Phospholipase Cδ1 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 cancers of cancer-related deaths worldwide, and Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations in CRC predict the ineffectiveness of EGFR receptor-targeted therapy. Previous transcriptional microarray analysis suggests the association between phospholipase Cδ1 (PLCδ1) expression and KRAS mutation status in CRC. However, both the roles and the regulatory mechanisms of PLCδ1 in CRC are not known. Here, we found that the expression of PLCδ1, 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 PLCδ1 in CRC cell lines that harbor an activating KRAS mutation. Ectopic expression of PLCδ1 in CRC cells induced the expression of E-cadherin, whereas knockdown of PLCδ1 repressed the expression of E-cadherin. Moreover, the overexpression of PLCδ1 suppressed the expression of several mesenchymal genes and reduced cell motility, invasiveness, and in vivo tumorigenicity of SW620 CRC cells. We also showed that PLCδ1 expression is repressed by the KRAS/mitogen-activated protein kinase kinase (MEK) pathway. Furthermore, PLCδ1 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 PLCδ1. These data indicate that PLCδ1 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

Significance

We found that expression of phospholipase Cδ1 (PLCδ1) is down-regulated in colorectal cancer (CRC) cells compared with normal colon epithelium. Ectopic expression of PLCδ1 in CRC cells induced expression of E-cadherin, a tumor-suppressive cell–cell adhesion molecule, whereas knockdown of PLCδ1 repressed E-cadherin expression. Moreover, PLCδ1 overexpression reduced the malignant phenotypes of CRC. We also identified that PLCδ1 expression is repressed by the Kirsten rat sarcoma viral oncogene homolog (KRAS)/mitogen-activated protein kinase (MEK) pathway, which is constitutively activated in many CRC cells. Furthermore, PLCδ1 expression suppressed the phosphorylation of ERK1/2, which is a MEK target, by E-cadherin induction. These data suggest that PLCδ1 has tumor-suppressive functions in CRC through E-cadherin induction and KRAS/MEK/ERK signaling suppression. 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.
Here, we have elucidated the roles of PLCδ in KRAS-mutant CRC cell lines. We found that PLCδ regulates expression of E-cadherin, suppression of EMT, cell motility, invasiveness, and tumorigenicity. Furthermore, KRAS/MEK signaling repressed PLCδ expression, whereas PLCδ suppressed the ERK1/2 phosphorylation by E-cadherin. These data indicate that PLCδ 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 PLCδ-mediated signals.

Results

PLCδ Is Down-Regulated in Colon Adenocarcinoma. Because the involvement of PLCδ in CRC remains virtually unknown, we first examined if PLCδ 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. PLCδ expression was observed strongly in normal colon surface epithelium and moderately in crypt cells as well as in the lamina propria (Fig. 1A). PLCδ expression was significantly diminished in CRC cells, but it was maintained in stromal cells (Fig. 1A and SI Fig. 1). No clinicopathological features, such as sex, age, differentiation, or stage, were found to be associated with PLCδ levels observed in adenocarcinoma tissues (Fig. S1B).

PLCδ Induces the Expression of E-Cadherin. To elucidate the role of PLCδ in CRC, we investigated PLCδ 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. 2A). Interestingly, we found that PLCδ expression was very low in SW620 cells, whereas it was relatively high in DLD-1 cells (Fig. 2A). Because of these results, we investigated the role of PLCδ in SW620 cells in association with the expression of E-cadherin. We found that PLCδ overexpression in SW620 significantly up-regulated E-cadherin protein levels (Fig. 2B). Similar results were also obtained by quantitative real-time PCR (qRT-PCR), showing that PLCδ induced E-cadherin expression transcriptionally (Fig. 2C). The E-cadherin and PLCδ mRNA expression levels in PLCδ-overexpressing clones were in the range between levels observed in SW480 and DLD-1 cells (Fig. S2), suggesting that the levels of PLCδ overexpression and restored E-cadherin are biologically relevant. Confocal microscopy showed that E-cadherin was localized at the cell–cell junctions in PLCδ-overexpressing cells, suggesting the functional restoration of E-cadherin by PLCδ (Fig. 2D). This restoration of E-cadherin was attenuated by PLCδ-targeting siRNA (Fig. 2E). 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. 2F).

