PLC $\delta$ 3 scarcely affected E-cadherin expression, even in the PLC $\delta$ 1-knockdown cells (Fig. 3*C* and Fig. S5*B* and *C*). These results suggest that endogenous PLC $\delta$ 3 hardly contributes to E-cadherin expression, at least in these cell lines, but our results of PLC $\delta$ 3 overexpression retain the possibility of PLC $\delta$ 3 contribution to E-cadherin expression in other cell context.

Moreover, E-cadherin down-regulation by PLC $\delta$ 1 knockdown was reversed by the coexpression of murine PLC $\delta$ 1 but not the PLC $\delta$ 1 lipase activity-dead construct, suggesting the functional redundancy of human and murine PLC $\delta$ 1 and the importance of lipase activity of PLC $\delta$ 1. (Fig. 3*D*).

**PLC $\delta$ 1 Represses the Expression of EMT-Associated Genes.** We next investigated the effect of PLC $\delta$ 1 on EMT-associated gene expression. Western blots showed that SW620 cells stably overexpressing PLC $\delta$ 1 express reduced levels of Vimentin, a mesenchymal gene, compared with vector-introduced control cells (Fig. 4*A*). Down-regulation of Vimentin in PLC $\delta$ 1-overexpressing cells was also observed by immunofluorescence microscopy (Fig. 4*B*) and qRT-PCR analysis (Fig. 4*C*). To investigate the expression of other EMT-related genes, qRT-PCR analysis was performed and showed that ectopic PLC $\delta$ 1 expression suppressed TGF- $\beta$ , Zeb1, Slug, and Snail1 mRNA expression (Fig. 4*D*). Previous reports show that increased E-cadherin expression results in the relocalization of  $\beta$ -catenin, a key factor in CRC progression (13, 14), from the nucleus to the membrane adherens junctions and also, causes a reduction in  $\beta$ -catenin-T-cell factor/lymphocyte enhancer factor (TCF/LEF) signaling as well as the expression of some of the transcription factors that promote EMT (15). To investigate if the



**Fig. 1.** PLC $\delta$ 1 was down-regulated in colon adenocarcinoma. (*A*) Immunohistochemistry with human colon carcinoma tissue arrays, which contain 36 matched normal and adenocarcinoma tissues, was performed with anti-PLC $\delta$ 1 antibody. *Insets in Upper* are shown magnified in *Lower*. Cr, crypt; E, surface epithelium; L, lamina propria; S, stroma. (Scale bar: 200  $\mu$ m.) (*B*) The expression level of PLC $\delta$ 1 in each sample was scored. As shown in *Right*, scores of 0–3 indicate complete loss, mild staining, moderate staining, and marked staining, respectively. The scored PLC $\delta$ 1 levels were assessed between normal and tumor samples (36 pairs) using the Wilcoxon signed rank test.



**Fig. 2.** PLC $\delta$ 1 induced the expression of E-cadherin. (A, Upper) The expressions of E-cadherin, PLC $\delta$ 1, and  $\beta$ -actin (as a loading control) in DLD-1, SW480, and SW620 cells were determined by Western blotting. (A, Lower) E-cadherin and nuclei were stained with anti-E-cadherin antibody and hoechst33342, respectively (blue, nuclei; green, E-cadherin). E-cad, E-cadherin. (Scale bar: 20  $\mu$ m.) (B) SW620 cells were transfected with either PLC $\delta$ 1-expression vector or the relevant empty vector and then selected with G418 treatment for 8 d. The expressions of E-cadherin, PLC $\delta$ 1, and  $\beta$ -actin (as loading control) in (Left) bulk or (Right) stable clone cells were determined by Western blotting. (C) PLC $\delta$ 1-overexpressing stable clones (PLC $\delta$ 1-1, -2, and -3) and the relevant control clones (Control-1 and -2) were assessed for E-cadherin mRNA expression by qRT-PCR analysis. The relative expression levels of E-cadherin, normalized to  $\beta$ -actin (as an internal control), are shown ( $n = 3$ ), with the value of Control-1 set as one. Statistical analysis was performed using the Tukey multiple comparison of means test.  $^{**}P < 0.005$ . (D) E-cadherin (green) and nuclei (blue) were observed by confocal microscopy. (Scale bar: 10  $\mu$ m.) (E) The PLC $\delta$ 1-overexpressing stable clone (PLC $\delta$ 1-1) and the relevant control clone (Control-1) were transfected with siRNA against PLC $\delta$ 1 (si $\delta$ 1) or non-target siRNA (siNeg) and assessed by Western blots for the indicated proteins. (F) The morphology of the cells in E is shown. (Scale bar: 10  $\mu$ m.)

