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ORIGINAL ARTICLE

Inhibition of protein kinase C delta leads to cellular senescence to induce anti-tumor effects in colorectal cancer

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Abstract

Protein kinase C delta (PKCδ) is a multifunctional serine–threonine kinase implicated in cell proliferation, differentiation, tumorigenesis, and therapeutic resistance. However, the molecular mechanism of PKCδ in colorectal cancer (CRC) remains unclear. In this study, we showed that PKCδ acts as a negative regulator of cellular senescence in p53 wild-type (wt-p53) CRC. Immunohistochemical analysis revealed that PKCδ levels in human CRC tissues were higher than those in the surrounding normal tissues. Deletion studies have shown that cell proliferation and tumorigenesis in wt-p53 CRC is sensitive to PKCδ expression. We found that PKCδ activates p21 via a p53-independent pathway and that PKCδ-kinase activity is essential for p21 activity. In addition, both repression of PKCδ expression and inhibition of PKCδ activity induced cellular senescence-like phenotypes, including increased senescence-associated β-galactosidase (SA-β-gal) staining, low LaminB1 expression, large nucleus size, and senescence-associated secretory phenotype (SASP) detection. Finally, a kinase inhibitor of PKCδ suppressed senescence-dependent tumorigenicity in a dose-dependent manner. These results offer a mechanistic insight into CRC survival and tumorigenesis. In addition, a novel therapeutic strategy for wt-p53 CRC is proposed.

KEYWORDS
cellular senescence, colorectal cancer, kinase inhibitor, protein kinase C delta, tumorigenesis

1 | INTRODUCTION

More than 1.9 million new cases of colorectal cancer (CRC) and 935,000 deaths reportedly occurred in 2020, representing approximately 1 in 10 cancer patients and deaths. Among cancers, colon cancer ranks third in terms of incidence and second in mortality.[1] CRC is a common malignancy worldwide. Epidemiological data show that the 5-year survival rate of patients with CRC is >90% for stage I patients; however, the survival rate is significantly lower for stage IV patients, at 10% survival.[2] Due to high recurrence and metastasis rates, patients with CRC have a poor prognosis, especially in more advanced stages. Although long-term survival rates for patients with CRC have improved due to advances in surgery and the development and advancement of chemoradiation, molecular

Abbreviations: CRC, colorectal cancer; GO, gene ontology; KD, knockdown; KO, knockout; HEPES, N-[2-hydroxyethyl]piperazine-N′-2-ethane-sulfonic acid; PI, propidium iodide; PKCδ, protein kinase C delta; qPCR, quantitative real-time polymerase chain reaction; SASP, senescence-associated secretory phenotype; SA-β-gal, senescence-associated β-galactosidase; UICC, Union for International Cancer Control.

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targeted therapy, and immunotherapy,[3–5] CRC outcomes remain unsatisfactory.

Kinase inhibitors for cancer therapy began to be approved about 20 years ago, and more aggressive development of kinase inhibitors has occurred in the last decade.[6–8] Kinases are critical factors in intracellular signaling pathways and are implicated in physiological processes such as homeostasis and development, as well as tumor formation, growth, invasion, and metastasis.[9–12] There are many cases of abnormal cell proliferation due to excessive protein kinase activity in cancer cells, and the development of precise molecular-targeted drugs acting only on specific protein kinases has been actively pursued.[13] Therefore, it is important to identify new protein kinases as target molecules to further understand the molecular pathogenesis of CRC and improve therapeutic outcomes.

Cellular senescence, first reported by Hayflick and Moorhead, is an irreversible cell cycle arrest caused by several factors[14] and is characterized by morphological changes such as hypertrophy and flattening, increased senescence-associated β-galactosidase (SA-β-gal) activity, and senescence-associated secretory phenotype (SASP).[15,16] Cellular senescence is predominant in normal tumor tissues and various precancerous lesions in humans and mice but is decreased in malignant tumors.[17] It is considered a physiological barrier to tumor development and progression.[18,19] Therefore, therapies that induce cellular senescence in tumors have potential as novel therapeutic strategies. Several studies have shown that genes aberrantly expressed in cancer cells, such as *AKAP95* and *ZNF768*, are involved in the regulation of tumor cell senescence and are potential therapeutic targets.[20–22] Additionally, p21 stability and activity are regulated by phosphorylation.[23,24] Thus, kinase activity may influence cellular senescence.

In this study, we identified protein kinase C delta (PKCδ) as a novel therapeutic target kinase for inducing cellular senescence in CRC. We found that PKCδ expression was higher in tumor tissues than in normal tissues of patients with CRC, and PKCδ expression was negatively correlated with p21 expression in p53 wild-type (wt-p53) CRC patients. Furthermore, we demonstrated that inhibition of PKCδ in wt-p53 CRC cell lines induces cellular senescence in a p21-dependent manner and significantly inhibits tumor growth.

2 | MATERIALS AND METHODS

2.1 | Patient data

Forty-five patients who underwent initial surgery for colorectal cancer at Jikei University Hospital between 2009 and 2012 were analyzed. In addition, right-sided colon cancer samples were collected from 71 patients who underwent initial surgery for right-sided colon cancer (cecal cancer to transverse colon cancer) at Jikei University Hospital between 2009 and 2016. The specimens were retrospectively reviewed using blocks stored in the hospital's pathology department. Colorectal cancer staging followed TNM staging according to the Union for International Cancer Control (UICC) 8th edition.

2.2 | Cell culture

The human CRC cell lines HCT116, RKO, LoVo, DLD-1, SW480, and SW620 were obtained from the JCRB Cell Bank or ATCC. HCT116 p53(−/−) cells were purchased from Horizon Discovery. All cells were maintained in DMEM (Nacalai Tesque) supplemented with 10% FBS (Biowest, Nuaillé, France) and 1% penicillin–streptomycin (Nacalai Tesque). Cells were cultured at 37°C in a 5% CO_2 incubator.

2.3 | CRISPR/Cas9-mediated knockout in HCT116 cells

PKCδ-knockout (KO) HCT116 cells were generated using the CRISPR/Cas9 system. Two independent guide RNAs for PKCδ were designed as described previously.[25]

2.4 | siRNA transfection

Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) was used to achieve transient knockdown with 20 nM siRNA according to the manufacturer's instructions (Table S1).

2.5 | Xenograft studies

Seven-week-old female nude mice were obtained from CLEA. The animals were maintained in a pathogen-free animal facility at Jikei University School of Medicine. The mice were randomly assigned to the indicated groups. The cell count was set to 1×10^6, suspended in Matrigel in a total of 100 μL, and injected subcutaneously into the backs of the mice. Tumor size was determined by caliper measurements of the largest (x) and smallest (y) vertical diameters and calculated according to the formula $V = \pi/6 \times xy^2$.

2.6 | Statistical analysis

Data are presented as the mean ± SD. Statistical significance of differences was evaluated using a two-tailed Student's t-test or one-way ANOVA. The relationship between clinicopathological factors and stainability was analyzed using the χ^2-test or Fisher's exact test. Differences were considered statistically significant at $p < 0.05$. Statistical analyses were performed using Prism 8 software (GraphPad, San Diego, CA, USA).

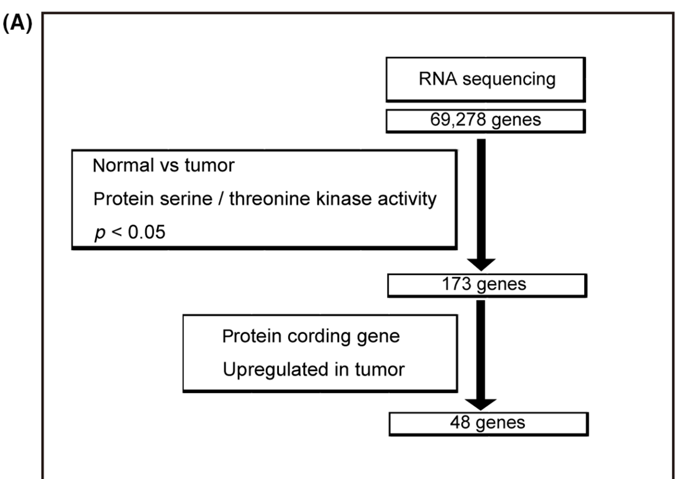
2.7 | Supplementary Material and Methods

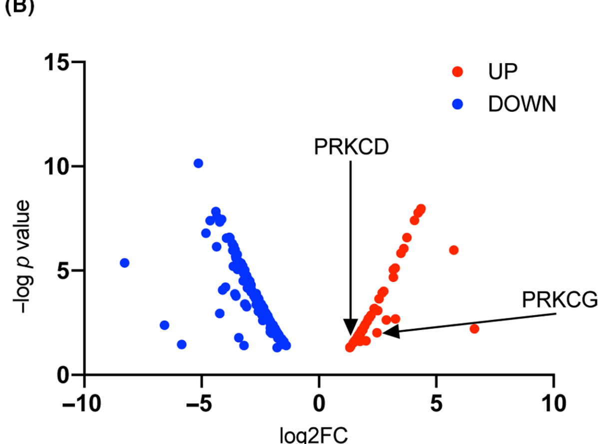
Details of other material and methods are clarified in Appendix S1.