Endogenous PLCδ Contributes to E-Cadherin Expression in a Lipase-Dependent Manner. Because PLCδ overexpression induced E-cadherin expression, we next tried to evaluate the more physiological effects of PLCδ on E-cadherin expression in CRC cell lines by silencing experiments. In SW620 and SW480 cells, siRNA-mediated PLCδ knockdown reduced E-cadherin expression (Fig. 3A and Fig. S3). In DLD-1 cells, PLCδ knockdown also reduced E-cadherin expression and junctional localization (Fig. 3B). Because functional redundancy between PLC isoforms is possible, we also examined whether another PLC isoform, PLCδ3, also regulates E-cadherin expression. Although, PLCδ3 expression was significantly down-regulated in CRC (Fig. S4) and ectopic expression of PLCδ3 in SW620 up-regulated E-cadherin expression (Fig. S5), knockdown of PLCδ3 scarcely affected E-cadherin expression, even in the PLCδ1-knockdown cells (Fig. 3C and Fig. S5 B and C). These results suggest that endogenous PLCδ3 hardly contributes to E-cadherin expression, at least in these cell lines, but our results of PLCδ3 overexpression retain the possibility of PLCδ3 contribution to E-cadherin expression in other cell context.

Moreover, E-cadherin down-regulation by PLCδ knockdown was reversed by the coexpression of murine PLCδ1 but not the PLCδ1 lipase activity-dead construct, suggesting the functional redundancy of human and murine PLCδ1 and the importance of lipase activity of PLCδ1. (Fig. 3D).

PLCδ Represses the Expression of EMT-Associated Genes. We next investigated the effect of PLCδ on EMT-associated gene expression. Western blots showed that SW620 cells stably overexpressing PLCδ1 expressed reduced levels of Vimentin, a mesenchymal gene, compared with vector-introduced control cells (Fig. 4A). Down-regulation of Vimentin in PLCδ1-overexpressing cells was also observed by immunofluorescence microscopy (Fig. 4B) and qRT-PCR analysis (Fig. 4C). To investigate the expression of other EMT-related genes, qRT-PCR analysis was performed and showed that ectopic PLCδ1 expression suppressed TGF-β, Zeb1, Slug, and Snail mRNA expression (Fig. 4D). Previous reports showed that induced E-cadherin expression results in the relocation of β-catenin, a key factor in CRC progression (13, 14), from the nucleus to the membrane adherens junctions and also, causes a reduction in β-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
of These results indicate that PLCδ1 clone (Control-1) were transfected with siRNA against PLCδ1 (siNeg) and assessed by Western blots for the indicated proliferation of SW620 cells at 24 h, but the proliferation rates of E-cadherin has been associated with CRC malignant phenotypes (16), the effects of PLCδ1 on CRC malignant phenotypes were assessed. Because E-cadherin mediates contact inhibition of proliferation (17), we investigated the role of PLCδ1 in CRC cell proliferation. Cells were plated in 24-well plates, and the cell number was counted every 24 h. Induction of PLCδ1 rarely affected the proliferation of SW620 cells at 24 h, but the proliferation rates of PLCδ1-overexpressing cells decreased at 48 and 72 h (Fig. S4), which could have been caused by E-cadherin-mediated contact inhibition. We next evaluated if PLCδ1 affected cell motility and invasiveness using transwell migration and invasion assays, respectively.

PLCδ1-induced E-cadherin is associated with the suppression of β-catenin-TCF/LEF-mediated transcription, a TCF4 transcriptional reporter (TOP-/FOP-FLASH; Upstate) assay was performed. PLCδ1-overexpressing cells showed TOP-/FOP-FLASH activity of about 50–60% compared with control cells (Fig. 4E). These results indicate that PLCδ1 suppressed the expression of EMT-promoting factors in association with the down-regulation of β-catenin-TCF/LEF signaling pathways.

**Role of PLCδ1 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 PLCδ1 on CRC malignant phenotypes were assessed. Because E-cadherin mediates contact inhibition of proliferation (17), we investigated the role of PLCδ1 on CRC cell proliferation. Cells were plated in 24-well plates, and the cell number was counted every 24 h. Induction of PLCδ1 hardly affected the proliferation of SW620 cells at 24 h, but the proliferation rates of PLCδ1-overexpressing cells decreased at 48 and 72 h (Fig. S4), which could have been caused by E-cadherin-mediated contact inhibition. We next evaluated if PLCδ1 affected cell motility and invasiveness using transwell migration and invasion assays, respectively.

The number of migrated SW620 cells, which stably expressed PLCδ1, was about 10–20% of control cells (Fig. 5F). The number of invaded SW620 cells, which stably expressed PLCδ1, was about 3–20% of control cells (Fig. 5C). These results suggest that PLCδ1 repressed the cell proliferation, migration, and invasion of CRC cells.