PLC $\delta$ 1-induced E-cadherin is associated with the suppression of  $\beta$ -catenin-TCF/LEF-mediated transcription, a TCF4 transcriptional reporter (TOP-/FOP-FLASH; Upstate) assay was performed. PLC $\delta$ 1-overexpressing cells showed TOP-/FOP-FLASH activity of about 50–60% compared with control cells (Fig. 4E). These results indicate that PLC $\delta$ 1 suppressed the expression of EMT-promoting factors in association with the down-regulation of  $\beta$ -catenin-TCF/LEF signaling pathways.

**Role of PLC $\delta$ 1 in Proliferation, Motility, and Invasiveness of CRC Cells.**

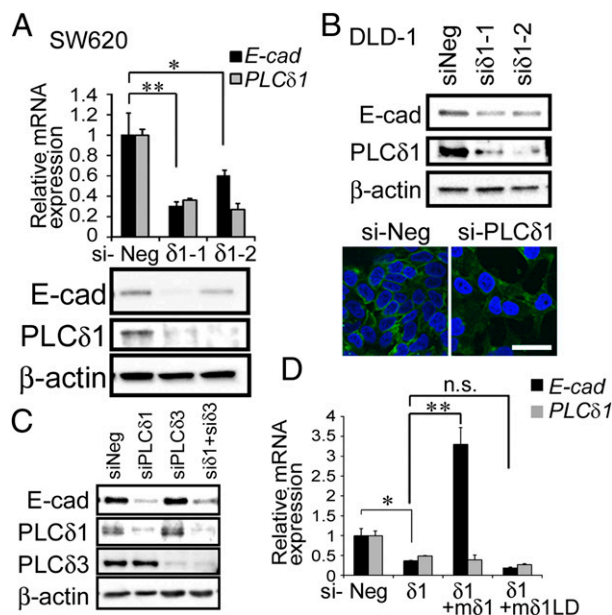
Because E-cadherin is a prognostic molecule and because the loss of E-cadherin has been associated with CRC malignant phenotypes (16), the effects of PLC $\delta$ 1 on CRC malignant phenotypes were assessed. Because E-cadherin mediates contact inhibition of proliferation (17), we investigated the role of PLC $\delta$ 1 on CRC cell proliferation. Cells were plated in 24-well plates, and the cell number was counted every 24 h. Induction of PLC $\delta$ 1 hardly affected the proliferation of SW620 cells at 24 h, but the proliferation rates of PLC $\delta$ 1-overexpressing cells decreased at 48 and 72 h (Fig. 5A), which could have been caused by E-cadherin-mediated contact inhibition. We next evaluated if PLC $\delta$ 1 affected cell motility and invasiveness using transwell migration and invasion assays, respectively.

The number of migrated SW620 cells, which stably expressed PLC $\delta$ 1, was about 10–20% of control cells (Fig. 5B). The number of invaded SW620 cells, which stably expressed PLC $\delta$ 1, was about 3–20% of control cells (Fig. 5C). These results suggest that PLC $\delta$ 1 repressed the cell proliferation, migration, and invasion of CRC cells.

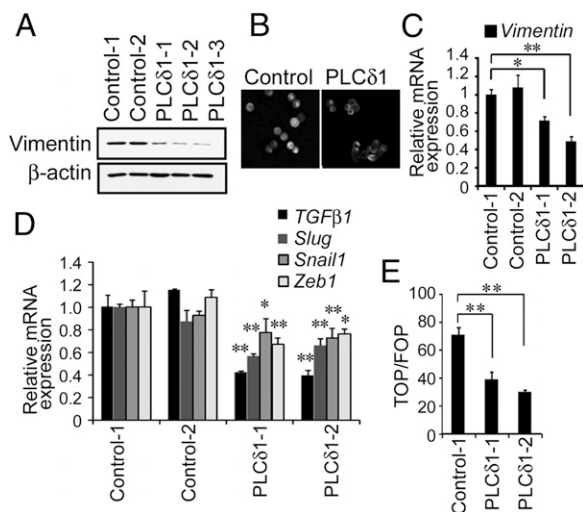
**PLC $\delta$ 1 Suppresses Anchorage-Independent Cell Growth and Tumorigenicity of CRC Cells.**