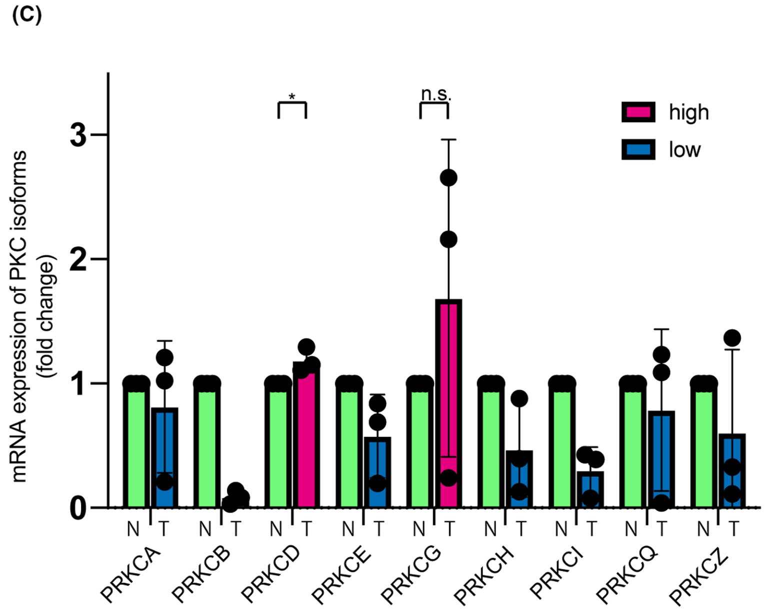
3 | RESULTS

3.1 | Protein kinase C delta is upregulated in human CRC tissues

To explore novel targets of protein kinases against CRC, we categorized serine/threonine kinase activity and examined genes with variable expression in GO analysis. We found 48 serine/threonine kinases upregulated in cancer tissues compared to normal tissues (Figure 1A,B; Table S2). Specifically, PKCδ showed significantly elevated expression and high reproducibility when compared among its isoforms (Figure 1C). Therefore, we examined the role of PKCδ in CRC. PKCδ expression was validated by immunostaining using matched pairs of tumors and adjacent normal tissues from patients







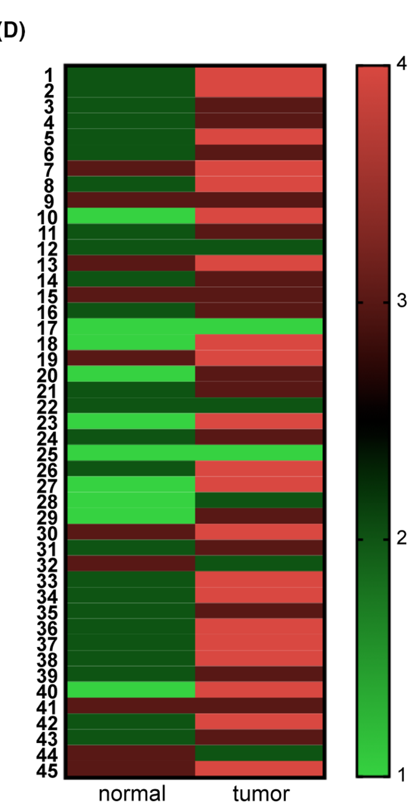


FIGURE 1 Protein kinase C delta (PKCδ) is identified as a highly expressed gene among serine–threonine kinases in colorectal cancer (CRC). (A) Representative extraction diagram showing candidate genes identified by RNA sequencing analysis. Gene expression was compared between normal and tumor tissues in the colon of one representative CRC patient with wt-p53. (B) Volcano plots of genes with variable expression in GO analysis categorized by serine/threonine kinase activity. Red plots show increased expression (48 genes) and blue plots show decreased expression (125 genes). (C) mRNA level as fold changes of PKC isoforms was calculated by relative comparison to normal tissue. N, normal tissue; T, tumor tissue. Data are presented as mean ± SD ($n = 3$). *$p = 0.0304$. n.s., not significant (paired two-tailed Student's t-test). (D) Normal and tumor tissues from patients with CRC were scored from 1 to 4 by immunohistochemistry and shown in a heatmap ($n = 45$).