**PLCδ1 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 PLCδ1 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 PLCδ1 overexpression on tumorigenicity. SW620 cells, which stably expressed PLCδ1, 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, PLCδ1-overexpressing cells had significantly reduced tumor volume (Fig. 5E). The weights of xenografts from PLCδ1-overexpressing cells were also significantly reduced (Fig. 5F). These results strongly suggest that PLCδ1 functions as a tumor suppressor.

**KRAS/MEK Signaling Suppressed the Expression of PLCδ1.** In CRC specimens, low levels of PLCδ1 mRNA expression have been

Fig. 2. PLCδ1 induced the expression of E-cadherin. (A, Upper) The expressions of E-cadherin, PLCδ1, and β-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 μm.) (B) SW620 cells were transfected with either PLCδ1-overexpression vector or the relevant empty vector and then selected with G418 treatment for 8 d. The expressions of E-cadherin, PLCδ1, and β-actin (as loading control) in (Left) bulk or (Right) stable clone cells were determined by Western blotting. (C) PLCδ1-overexpressing stable clones (PLCδ1-1, -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 β-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 μm.) (E) The PLCδ1-overexpressing stable clone (PLCδ1-1) and the relevant control clone (Control-1) were transfected with siRNA against PLCδ1 (siδ1) 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 μm.)

Fig. 3. Knockdown of PLCδ1 but not PLCδ3 repressed the expression of E-cadherin in CRC cells. (A) Cells were transfected with negative control siRNA (siNeg) or siRNA for PLCδ1 (siδ1-1 or -2). After 7 d, the expression levels of E-cadherin and PLCδ1 were determined by qRT-PCR (n = 3). The relative mRNA expression, normalized by β-actin, is shown. Western blots for the expression levels of E-cadherin, PLCδ1, and β-actin (as loading control) are shown below. E-cad, E-cadherin. (B) DLD-1 cells transfected with siNeg, siδ1-1, or siδ1-2 were assessed by Western blots for E-cadherin, PLCδ1, and β-actin. Confocal microscope images of these cells stained with E-cadherin antibody (green) and hoechst33342 (blue) are also shown. (Scale bar: 30 μm.) (C) The expression levels of E-cadherin, PLCδ1, and PLCδ3 in SW620 cells transfected with siNeg, siδ1, or siRNA for PLCδ3 (siδ3) as indicated were determined by Western blots. (D) The expression levels of E-cadherin and PLCδ1 (human) mRNA in SW620 cells transfected with siNeg, siδ1 (human), and siδ3 (human) with mouse PLCδ1 (m1), or lipase activity-dead PLCδ1 (m31LD) expression plasmid were determined by qRT-PCR (n = 3). The relative mRNA expression, normalized by β-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.
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 PLCδ1 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 PLCδ1. MEK inhibitor treatment increased both PLCδ1 and E-cadherin transcriptional levels about two- to threefold in a dose-dependent manner (Fig. 6A). MEK inhibitor treatment also increased PLCδ1 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 PLCδ1 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 PLCδ1 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 PLCδ1 expression. The EGF-mediated PLCδ1 suppression in HEK293 cells was recovered by cotreatment with the MEK inhibitor (Fig. 6D). However, EGF did not suppress PLCδ1 expression in SW620 cells in which the KRAS/MEK signal is constitutively active because of the KRAS G13D mutation, whereas UO126 treatment up-regulated PLCδ1 expression (Fig. 6D). Knockdown of KRAS increased PLCδ1 and E-cadherin genes expression in SW620, HCT116, and DLD-1 cells, showing the involvement of KRAS in PLCδ1 repression (Fig. 6E). These results clearly indicated that activation of the KRAS/MEK signaling pathway suppressed PLCδ1 expression. In these experiments, E-cadherin expression was also assessed and shown to be changed similarly to PLCδ1 expression, but the expression of PLCδ1 tended to precede E-cadherin expression, because only PLCδ1 expression was up-regulated with a 4-h treatment of the MEK inhibitor in SW620 cells (Fig. 6D, SW620), although both E-cadherin and PLCδ1 expressions were up-regulated with a 48-h treatment with the MEK inhibitor (Fig. 6A).

**PLCδ1 Suppressed ERK1/2 Phosphorylation Through E-Cadherin.** Finally, we examined if PLCδ1 affects the KRAS/MEK/ERK signaling pathway. ERK1/2 phosphorylation levels were assessed by Western blots in control or PLCδ1-overexpressing stable cells. As shown in Fig. 7A, phosphorylated ERK1/2 was reduced in PLCδ1-overexpressing cells. The reduction was mitigated by siRNA-mediated PLCδ1 knockdown (Fig. 7B). Because MEK/ERK signaling is reportedly attenuated by E-cadherin (20), we assessed if suppression of ERK phosphorylation by ectopic PLCδ1 could be mediated by E-cadherin. As shown in Fig. 7C, the decreased ERK1/2 phosphorylation levels in PLCδ1-overexpressing cells were restored by E-cadherin knockdown to the levels of control cells. These results clearly indicate that PLCδ1 suppresses ERK1/2 phosphorylation by restoring E-cadherin expression.