The anchorage-independent growth of cancer cells is one of the hallmarks of malignant phenotypes and promoted by loss of E-cadherin (18). Soft agar assays revealed that PLC $\delta$ 1 reduced the anchorage-independent cell growth of SW620 cells (Fig. 5D). Anchorage-independent growth in soft agar often relates to the tumorigenic potential of tumor cells (19). We next performed in vivo experiments to evaluate the effect of PLC $\delta$ 1 overexpression on tumorigenicity. SW620 cells, which stably expressed PLC $\delta$ 1, and the relevant control cells were inoculated into the flanks of nude mice, and the established xenograft volumes were assessed one time per week. As a result, PLC $\delta$ 1-overexpressing cells had significantly reduced tumor volume (Fig. 5E). The weights of xenografts from PLC $\delta$ 1-overexpressing cells were also significantly reduced (Fig. 5F). These results strongly suggest that PLC $\delta$ 1 functions as a tumor suppressor.

**KRAS/MEK Signaling Suppressed the Expression of PLC $\delta$ 1.** In CRC specimens, low levels of PLC $\delta$ 1 mRNA expression have been



**Fig. 3.** Knockdown of PLC $\delta$ 1 but not PLC $\delta$ 3 repressed the expression of E-cadherin in CRC cells. (A) Cells were transfected with negative control siRNA (siNeg) or siRNA for PLC $\delta$ 1 (si $\delta$ 1-1 or -2). After 7 d, the expression levels of E-cadherin and PLC $\delta$ 1 were determined by qRT-PCR ( $n = 3$ ). The relative mRNA expression, normalized by  $\beta$ -actin, is shown. Western blots for the expression levels of E-cadherin, PLC $\delta$ 1, and  $\beta$ -actin (as loading control) are shown below. E-cad, E-cadherin. (B) DLD-1 cells transfected with siNeg, si $\delta$ 1-1, or si $\delta$ 1-2 were assessed by Western blots for E-cadherin, PLC $\delta$ 1, and  $\beta$ -actin. Confocal microscope images of these cells stained with E-cadherin antibody (green) and hoechst33342 (blue) are also shown. (Scale bar: 30  $\mu$ m.) (C) The expression levels of E-cadherin, PLC $\delta$ 1, and PLC $\delta$ 3 in SW620 cells transfected with siNeg, si $\delta$ 1, or siRNA for PLC $\delta$ 3 (si $\delta$ 3) as indicated were determined by Western blots. (D) The expression levels of E-cadherin and PLC $\delta$ 1 (human) mRNA in SW620 cells transfected with siNeg, si $\delta$ 1 (human), and si $\delta$ 1 (human) with mouse PLC $\delta$ 1 (m $\delta$ 1) or lipase activity-dead PLC $\delta$ 1 (m $\delta$ 1LD) expression plasmid were determined by qRT-PCR ( $n = 3$ ). The relative mRNA expression, normalized by  $\beta$ -actin, is shown. Statistical analysis was performed using the (A) Dunnett or (D) Tukey multiple comparison of means test. n.s., Not significant.  $^{*}P < 0.05$ ;  $^{**}P < 0.005$ .