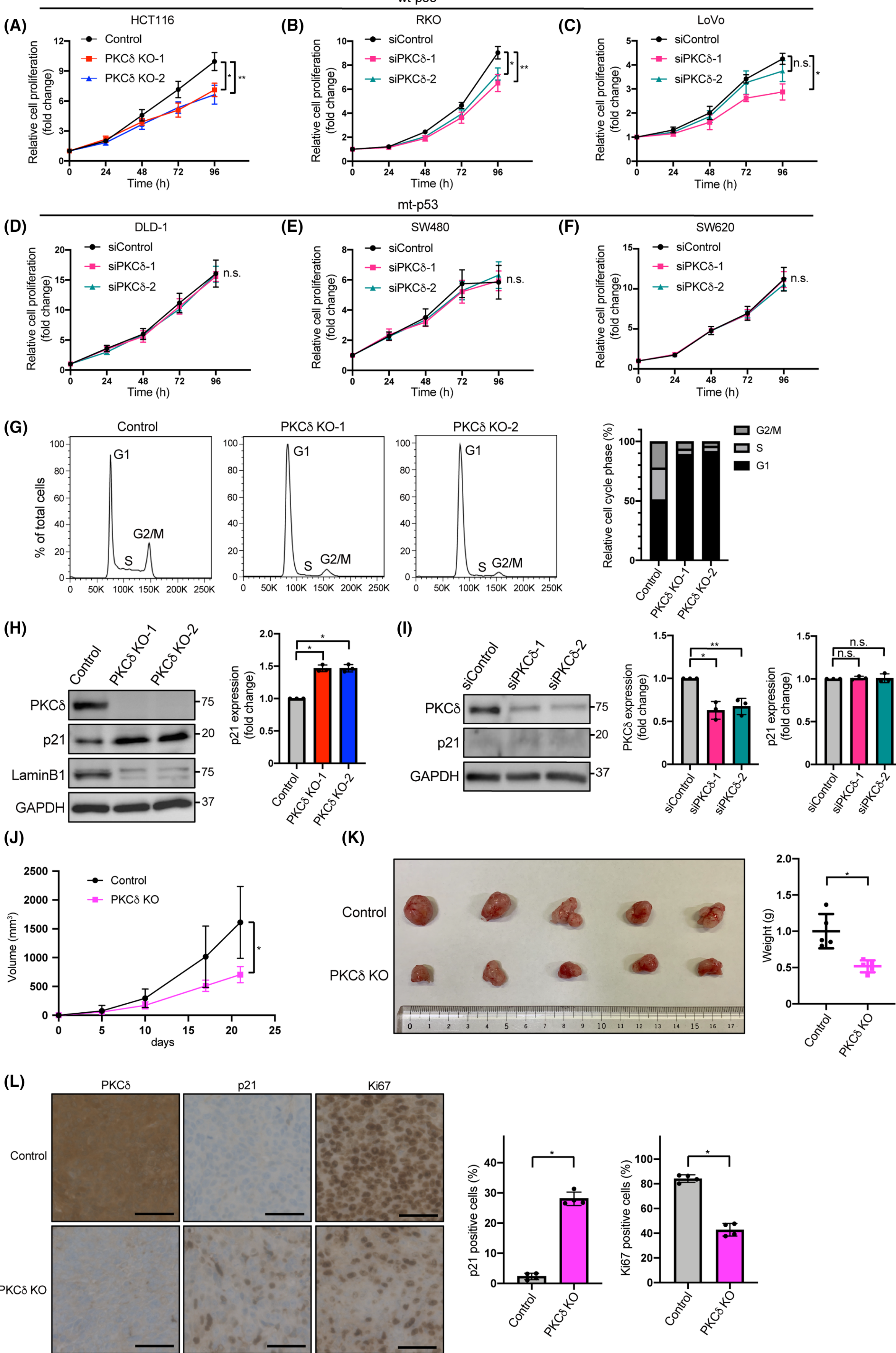

wt-p53
(A)
HCT116
Control
PKCδ KO-1
PKCδ KO-2
Relative cell proliferation (fold change)
Time (h)
(B)
RKO
siControl
siPKCδ-1
siPKCδ-2
(C)
LoVo
n.s.
mt-p53
(D)
DLD-1
(E)
SW480
(F)
SW620
(G)
G1
S
G2/M
% of total cells
Relative cell cycle phase (%)
(H)
PKCδ
p21
LaminB1
GAPDH
p21 expression (fold change)
(I)
PKCδ expression (fold change)
(J)
Volume (mm³)
days
PKCδ KO
(K)
Weight (g)
(L)
Ki67
p21 positive cells (%)
Ki67 positive cells (%)

FIGURE 2 Protein kinase C delta (PKCδ) is involved in tumor growth in wt-p53 colorectal cancer (CRC) cell lines. (A) Cell proliferation assay of control (parental HCT116) cells and PKCδ-KO (PKCδ KO-1, PKCδ KO-2) cells. Cell proliferation was shown by MTS assay. Data are presented as mean ± SD ($n = 4$). *$p = 0.0021$, **$p = 0.0007$ (one-way ANOVA followed by Bonferroni's multiple comparisons test). (B) Cell proliferation assay of RKO cells treated with control non-targeting siRNA (siControl) or PKCδ-specific siRNA (siPKCδ-1 and siPKCδ-2). Cell proliferation was shown by MTS assay. Data are shown as mean ± SD ($n = 4$). *$p = 0.0041$; **$p = 0.0004$ (one-way ANOVA followed by Bonferroni's multiple comparisons test). (C) Cell proliferation assay of LoVo cells treated with control non-targeting siRNA (siControl) or PKCδ-specific siRNA (siPKCδ-1 and siPKCδ-2). Cell proliferation was measured by MTS assay. Data are shown as mean ± SD ($n = 4$). *$p = 0.0006$. n.s., not significant (one-way ANOVA followed by Bonferroni's multiple comparisons test). (D) Cell proliferation assay of DLD-1 cells treated with control non-targeting siRNA (siControl) or PKCδ-specific siRNA (siPKCδ-1 and siPKCδ-2). Cell proliferation was measured by MTS assay. Data are shown as mean ± SD ($n = 4$). n.s., not significant (one-way ANOVA followed by Bonferroni's multiple comparisons test). (E) Cell proliferation assay of SW480 cells treated with control non-targeting siRNA (siControl) or PKCδ-specific siRNA (siPKCδ-1 and siPKCδ-2). Cell proliferation was measured by MTS assay. Data are shown as mean ± SD ($n = 4$). n.s., not significant (one-way ANOVA followed by Bonferroni's multiple comparisons test). (F) Cell proliferation assay of SW620 cells treated with control non-targeting siRNA (siControl) or PKCδ-specific siRNA (siPKCδ-1 and siPKCδ-2). Cell proliferation was measured by MTS assay. Data are shown as mean ± SD ($n = 4$). n.s., not significant (one-way ANOVA followed by Bonferroni's multiple comparisons test). (G) Cell cycle analysis by flow cytometry with propidium iodide (PI) staining in control and PKCδ-KO cells in HCT116. (H) Immunoblot analysis of PKCδ, p21, LaminB1, and GAPDH (loading control) in control and PKCδ-KO cell lysates. Migration of molecular weight markers is shown on the right (kDa). Protein level as fold change of p21 was calculated by comparing protein levels relative to those of control cells after normalization to GAPDH. Data are shown as mean ± SD ($n = 3$). *$p < 0.0001$ (one-way ANOVA followed by Bonferroni's multiple comparisons test). (I) Immunoblot analysis of PKCδ, p21, and GAPDH (loading control) in lysates of DLD-1 cells treated with control non-targeting siRNA (siControl) or PKCδ specific siRNA (siPKCδ-1 and siPKCδ-2). Migration of molecular weight markers is shown on the right (kDa). Protein level as fold changes of PKCδ and p21 was calculated by comparing protein levels relative to those of siControl cells after normalization to GAPDH. Data are shown as mean ± SD ($n = 3$). *$p = 0.0027$, **$p = 0.0052$. n.s., not significant (one-way ANOVA followed by Bonferroni's multiple comparisons test). (J) Tumor volumes were measured at indicated time points. Data are shown as mean ± SD ($n = 5$ per group). *$p = 0.0132$ (paired two-tailed Student's *t*-test). (K) Images of tumors at day 21 necropsy of nude mice are shown (left), and tumor mass was measured at that time (right). Data are shown as mean ± SD ($n = 5$ per group). *$p = 0.0025$ (paired two-tailed Student's *t*-test). (L) Representative images of immunohistochemistry staining of PKCδ, p21, and Ki67 in xenograft tumor tissue are shown. Scale bar represents 50 μm. The percentage of cells positive for p21 and PKCδ was calculated from four different magnified fields of view as a percentage of the total number of positive cells in the field of view. Data are shown as mean ± SD ($n = 4$). *$p < 0.0001$ (paired two-tailed Student's *t*-test).

who had undergone surgery for CRC ($n = 45$) (Table S3). According to the scores of immunostaining degree (Figure S1), PKCδ expression was significantly higher in cancer tissues than that in normal tissues (Figure 1D). Additionally, we confirmed the expression of PKCδ at the protein level in various human CRC cell lines (Figure S2). Bioinformatic analysis of overall survival using the Kaplan–Meier plotter (http://kmplot.com/analysis/index.php?p=background) showed a relationship between PKCδ and CRC (Figure S3). These results suggest that PKCδ expression is involved in CRC progression.

3.2 | Protein kinase C delta is involved in tumor growth in wt-p53 CRC cell lines

To investigate the biological effects of PKCδ on cancer, we generated PKCδ-KO cells (PKCδ KO-1 and PKCδ KO-2) using the wt-p53 human CRC cell line HCT116 by the CRISPR knockout system. Cell proliferation assays showed that cell growth was significantly inhibited in PKCδ-KO cells compared to that in control cells (Figure 2A). Similar results were obtained from PKCδ-knockdown (KD) cells (Figure S4A). For other wt-p53 CRC cell lines, RKO and LoVo, PKCδ-KD cells also showed significant suppression of cell growth (Figures 2B,C and S4B,C). However, for the p53 mutant-type (mt-p53) CRC cell lines, DLD-1, SW480, and SW680, PKCδ-KD cells showed no change in tumor cell growth (Figures 2D–F and S4D,E). To examine the mechanism by which cell proliferation is suppressed in wt-p53 CRC cell lines, cell-cycle analysis was performed using HCT116 cells. In HCT116 PKCδ-KO cells, the percentage of G1-phase cells increased and the percentage of S-phase and GS/M-phase cells decreased compared to that in control cells (Figure 2G). These results suggest that the inhibitory effect of the suppression of PKCδ expression on tumor cell growth is characteristic of wt-p53 CRC and is due to G1 phase arrest. Subsequent protein expression analysis with HCT116 PKCδ-KO cells revealed significant upregulation of p21, a cyclin-dependent kinase inhibitor (Figure 2H). Similar results were obtained in HCT116 PKCδ-KD cells, such as increased expression of p21 and altered expression of factors involved in the cell cycle (Figure S4F). However, for the mt-p53 CRC cell line, DLD-1, PKCδ-KD cells showed no change in p21 expression (Figure 2I). These results suggest that PKCδ-induced inhibition of cell-growth inhibition is p21-dependent.