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**Fig. 4.** PLCδ1 repressed EMT-associated gene expression. (A) The expressions of Vimentin and β-actin (as loading control) in SW620 cells that stably overexpress PLCδ1 (PLCδ1-1, -2, and -3) and control cells (Control-1 and -2) were determined by Western blotting. (B) Vimentin in control cells and PLCδ1-overexpressing SW620 cells was stained using an anti-Vimentin antibody and observed by immunofluorescence microscopy. (C and D) The mRNA expressions of Vimentin, TGF-β, Slug, Snail, and Zeb1 in SW620 cells that stably overexpresses PLCδ1 (PLCδ1-1 and -2) and control cells (Control-1 and 2) were determined by qRT-PCR (n = 3). The relative expression levels, normalized to β-actin expression, are shown. (E) Control or PLCδ1-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).

**Fig. 5.** The roles of PLCδ1 on malignant phenotypes of CRC cells. (A) Control or PLCδ1-overexpressing SW620 cells (Control-1 and -2 or PLCδ1-1 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 PLCδ1-overexpressing SW620 cells as described in Materials and Methods. The representative images of the colonies are shown in Fig. S5). (D) Control or PLCδ1-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 PLCδ1-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).
**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 PLCδ1 down-regulation.

To the best of our knowledge, we show here for the first time that PLCδ1 ectopic expression induced E-cadherin expression, whereas PLCδ1 suppression decreased E-cadherin expression in CRC. To investigate the link between the expressions of PLCδ1 and E-cadherin in other cancer types, we assessed PLCδ1 and E-cadherin levels 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 PLCδ1 and the link between total E-cadherin levels and PLCδ1 may be cell-content dependent, E-cadherin is regulated by PLCδ1 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-related genes, whereas E-cadherin expression was reduced by PLCδ1 knockdown. These results suggest that E-cadherin maintenance by PLCδ1 is not always correlated with these EMT inducers.

PLCδ1 is important for the intracellular Ca\(^{2+}\) maintenance in epithelial cells, especially keratinocytes. PLCδ1-KO mice have significantly decreased intracellular Ca\(^{2+}\) levels, resulting in the abnormal differentiation of epidermal and hair follicles (26). Ca\(^{2+}\) 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, PLCδ1 may contribute to intracellular Ca\(^{2+}\) maintenance, which is necessary for E-cadherin expression in CRC. Ca\(^{2+}\) activates several signaling molecules, including calcineurin/nuclear factor of activated T cells and PKC. Additional study to understand the molecules downstream of PLCδ1 that are necessary for its tumor-suppressive function is needed in future works.

In conclusion, we clarified that KRAS/MEK signaling represses PLCδ1 expression. Furthermore, we first elucidated that PLCδ1 protein levels were significantly reduced in CRC specimens.
concurred with the normal colonic epithelium by tissue microarray analysis. In this analysis, no association between PLCδ down-regulation. Because KRAS mutation is observed during the early stages in multistep processes of carcinogenesis (28), PLCδ down-regulation by KRAS/MEK signaling may also occur in the early stages of colorectal carcinogenesis. We also showed that PLCδ suppressed the KRAS/MEK/ERK pathway, suggesting a negative regulatory loop between KRAS/MEK/ERK signaling and PLCδ (Fig. 7D). From these results, we speculate that activation of PLCδ or PLCδ 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 PLCδ expression, enhancing E-cadherin expression, and attenuating KRAS/MEK/ERK signal attenuation. It is worth noting that PLCδ expression and tumor grade or stage was observed. This phe-

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 procedures for human CRC tissues were performed with a mouse antihuman E-cadherin Rabbit ABC Kit (Vector Laboratories) with anti-PLCδ 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 COLO205 cell lines were obtained from the Japanese Collection of Research Resources Cell Bank (National Institute of Health Sciences). These cells were maintained at 37 °C in a 5% (vol/vol) CO2 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 employed 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-β-actin antibody (Sigma), α-tubulin (GeneTex), Vimentin (Santa Cruz), and PLCδ (Santa Cruz) were used.

Migration and Invasion Assay. Migration assays were performed with cell culture insert with 8-µm-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 50 µL (2.5 mg/mL) BD Matrigel Basement Membrane Matrix Growth Factor Reduced (BD Biosciences) as described previously (31). Materials and methods for plasmin; 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.).