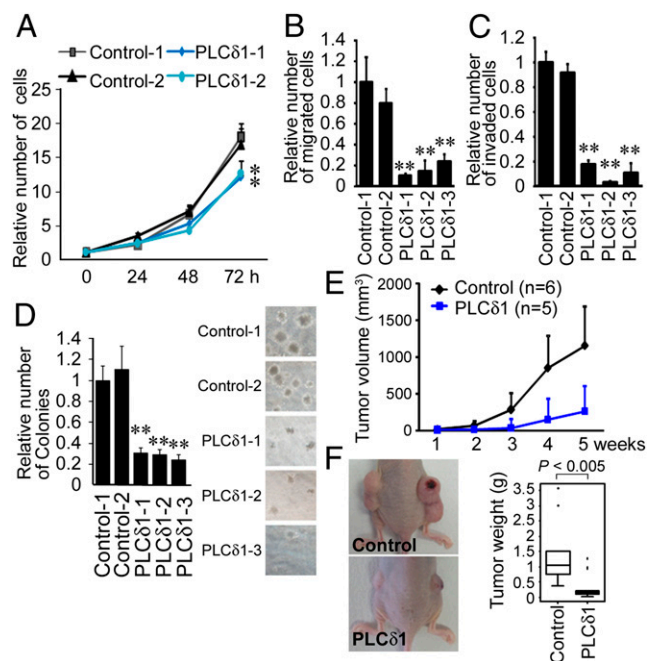


**Fig. 4.** PLC $\delta$ 1 repressed EMT-associated gene expression. (A) The expressions of Vimentin and  $\beta$ -actin (as loading control) in SW620 cells that stably overexpress PLC $\delta$ 1 (PLC $\delta$ 1-1, -2, and -3) and control cells (Control-1 and -2) were determined by Western blotting. (B) Vimentin in control cells and PLC $\delta$ 1-overexpressing SW620 cells was stained using an anti-Vimentin antibody and observed by immunofluorescence microscopy. (C and D) The mRNA expressions of Vimentin, TGF- $\beta$ , Slug, Snail, and Zeb1 in SW620 cells that stably overexpress PLC $\delta$ 1 (PLC $\delta$ 1-1 and -2) and control cells (Control-1 and 2) were determined by qRT-PCR ( $n = 3$ ). The relative expression levels, normalized to  $\beta$ -actin expression, are shown. (E) Control or PLC $\delta$ 1-overexpressing cells were transfected with TOP- or FOP-FLASH reporter plasmids and pRL-TK control plasmid in triplicate. The relative values of firefly luciferase activity, normalized by Renilla luciferase activity, are shown. Statistical analysis was performed using Tukey multiple comparison of means test. \* $P < 0.05$ ; \*\* $P < 0.005$  (vs. Control-1).

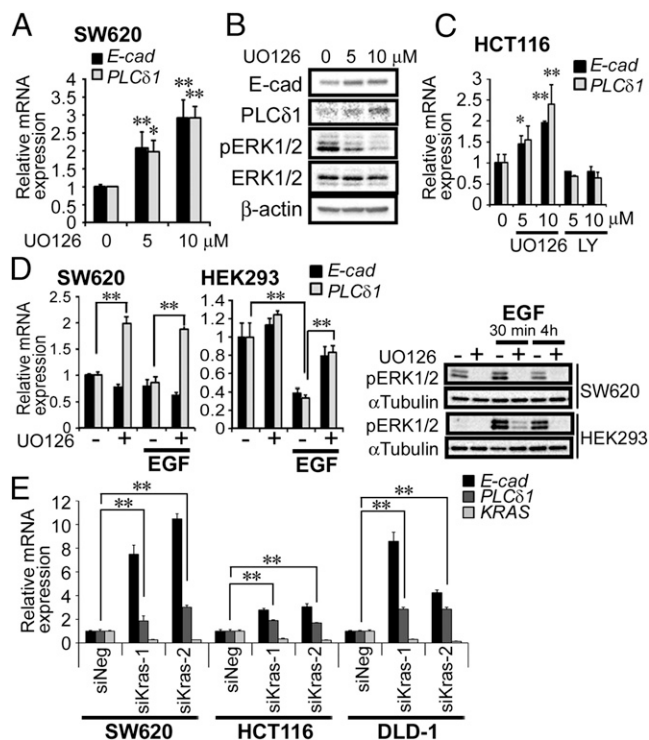
shown to correlate with the presence of KRAS mutations (8). Therefore, we examined if two downstream signaling pathways of KRAS (MEK/ERK and PI3K) affect the expression of PLC $\delta$ 1 using cells with mutant KRAS (SW620 and HCT116) and cells with WT KRAS (HEK293 and HeLa). SW620 cells (with KRAS G12V) were treated with the MEK inhibitor UO126, and we assessed the expression of PLC $\delta$ 1. MEK inhibitor treatment increased both PLC $\delta$ 1 and E-cadherin transcriptional levels about two- to threefold in a dose-dependent manner (Fig. 6A). MEK inhibitor treatment also increased PLC $\delta$ 1 and E-cadherin protein levels, whereas phosphorylation of the MEK downstream effectors ERK1/2 was decreased in a dose-dependent manner, indicating the effectiveness of UO126 (Fig. 6B). MEK inhibitor treatment also induced PLC $\delta$ 1 expression about 1.5- to 2.5-fold in HCT116 cells (with KRAS G13D) but not HEK293 (KRAS WT) and HeLa cells (KRAS WT), whereas the PI3K inhibitor LY294002 did not enhance the expression of PLC $\delta$ 1 in any of these cell lines (Fig. 6C and Fig. S6). The phosphorylation statuses of the downstream effectors of MEK/ERK and PI3K signaling (ERK1/2 and AKT, respectively) were reduced by these inhibitor treatments in these cell lines (Fig. S6C). Furthermore, EGF treatment, which promotes the RAS/MEK pathway and the phosphorylation of ERK1/2 in HEK293 cells, suppressed PLC $\delta$ 1 expression. The EGF-mediated PLC $\delta$ 1 suppression in HEK293 cells was recovered by cotreatment with the MEK inhibitor (Fig. 6D). However, EGF did not suppress PLC $\delta$ 1 expression in SW620 cells in which the KRAS/MEK signal is constitutively active because of the KRAS G13D mutation, whereas UO126 treatment up-regulated PLC $\delta$ 1 expression (Fig. 6D). Knockdown of KRAS increased PLC $\delta$ 1 and E-cadherin gene expression in SW620, HCT116, and DLD-1 cells, showing the involvement of KRAS in PLC $\delta$ 1 repression (Fig. 6E). These results clearly indicated that activation of the KRAS/MEK signaling pathway suppressed PLC $\delta$ 1 expression. In these experiments, E-cadherin