To investigate the biological impact of PKCδ on CRC, we subcutaneously inoculated HCT116 PKCδ-KO cells into mice. We evaluated tumor size in control and PKC-KO xenografts at necropsy and found that PKC-KO cell-bearing tumors were significantly smaller than control xenografts (Figure 2J). Similarly, the tumor weight of PKCδ-KO xenografts was significantly lesser than that of the control xenografts (Figure 2K). Immunohistochemical analysis of xenograft tumors revealed that PKCδ and p21 expression were inversely correlated, and Ki67 expression (a marker for active cell proliferation) in PKCδ-KO xenograft tumors was significantly downregulated compared to the control xenograft tumors (Figure 2L). These results indicate that PKCδ is involved in the tumor growth of wt-p53 CRC.

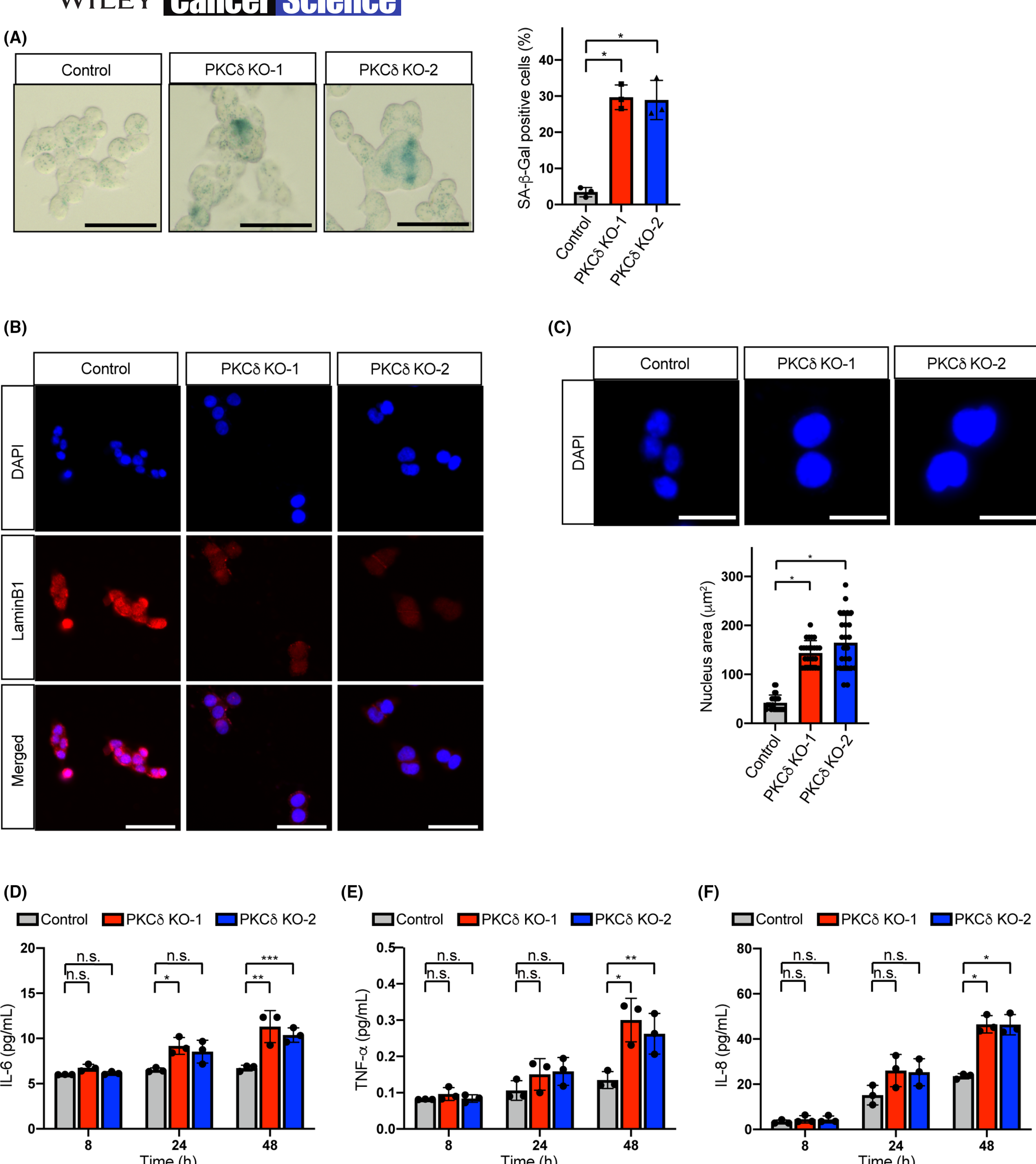


FIGURE 3 Suppression of protein kinase C delta (PKCδ) expression induces cellular senescence. (A) Representative images of SA-β-gal staining (blue) in control and PKCδ-KO cells are shown. Scale bar represents 50 μm. The percentage of SA-β-gal positive cells to the total number of cells/well was evaluated in three independent experiments. Data are shown as mean ± SD ($n = 3$). *$p = 0.0003$ (one-way ANOVA followed by Bonferroni's multiple comparisons test). (B) Control and PKCδ-KO cells were fixed and immunofluorescently stained with anti-LaminB1 antibody (red). Nuclei were stained with DAPI (blue). Scale bars, 50 μm. (C) Nuclei of control and PKCδ-KO cells were stained with DAPI; scale bars, 20 μm. The area of nuclei of control and PKCδ-KO cells was measured. Data are presented as mean ± SD ($n = 25$). *$p < 0.0001$ (one-way ANOVA followed by Bonferroni's multiple comparisons test). (D–F) Control and PKCδ-KO cells were seeded, respectively, and the concentration of SASP in the cell culture medium was measured by ELISA at 8, 24 and 48 h. Data are presented as mean ± SD ($n = 3$). The concentration of IL-6 in the culture medium was indicated (D). *$p = 0.00241$; **$p = 0.0053$; ***$p = 0.0155$; n.s., not significant. The concentration of TNF-α in culture medium was indicated (E). *$p = 0.0124$; **$p = 0.0380$. n.s., not significant. The concentration of IL-8 in culture medium was indicated (F). *$p = 0.0004$. n.s., not significant (one-way ANOVA followed by Bonferroni's multiple comparisons test).