expression was also assessed and shown to be changed similarly to PLC $\delta$ 1 expression, but the expression of PLC $\delta$ 1 tended to precede E-cadherin expression, because only PLC $\delta$ 1 expression was up-regulated with a 4-h treatment of the MEK inhibitor in SW620 cells (Fig. 6D, SW620), although both E-cadherin and PLC $\delta$ 1 expressions were up-regulated with a 48-h treatment with the MEK inhibitor (Fig. 6A).

**PLC $\delta$ 1 Suppressed ERK1/2 Phosphorylation Through E-Cadherin.** Finally, we examined if PLC $\delta$ 1 affects the KRAS/MEK/ERK signaling pathway. ERK1/2 phosphorylation levels were assessed by Western blots in control or PLC $\delta$ 1-overexpressing stable cells. As shown in Fig. 7A, phosphorylated ERK1/2 was reduced in PLC $\delta$ 1-overexpressing cells. The reduction was mitigated by siRNA-mediated PLC $\delta$ 1 knockdown (Fig. 7B). Because MEK/ERK signaling is reportedly attenuated by E-cadherin (20), we assessed if suppression of ERK phosphorylation by ectopic PLC $\delta$ 1 could be mediated by E-cadherin. As shown in Fig. 7C, the decreased ERK1/2 phosphorylation levels in PLC $\delta$ 1-overexpressing cells were restored by E-cadherin knockdown to the levels of control cells. These results clearly indicate that PLC $\delta$ 1 suppresses ERK1/2 phosphorylation by restoring E-cadherin expression.



**Fig. 5.** The roles of PLC $\delta$ 1 on malignant phenotypes of CRC cells. (A) Control or PLC $\delta$ 1-overexpressing SW620 cells (Control-1 and -2 or PLC $\delta$ 1-1 and -2) were plated at 10,000 cells/well in 24-well plates. Cells were dissociated by trypsinization, and the total number of cells was determined every 24 h ( $n = 3$ ). (B and C) Transwell migration or invasion assays were performed with control or PLC $\delta$ 1-overexpressing SW620 cells as described in *Materials and Methods*. The relative numbers of migrated or invaded cells are shown ( $n = 3$ ). (D) Control or PLC $\delta$ 1-overexpressing SW620 cells were plated in six-well plates with soft agar. After 14 d, the numbers of colonies were counted and are shown in a bar graph ( $n = 3$ ). The representative images of colonies are shown in *Right*. (E) Control ( $n = 6$ ) or PLC $\delta$ 1-overexpressing SW620 ( $n = 5$ ) cells were inoculated into the flanks of nude mice. The volume of xenografts was determined as described in *Materials and Methods*. (F) Representative images of the mice with xenografts are shown in *Left*. After 5 wk, the weights of the xenografts were assessed. The statistical difference was determined by Mann-Whitney  $U$  test. (B–D) Statistical analysis was performed by Tukey multiple comparison of means test. \*\* $P < 0.005$  (vs. Control-1).



**Fig. 6.** KRAS/MEK signaling suppressed the expression of PLC $\delta$ 1. (A) SW620 cells were treated with the MEK inhibitor UO126 (5 or 10  $\mu$ M) for 48 h. Cells were harvested, and the mRNA expression levels of PLC $\delta$ 1, E-cadherin, and  $\beta$ -actin (as internal control) were determined by qRT-PCR analysis ( $n = 3$ ). The relative expression levels of PLC $\delta$ 1 and E-cadherin, normalized by  $\beta$ -actin, are shown. E-cad, E-cadherin. (B) SW620 cells treated as in A were assessed by Western blots with the indicated antibodies. (C) HCT116 cells were treated with UO126 (5 or 10  $\mu$ M) or LY294002 (5 or 10  $\mu$ M) for 24 h, and then, the mRNA expression levels were determined ( $n = 3$ ). The relative expression levels of PLC $\delta$ 1 and E-cadherin, normalized by  $\beta$ -actin, are shown. (D, Right) Cells were also assessed by Western blots for the indicated proteins. (E) SW620, HCT116, and DLD-1 cells were transfected with negative control siRNA (siNeg) or siRNA targeting KRAS (siKras-1 or -2) for 48 h. The relative mRNA expression levels of PLC $\delta$ 1, E-cadherin, and KRAS, normalized by  $\beta$ -actin, are shown ( $n = 3$ ). Statistical analysis was performed using Tukey multiple comparison of means test. \* $P < 0.05$ ; \*\* $P < 0.005$ .

## Discussion

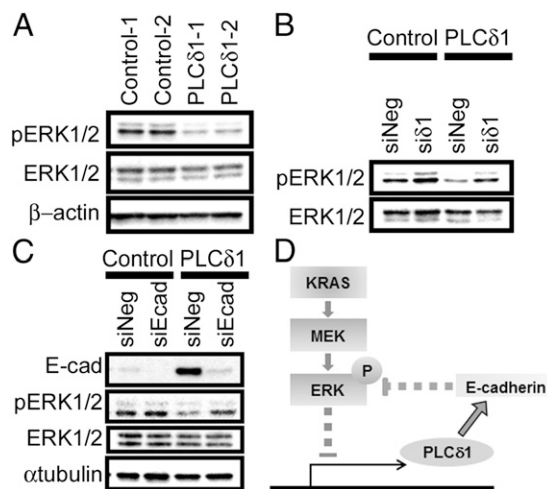
Loss of the epithelial adhesive molecule E-cadherin promotes CRC growth and invasiveness and is associated with CRC metastasis and poor prognosis (21, 22). Loss of E-cadherin is promoted by many tumor-promoting factors, including EMT inducers, cytokines, and several tumor-promoting mutant genes, such as p53 (23) or KRAS (24). In this study, we clarified the notable mechanisms of E-cadherin suppression and CRC progression mediated by PLC $\delta$ 1 down-regulation.

To the best of our knowledge, we show here for the first time that PLC $\delta$ 1 ectopic expression induced E-cadherin expression, whereas PLC $\delta$ 1 suppression decreased E-cadherin expression in CRC. To investigate the link between the expressions of PLC $\delta$ 1 and E-cadherin in other cancer types, we assessed PLC $\delta$ 1 and E-cadherin levels in hepatocellular carcinoma cell lines HepG2 (with KRAS mutation), SK-Hep-1, and HLE and breast cancer cell lines MDA-MB-231 (with KRAS mutation) and MCF-7. As shown in Fig. S7A, we observed little correlation in these cells. Interestingly, knockdown of PLC $\delta$ 1 significantly decreased E-cadherin expression in hepatocellular carcinoma cell lines