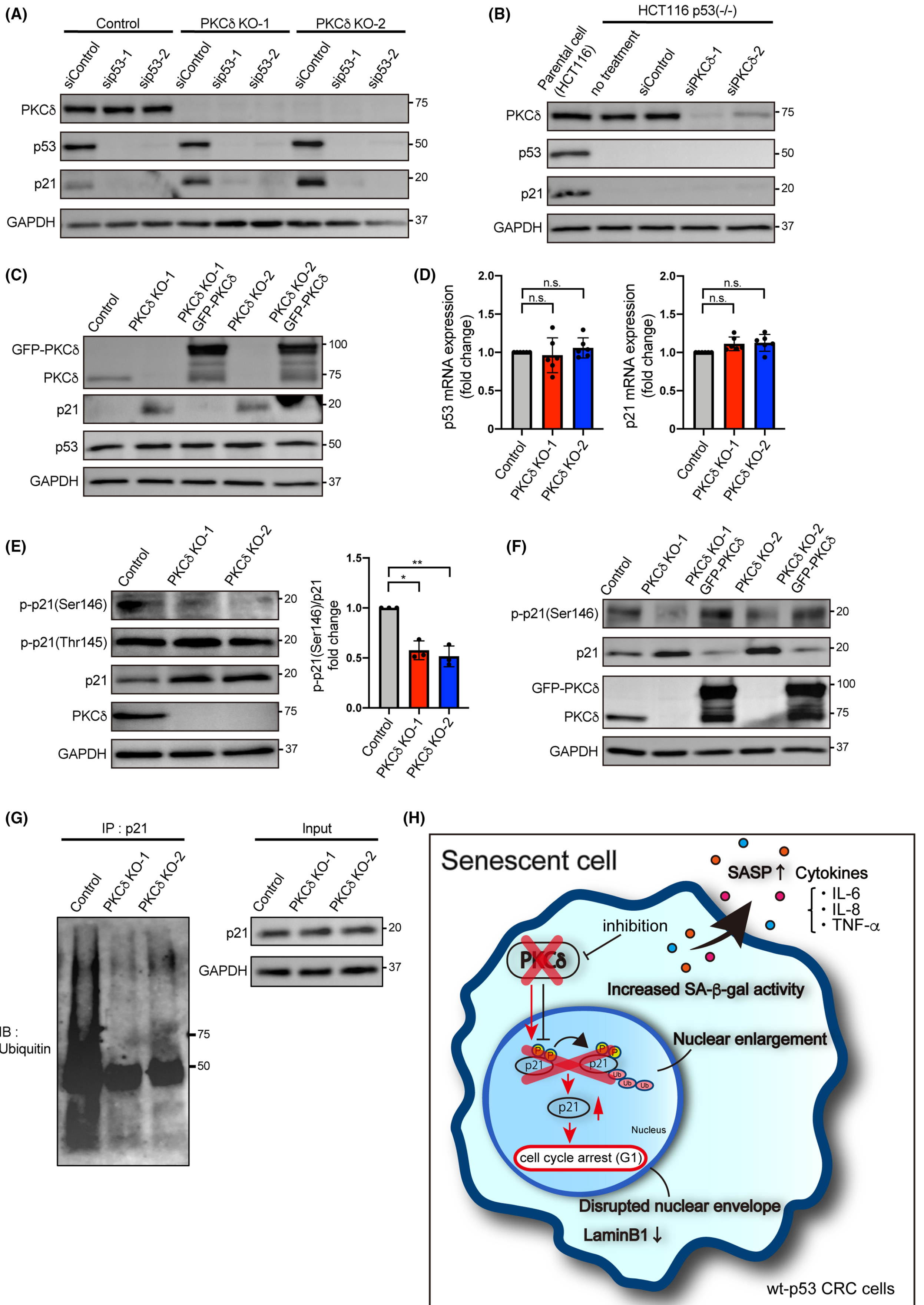


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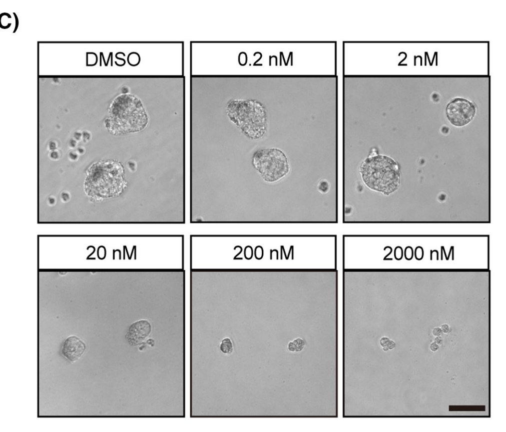
FIGURE 4 Protein kinase C delta (PKCδ) phosphorylates p21 independent of p53 and degrades p21 in the ubiquitin-proteasome system. (A) Control cells and PKCδ-knockout (KO) cells transformed with control non-targeting siRNA (siControl) or p53-specific siRNA (sip53-1 and sip53-2) were lysed and analyzed for PKCδ, p53, p21, and GAPDH (loading control) by immunoblotting. Migration of molecular weight markers is shown on the right (kDa). (B) No-treatment HCT116 p53(−/−) cells or HCT116 p53(−/−) cells transformed with control non-targeting siRNA (siControl) or PKCδ-specific siRNA (siPKCδ-1 and siPKCδ-2) were lysed and analyzed for PKCδ, p53, p21, and GAPDH (loading control) by immunoblotting. Parental HCT116 cells were also used as positive controls. Migration of molecular weight markers is shown on the right (kDa). (C) Control and PKCδ-KO cells and GFP-PKCδ transfected PKCδ-KO cells were lysed and subjected to immunoblot analysis for PKCδ, p21, p53, and GAPDH (loading control). Migration of molecular weight markers is shown on the right (kDa). (D) mRNA expression of p53 and p21 in control and PKCδ-KO cells was measured by qPCR. HPRT1 was used as an internal standard, and fold change was calculated by comparing mRNA expression levels relative to those of control cells. Data are shown as mean ± SD ($n = 6$). n.s., not significant (one-way ANOVA followed by Bonferroni's multiple comparisons test). (E) Immunoblot analysis of phospho-p21 (Ser146), phospho-p21 (Thr145), p21, PKCδ, and GAPDH (loading control) in lysates from control and PKCδ-KO cells. Migration of molecular weight markers is shown on the right (kDa). Protein levels of p-p21(Ser146) and p21 were normalized with those of GAPDH, and the amount of change was calculated by comparing p-p21 (Ser146)/p21 in PKCδ-KO cells relative to that in control cells. Data are shown as mean ± SD ($n = 3$). *$p = 0.0014$; **$p = 0.0007$ (one-way ANOVA followed by Bonferroni's multiple comparisons test). (F) Control and PKCδ-KO cells transfected with GFP-PKCδ were lysed and immunoblot analyzed for phospho-p21 (Ser146), p21, PKCδ, and GAPDH (loading control). Migration of molecular weight markers is shown on the right (kDa). (G) Immunoprecipitation analysis of control and PKCδ-KO cells transfected with HA-tagged ubiquitin. Accumulation of polyubiquitinated proteins was induced by MG132 treatment (5 μM, 5 h) prior to cell lysis. Cells were analyzed by immunoprecipitation and immunoblotting using the antibodies shown. Migration of molecular weight markers is shown on the right (kDa). (H) Inhibition of genetic targeting of PKCδ in HCT116 colorectal cancer (CRC) cells (wt-p53) causes not only cell cycle arrest but also induction of cell senescence and even secretion of SASP, resulting in cell growth inhibition.

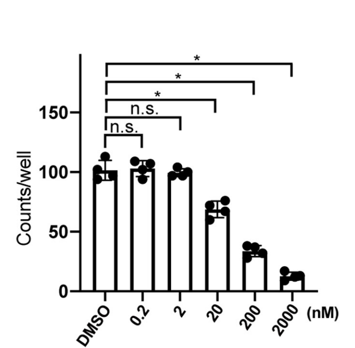
3.3 | Suppression of protein kinase C delta expression induces cellular senescence

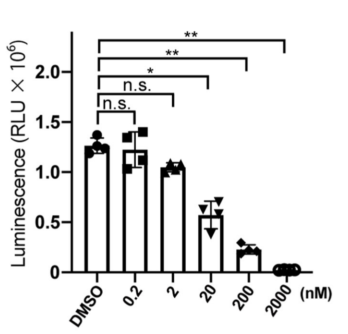
To verify whether PKCδ-KO cells affect cellular senescence, we performed SA-β-gal staining and found that the ratio of SA-β-gal staining-positive cells in PKCδ-KO cells was significantly higher than that in control cells (Figure 3A). Protein expression analysis of senescence markers in PKCδ-KO cells showed that the expression of LaminB1 was significantly decreased (Figure 2H). This was confirmed by immunofluorescence, where we observed a decrease in LaminB1 staining in the PKCδ-KO cells compared to the control CRC cells (Figure 3B). DAPI staining also revealed marked nuclear enlargement in PKCδ-KO cells (Figure 3C). We also confirmed that the cellular senescence phenotype induced by PKCδ-KO is reversed by p21-KD (Figure S5A–C). To examine SASPs, we subsequently measured the amounts of representative SASP factors—IL-6, TNF-α, and IL-8—in culture supernatants by ELISA. There was a significant increase in secretion over time in the PKCδ-KO cells compared to that in the control CRC cells (Figure 3D-F). In addition, we found increased secretion of SASP at the mRNA level (Figure S6A–C). These results are consistent with senescence changes, indicating that suppression of PKCδ expression induces cellular senescence.