HepG2 and HLE but not MCF-7 (Fig. S7B). These results suggest that, although the total expression level of E-cadherin is regulated by multiple factors in addition to PLC $\delta$ 1 and the link between total E-cadherin levels and PLC $\delta$ 1 may be cell-content dependent, E-cadherin is regulated by PLC $\delta$ 1 in some cancer cell types. Down-regulation of E-cadherin has been reported to be mediated by EMT inducers, and E-cadherin restoration can suppress the expression of EMT inducers (15, 25). Among EMT inducers, we found here that the expressions of TGF- $\beta$ , Slug, Zeb1, and Snail1 were down-regulated with ectopic PLC $\delta$ 1 expression. In contrast, transient PLC $\delta$ 1 knockdown rarely up-regulated the expression of EMT-related genes, whereas E-cadherin expression was reduced by PLC $\delta$ 1 knockdown. These results suggest that E-cadherin maintenance by PLC $\delta$ 1 is not always correlated with these EMT inducers.

PLC $\delta$ 1 is important for the intracellular Ca<sup>2+</sup> maintenance in epithelial cells, especially keratinocytes. PLC $\delta$ 1-KO mice have significantly decreased intracellular Ca<sup>2+</sup> levels, resulting in the abnormal differentiation of epidermal and hair follicles (26). Ca<sup>2+</sup> and the calcium-sensing receptor are reported to be important for differentiation and E-cadherin expression in colonic epithelial cells, and disruption of calcium-sensing receptor system contributes to abnormal differentiation and malignant progression (27). Therefore, PLC $\delta$ 1 may contribute to intracellular Ca<sup>2+</sup> maintenance, which is necessary for E-cadherin expression in CRC. Ca<sup>2+</sup> activates several signaling molecules, including calcineurin/nuclear factor of activated T cells and PKC. Additional study to understand the molecules downstream of PLC $\delta$ 1 that are necessary for its tumor-suppressive function is needed in future works.

In addition, we clarified that KRAS/MEK signaling represses PLC $\delta$ 1 expression. Furthermore, we first elucidated that PLC $\delta$ 1 protein levels were significantly reduced in CRC specimens



**Fig. 7.** PLC $\delta$ 1 suppressed the phosphorylation of ERK1/2 by E-cadherin. (A) Control or PLC $\delta$ 1-overexpressing SW620 cells (Control-1 and -2 or PLC $\delta$ 1-1 and -2) were assessed for the protein levels of phosphorylated ERK1/2, ERK1/2, and  $\beta$ -actin (as a loading control) by Western blots. (B) Control or PLC $\delta$ 1-overexpressing SW620 cells (Control-1 or PLC $\delta$ 1-1) were transfected with siRNA for PLC $\delta$ 1 (si $\delta$ 1) or negative control siRNA (siNeg). After 5 d, cells were harvested and assessed for protein levels with the indicated antibodies by Western blots. (C) Control or PLC $\delta$ 1-overexpressing SW620 cells (Control-1 or PLC $\delta$ 1-1) were transfected with E-cadherin siRNA (siEcad) or siNeg. After 5 d, cells were harvested and assessed for the protein levels with the indicated antibodies by Western blots. E-cad, E-cadherin. (D) The regulatory loop of KRAS/MEK/ERK signaling and PLC $\delta$ 1 in CRC. KRAS/MEK signaling, which is constitutively promoted by KRAS mutations in many CRC patients, suppressed the expression of PLC $\delta$ 1. Because PLC $\delta$ 1 promotes the expression of E-cadherin, one of major tumor-suppressive molecules that also suppresses MEK/ERK signaling, PLC $\delta$ 1 down-regulation by KRAS mutation accelerates tumor progression. P indicates phosphorylation.