3.4 | Protein kinase C delta phosphorylates p21 in a p53-independent manner and degrades p21 in the ubiquitin-proteasome pathway

To elucidate the mechanism by which p21 was upregulated by the suppression of PKCδ expression, we first focused on p53, a tumor suppressor gene product upstream of p21. p53-KD in control and PKCδ-KO cells also suppressed p21 expression (Figure 4A). p21 expression was not observed in control or PKCδ-KD cells in HCT116 p53(−/−) cells (Figure 4B). These results indicate that p53 is required for p21 expression. Next, the role of PKCδ in p21 regulation was verified by transfecting GFP-PKCδ into PKCδ-KO cells. Transfection of PKCδ-KO cells with GFP-PKCδ resulted in a return of p21 expression but no change in p53 expression levels (Figure 4C). We then analyzed the expression of p53 and p21 at the transcriptional level. On comparing control cells with PKCδ-KO cells, we found little, if any, difference in the mRNA expression levels of both p53 and p21 (Figure 4D). These results indicate that PKCδ acts on p21 at the post-translational level in a p53-independent manner. In this regard, phospho-p21 (Ser146) expression was suppressed in the PKCδ-KO cells (Figure 4E). GFP-PKCδ transfection into PKCδ-KO cells restored the levels of p21, and phospho-p21 (Ser146) levels were also increased (Figure 4F). HA-ubiquitin was co-expressed in control and PKCδ-KO cells, and immunoprecipitation with an anti-p21 antibody and immunoblot analysis with an anti-ubiquitin antibody was performed. Immunoprecipitation analysis demonstrated that PKCδ-KO cells lacked the ability to bind to p21 and ubiquitin (Figure 4G). To further explore protein stability and ubiquitination by phosphorylation, HCT116 cells were transfected with p21_WT, p21_Ser146D (the phospho-mimic mutant), or p21_Ser146A (the phospho-deficient mutant). Immunoprecipitation with anti-p21 antibody and immunoblot analysis with anti-ubiquitin antibody were performed. Immunoprecipitation analysis revealed that cells transfected with p21_Ser146A markedly lacked the ability to bind p21 and ubiquitin (Figure S7). These results suggest that PKCδ phosphorylates p21 (Ser146) and degrades p21 via the ubiquitin–proteasome system. They also show that suppression of PKCδ expression in wt-p53 HCT116 inhibits p21 (Ser146) phosphorylation and prevents p21 degradation, which allows p21 accumulation and cell cycle arrest, as well as cellular senescence (Figure 4H).







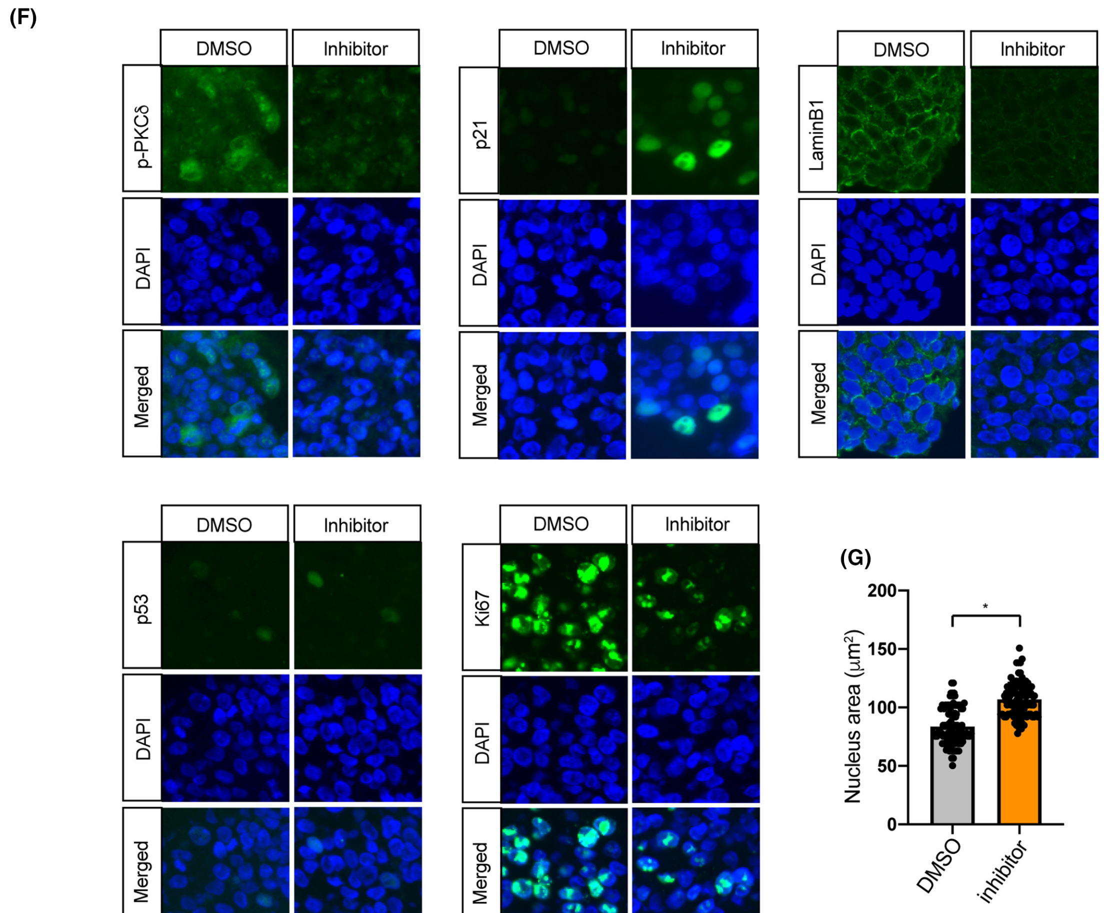


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FIGURE 5 VTX-27 suppresses tumorigenesis in a p21-dependent way. (A) Immunoblot analysis of phospho-PKCδ (Thr505), phospho-p21 (ser146), p21, and GAPDH (loading control) in HCT116 colorectal cancer (CRC) cell lysates treated with DMSO or VTX-27 for 48 hours. Migration of molecular weight markers is shown on the right (kDa). (B) Cell proliferation assay of HCT116 cells treated with DMSO or VTX-27. Cell proliferation was measured by the MTS assay. Data are shown as mean ± SD ($n = 3$). *$p = 0.0015$; **$p < 0.0001$. n.s., not significant (one-way ANOVA followed by Bonferroni's multiple comparisons test). (C) three-dimensional multicellular spheroid formation after 7 days of culture of HCT116 cells on ultra-low adhesion plates and treatment with DMSO or VTX-27. Scale bar indicates 50 μm. (D) The number of spheroids larger than 40 μm per well was counted. Data are shown as mean ± SD ($n = 4$). *, $p < 0.0001$. n.s., not significant (one-way ANOVA followed by Bonferroni's multiple comparisons test). (E) Cell viability of spheroids was measured by ATP luminescence. The 3D Cell Viability Assay was used to measure cell viability. Data are presented as mean ± SD ($n = 4$). *$p < 0.0345$; **$p < 0.0001$. n.s., not significant (one-way ANOVA followed by Bonferroni's multiple comparisons test). (F) Spheroids formed by treatment with DMSO or VTX-27200 nM were immunofluorescently stained with anti-phospho-PKCδ (Thr505), anti-p21, anti-LaminB1, anti-p53, and anti-Ki67 antibodies (green). Nuclei were stained with DAPI (blue). Scale bar indicates 50 μm. (G) The area of nuclei of control (DMSO) cells and cells treated with VTX-27 was measured. Data are presented as mean ± SD ($n = 100$). *$p < 0.0001$ (paired two-tailed Student's *t*-test).

3.5 | VTX-27 suppresses tumorigenesis in a p21-dependent way

To investigate the biological effects of PKCδ inhibitors on senescence in CRC cells, we used PKCδ inhibitors. Among various PKCδ inhibitors, including rottlerin, we selected VTX-27, an inhibitor particularly selective for PKCδ.[26] We confirmed a concentration-dependent decrease in phospho-PKCδ (Thr505) by VTX-27 at the protein level, a decrease in phospho-p21 (Ser146) expression, and an increase in p21 expression (Figure 5A). We evaluated the IC50 in HCT116 cells (Figure S8A) and demonstrated a concentration-dependent decrease in the proliferative potential of VTX-27 in the proliferation assay (Figure 5B). To investigate this phenomenon in an environment similar to the in vivo environment, we performed spheroid formation assays. A significant concentration-dependent reduction in spheroid size was observed at concentrations with VTX-27 above 20 nM (Figures 5C and S8B). Cell count and luminescence measurements also confirmed the effect of this inhibitor on tumor formation (Figure 5D,E). We performed apoptosis assays to assess the effect of VTX-27 on cell death on HCT116 cells but found no apparent apoptosis (Figure S8C). We subsequently performed immunofluorescence staining of spheroids. We confirmed decreased expression of phospho-PKCδ (Thr505) by VTX-27 and increased expression of p21 and decreased expression of LaminB1 (Figure 5F). We also revealed nuclear enlargement in cells treated with VTX-27 (Figure 5G). Contrastingly, no change in p53 expression was observed (Figure S9A). The decrease in Ki67 expression by VTX-27 was also indicative of decreased proliferative capacity (Figure S9B). These data demonstrate that the suppression of PKCδ activity in a near-in vivo environment induces p53-independent cellular senescence and leads to antitumor effects.

3.6 | Protein kinase C delta in human wt-p53 colorectal cancer tissues correlates positively with cancer progression and negatively with p21 expression

The incidence of p53 mutations varies depending on the site of tumor development in the colon, and their frequency has been reported to be higher in left-sided (distal) CRC than in right-sided (proximal) CRC.[27,28] To collect cases of wt-p53 in human cancer tissue, 71 cases of right-sided colon cancer were randomly selected and their p53 status was determined by immunostaining patterns (Figure S10A; refs. [29]). Of the 71 cases, 32 had wt-p53 CRC and 37 had mt-p53 CRC. Interestingly, clinical stage and T classification were positively correlated with staining intensity in wt-p53 (Figure 6A; Table S4). However, there was no obvious difference in mt-p53 (Figure 6A; Table S5). These results suggest that PKCδ functions in a tumor-promoting manner in wt-p53 CRC. Based on these findings, we evaluated p21 expression in wt-p53 CRC. Intriguingly, the weaker the staining of PKCδ, the higher the expression of p21; the stronger the staining of PKCδ, the lower the expression of p21 (Figures 6B and S10B). These results suggest that the positive correlation between PKCδ expression and cancer progression in wt-p53 CRC is due to the abrogation of G1 phase arrest mediated by p21. Data from human tissues are consistent with those from in vitro experiments.

4 | DISCUSSION

We concentrated on the PKC family, which functions differently in different types of cancer[30] and PKCδ, which was significantly upregulated. Intriguingly, some reports show the low expression of PKCδ, while recent reports show its high expression, indicating controversial findings on PKCδ expression in CRC.[31–34] Additionally, we confirmed that PKCδ expression was significantly elevated in CRC tissues based on immunostaining analysis.

In functional analysis of PKCδ, PKCδ inhibition increased p21 protein levels. p21 is a key player in the PKCδ-dependent cellular senescence. Interestingly, this phenomenon appears to be p53-independent. Pharmacological inhibition of PKCδ in wt-p53 cell lines increased p21 protein levels in a p53-independent manner, whereas restoring PKCδ function reversed the increase in p21 protein levels. This mechanism was supported by the result of the suppression of phosphorylation of p21 by PKCδ, which suppressed ubiquitin-proteasomal degradation of p21 and resulted in p21 accumulation. Although direct involvement of p21 in PKCδ-mediated biological effects has been reported,[35] its role in cellular senescence in CRC

FIGURE 6 In wt-p53 colorectal cancer, protein kinase C delta (PKCδ) expression is positively and negatively correlated with cancer progression and p21 expression, respectively. (A) Tumor tissues from colorectal cancer patients were immunohistochemically stained with anti-PKCδ antibody, divided into high and low staining, and the correlation between stage classification and staining intensity and T classification and staining intensity were plotted (wt-p53; $n = 32$, mt-p53; $n = 39$). (B) Immunohistochemical staining of wt-p53 colorectal tumor tissue with anti-PKCδ and anti-p21 antibodies. Representative samples with positive and negative p21 expression are presented. Scale bar indicates 100 μm. The percentage of cells positive for p21 is the ratio of the number of positive cells to the total number of cells in the field of view and was calculated from four different magnified fields of view. Data are shown as mean ± SD ($n = 6$). $^{*}p < 0.0001$ (paired two-tailed Student's t-test).

has never been elucidated. Our findings show that PKCδ induces cellular senescence through its involvement in phosphorylation of p21, which is the first report to establish direct regulation of p21 by PKCδ.

Cellular senescence, defined as a state of cell cycle arrest, has a potent inhibitory effect on tumorigenesis. Thus, molecules that promote senescence have long been expected to be used as anticancer agents.[19,36] In this study, we showed that PKCδ plays an important role in cellular senescence in wt-p53 CRC. In the CRC cell line HCT116, inhibition of PKCδ induced marked cellular senescence, resulting in the production of several cytokines involved in SASP. This finding provides evidence that PKCδ-dependent cellular senescence is induced by cyclin-dependent kinase inhibitor p21-mediated senescence.

Protein kinase C delta, a PKC isoform, activates p21 in a p53-dependent manner in response to DNA damage and cell cycle.[37–40] Hence, PKCδ functions as a tumor suppressor. Conversely, high PKCδ expression has been correlated with poor prognosis, and PKCδ has been reported to have tumor-promoting functions in liver and breast cancers.[41–43] PKCδ is known to be associated with anticancer drug resistance in lung cancer.[44] In this study, PKCδ was highly expressed in CRCs. We also found that it suppresses senescence in wt-p53 CRC cells and contributes to tumor enhancement. This suggests that PKCδ may function as a tumor-promoting factor in CRC, whereas PKCδ functions as a tumor suppressor in precancerous lesions, which are susceptible to DNA damage and may function progressively against cancer after cancer transformation.

Cellular senescence involves SASPs that secrete cytokines and chemokines, which act in an autocrine manner and enhance cellular senescence by CXCR2 and its ligand factors.[45] In contrast, it has been shown to act on the surrounding cells in a paracrine manner. However, the paracrine action of SASP not only enhances cellular senescence but also has negative effects, many of which have undesirable effects on the organism, such as carcinogenesis and inflammation.[46] In the present study, we observed a marked increase in the SASP factors IL-6, IL-8, and TNF-α in the medium of PKCδ-KO HCT116 cells. This phenomenon is consistent with cellular senescence; however, whether this is an autocrine enhancement of CXCR2-mediated senescence or a paracrine enhancement of cancer-promoting effects is a subject for future studies on SASP factor inhibition. If SASP secretion is associated with PKCδ inhibition, it may affect carcinogenesis and cancer progression in a paracrine manner. We believe that the combination of PKCδ inhibition with anti-chemokine antibodies may have further anti-tumor effects.

Although cellular senescence is regulated by the p53 and p16-pRb pathways, several studies have shown that this process is cell type-specific and can be regulated by different senescence pathways independent of p53 and p16.[47,48] In the present study, p16 did not appear to be involved in PKCδ-induced cellular senescence because suppression of PKCδ expression in HCT116 cells induced p21 and SA-β-gal expression regardless of p16 expression status (Figure S4F). Contrastingly, suppression of PKCδ expression upregulated pRb expression, implying that Rb gene activation is regulated by a p21-dependent pathway rather than the p16-pRb pathway, which requires further clarification.

We found that PKCδ was upregulated in CRC tissues regardless of p53 status. Additionally, a positive correlation was found between PKCδ and tumor progression only in wt-p53 CRC. Although we could not find a relationship between PKCδ and cellular senescence in mt-p53 CRC cell lines in the present study, the expression of PKCδ was upregulated in mt-p53 CRC from an early stage, and the involvement should be investigated from a tumorigenesis perspective in a future study. Finally, we propose that PKCδ is a promising biomarker and therapeutic target for colorectal cancer in wt-p53 CRC.

AUTHOR CONTRIBUTIONS

Yuya Shimoyama: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing-Original draft, Writing-Review & Editing. **Kohji Yamada:** Conceptualization, Methodology, Validation, Resources, Data curation, Writing-Review & Editing, Funding acquisition. **Saishu Yoshida:** Methodology, Investigation, Resources. **Akira Kawamura:** Investigation. **Yoshito Hannya:** Investigation. **Yuta Imaizumi:** Investigation. **Tomotaka Kumamoto:** Investigation. **Yasuhiro Takeda:** Investigation, Funding acquisition. **Masayuki Shimoda:** Methodology, Formal analysis. **Ken Eto:** Data curation, Supervision, Funding acquisition. **Kiyotsugu Yoshida:** Conceptualization, Resources, Data curation, Writing-Review & Editing, Supervision, Project administration, Funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The unprocessed source data and the statistical source data that support the findings of this study are available, and correspondence and requests for materials should be addressed to kyoshida@jikei.ac.jp.