compared with the normal colonic epithelium by tissue microarray analysis. In this analysis, no association between PLC $\delta$ 1 expression and tumor grade or stage was observed. This phenomenon is likely caused by KRAS/MEK signaling-mediated PLC $\delta$ 1 down-regulation. Because KRAS mutation is observed during the early stages in multistep processes of carcinogenesis (28), PLC $\delta$ 1 down-regulation by KRAS/MEK signaling may also occur in the early stages of colorectal carcinogenesis. We also showed that PLC $\delta$ 1 suppressed the KRAS/MEK/ERK pathway, suggesting a negative regulatory loop between KRAS/MEK/ERK signaling and PLC $\delta$ 1 (Fig. 7D). From these results, we speculate that activation of PLC $\delta$ 1 or PLC $\delta$ 1 downstream signaling, which restores E-cadherin and suppresses tumor malignancy, could be a novel strategy for CRC treatments. Notably, this strategy may lead to a virtuous cycle of restoring PLC $\delta$ 1 expression, enhancing E-cadherin expression, and attenuating KRAS/MEK/ERK signaling, which may, ultimately, inhibit cancer malignancy.

In this study, we found a significant reduction in PLC $\delta$ 1 expression in CRC cells in clinical specimens and clarified the roles of PLC $\delta$ 1 in KRAS-mutant CRC cell lines. We revealed that PLC $\delta$ 1 was responsible for E-cadherin expression, suppression of EMT, cell motility, invasiveness, and tumorigenicity. Furthermore, the expression of PLC $\delta$ 1 was repressed by KRAS/MEK signaling, whereas PLC $\delta$ 1 suppressed the phosphorylation of ERK1/2 through E-cadherin. These data indicate that PLC $\delta$ 1 has tumor-suppressive functions in CRC through E-cadherin induction and KRAS/MEK/ERK signal attenuation. It is worth noting that KRAS knockdown increased E-cadherin and PLC $\delta$ 1 expressions in CRC. Because the level of E-cadherin and malignancy of CRC are changeable by PLC $\delta$ 1, PLC $\delta$ 1-inducible compounds may be a convincing candidate for CRC drugs. Our results provide a valuable perspective on the therapeutic approaches to KRAS-mutated CRC.

## Materials and Methods

**Immunohistochemistry.** Human colon carcinoma tissue arrays with matched adjacent normal colon tissue were purchased from Biomax (US Biomax). The immunostaining was performed as described previously (29). Immunohistochemical assays for human PLC $\delta$ 1 were performed with a Vectastain Elite Rabbit ABC Kit (Vector Laboratories) with anti-PLC $\delta$ 1 antibody (Sigma) followed by light counterstaining with Mayer's hematoxylin (Wako). Sections were examined under a BX51 microscope (Olympus).

**Cell Culture.** The colorectal adenocarcinoma cell lines SW620 and HCT116 were obtained from the American Type Culture Collection. DLD-1 and HEK293 cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank (National Institute of Health Sciences). These cells were maintained at 37 °C in a 5% (vol/vol) CO $_2$  humidified atmosphere in RPMI medium 1640 (Invitrogen) supplemented with 10% (vol/vol) FBS. SW480 and HeLa cells were cultured as described previously (30).

**Western Blot Analysis.** Western blot analysis was performed as described previously (29) with some modifications. Primary antibodies for E-cadherin (BD Biosciences), GAPDH, phospho-Akt (Ser473), Akt, phospho-ERK1/2 (Thr202/204), ERK1/2 (Cell Signaling), anti- $\beta$ -actin antibody (Sigma),  $\alpha$ -tubulin (GeneTex), Vimentin (Santa Cruz), and PLC $\delta$ 1 (Santa Cruz) were used.

**Migration and Invasion Assay.** Migration assays were performed using cell culture insert with 8- $\mu$ m-sized pores (BD Biosciences) in a 24-well plate with RPMI Medium 1640 containing 10% (vol/vol) FBS. For invasion assays, the cell culture inserts were added with 60  $\mu$ L (2.5 mg/mL) BD Matrigel Basement Membrane Matrix Growth Factor Reduced (BD Biosciences) as described previously (31). Materials and methods for plasmids; siRNA and transfection; immunofluorescence microscopy; RNA isolation, cDNA synthesis, and qRT-PCR; luciferase reporter assay; cell proliferation assay; soft agar colony formation assay; animal experiments; and statistical analysis are provided in *SI Materials and Methods*.

**ACKNOWLEDGMENTS.** We thank Dr. Y. Nakamura, and Dr. A. Yoneda for fruitful discussions. This work was supported by the Funding Program for the Next Generation World-Leading Researchers (to K.F.).

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