ETHICS APPROVAL STATEMENT

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Review

Committee of the Jikei University School of Medicine (ethics approval license: 33–149 [10764]).

ANIMAL STUDIES

All animal experiments were approved by the Animal Welfare Committee of the Jikei University School of Medicine (ethics approval license: 2020–049) and were performed in accordance with the animal experimental guidelines established by the Jikei University School of Medicine.

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REFERENCES

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209-249. doi:10.3322/caac.21660
2. Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Abate D, et al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2017: a systematic analysis for the global burden of disease study. JAMA. *Oncologia*. 2019;5(12):1749-1768. doi:10.1001/jamaoncol.2019.2996
3. Ganesh K, Stadler ZK, Cercek A, et al. Immunotherapy in colorectal cancer: rationale, challenges and potential. *Nat Rev Gastroenterol Hepatol*. 2019;16(6):361-375. doi:10.1038/s41575-019-0126-x
4. Rejhová A, Opattová A, Čumová A, Slíva D, Vodička P. Natural compounds and combination therapy in colorectal cancer treatment. *Eur J Med Chem*. 2018;144:582-594. doi:10.1016/j.ejmech.2017.12.039
5. Kuipers EJ, Grady WM, Lieberman D, et al. Colorectal cancer. *Colorectal Cancer Nat Rev Dis Primers*. 2015;1:15065. doi:10.1038/nrdp.2015.65
6. Roskoski R Jr. Classification of small molecule protein kinase inhibitors based upon the structures of their drug-enzyme complexes. *Pharmacol Res*. 2016;103:26-48. doi:10.1016/j.phrs.2015.10.021
7. Wu P, Nielsen TE, Clausen MH. Small-molecule kinase inhibitors: an analysis of FDA-approved drugs. *Drug Discov Today*. 2016;21(1):5-10. doi:10.1016/j.drudis.2015.07.008
8. Rask-Andersen M, Zhang J, Fabbro D, Schiöth HB. Advances in kinase targeting: current clinical use and clinical trials. *Trends Pharmacol Sci*. 2014;35(11):604-620. doi:10.1016/j.tips.2014.09.007
9. Cohen P. The origins of protein phosphorylation. *Nat Cell Biol*. 2002;4(5):E127-E130. doi:10.1038/ncb0502-e127
10. Johnson LN, Lewis RJ. Structural basis for control by phosphorylation. *Chem Rev*. 2001;101(8):2209-2242. doi:10.1021/cr000225s
11. Sulzmaier FJ, Ramos JW. RSK isoforms in cancer cell invasion and metastasis. *Cancer Res*. 2013;73(20):6099-6105. doi:10.1158/0008-5472.CAN-13-1087
12. Lu Z, Jiang G, Blume-Jensen P, Hunter T. Epidermal growth factor-induced tumor cell invasion and metastasis initiated by dephosphorylation and downregulation of focal adhesion kinase. *Mol Cell Biol*. 2001;21(12):4016-4031. doi:10.1128/MCB.21.12.4016-4031.2001
13. Shams R, Matsukawa A, Ochi Y, Ito Y, Miyatake H. In silico and in cell hybrid selection of Nonrapalog ligands to allosterically inhibit the kinase activity of mTORC1. *J Med Chem*. 2022;65(2):1329-1341. doi:10.1021/acs.jmedchem.1c00536
14. Campisi J. Aging, cellular senescence, and cancer. *Annu Rev Physiol*. 2013;75:685-705. doi:10.1146/annurev-physiol-030212-183653
15. Muñoz-Espín D, Serrano M. Cellular senescence: from physiology to pathology. *Nat Rev Mol Cell Biol*. 2014;15(7):482-496. doi:10.1038/nrm3823
16. Coppé JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol*. 2010;5:99-118. doi:10.1146/annurev-pathol-121808-102144
17. Lan L, Holland JD, Qi J, et al. Shp2 signaling suppresses senescence in PyMT-induced mammary gland cancer in mice. *EMBO J*. 2015;34(11):1493-1508. doi:10.15252/embj.201489004
18. Kim YH, Park TJ. Cellular senescence in cancer. *BMB Rep*. 2019;52(1):42-46. doi:10.5483/BMBRep.2019.52.1.295
19. Lee S, Lee JS. Cellular senescence: a promising strategy for cancer therapy. *BMB Rep*. 2019;52(1):35-41. doi:10.5483/BMBRep.2019.52.1.294
20. Li W, Hu J, Shi B, et al. Biophysical properties of AKAP95 protein condensates regulate splicing and tumorigenesis. *Nat Cell Biol*. 2020;22(8):960-972. doi:10.1038/s41556-020-0550-8
21. Villot R, Poirier A, Bakan I, et al. ZNF768 links oncogenic RAS to cellular senescence. *Nat Commun*. 2021;12(1):4841. doi:10.1038/s41467-021-24932-w
22. Acosta JC, Gil J. Senescence: a new weapon for cancer therapy. *Trends Cell Biol*. 2012;22(4):211-219. doi:10.1016/j.tcb.2011.11.006
23. Rodríguez-Vilarrupla A, Díaz C, Canela N, Rahn HP, Bachs O, Agell N. Identification of the nuclear localization signal of p21(cip1) and consequences of its mutation on cell proliferation. *FEBS Lett*. 2002;531(2):319-323. doi:10.1016/s0014-5793(02)03549-4
24. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol*. 2001;3(3):245-252. doi:10.1038/35060032
25. Yamada K, Oikawa T, Kizawa R, et al. Unconventional secretion of PKCδ exerts tumorigenic function via stimulation of ERK1/2 signaling in liver cancer. *Cancer Res*. 2021;81(2):414-425. doi:10.1158/0008-5472.CAN-20-2009
26. Jimenez JM, Boyall D, Brenchley G, et al. Design and optimization of selective protein kinase C θ (PKCθ) inhibitors for the treatment of autoimmune diseases. *J Med Chem*. 2013;56(5):1799-1810. doi:10.1021/jm301465a
27. Hamelin R, Laurent-Puig P, Olschwang S, et al. Association of p53 mutations with short survival in colorectal cancer. *Gastroenterology*. 1994;106(1):42-48. doi:10.1016/s0016-5085(94)94217-x
28. Goh HS, Elnatan J, Low CH, Smith DR. p53 point mutation and survival in colorectal cancer patients: effect of disease dissemination and tumour location. *Int J Oncol*. 1999;15(3):491-498. doi:10.3892/ijo.15.3.491
29. Yemelyanova A, Vang R, Kshirsagar M, et al. Immunohistochemical staining patterns of p53 can serve as a surrogate marker for TP53 mutations in ovarian carcinoma: an immunohistochemical and nucleotide sequencing analysis. *Mod Pathol*. 2011;24(9):1248-1253. doi:10.1038/modpathol.2011.85
30. Reyland ME. Protein kinase C isoforms: multi-functional regulators of cell life and death. *Front Biosci (Landmark Ed)*. 2009;14(6):2386-2399. doi:10.2741/3385
31. Suga K, Sugimoto I, Ito H, Hashimoto E. Down-regulation of protein kinase C-alpha detected in human colorectal cancer. *Biochem Mol Biol Int*. 1998;44(3):523-528. doi:10.1080/15216549800201552
32. Kuranami M, Powell CT, Hug H, Zeng Z, Cohen AM, Guillem JG. Differential expression of protein kinase C isoforms in human colorectal cancers. *J Surg Res*. 1995;58(2):233-239. doi:10.1006/jsre.1995.1036
33. Doller A, Winkler C, Azrilian I, et al. High-constitutive HuR phosphorylation at Ser 318 by PKC{delta} propagates tumor relevant functions in colon carcinoma cells. *Carcinogenesis*. 2011;32(5):676-685. doi:10.1093/carcin/bgr024

34. Park M, Kim WK, Song M, et al. Protein kinase C-δ-mediated recycling of active KIT in colon cancer. *Clin Cancer Res*. 2013;19(18):4961-4971. doi:10.1158/1078-0432.CCR-13-0131
35. Oh YT, Chun KH, Park BD, Choi JS, Lee SK. Regulation of cyclin-dependent kinase inhibitor p21WAF1/CIP1 by protein kinase Cdelta-mediated phosphorylation. *Apoptosis*. 2007;12(7):1339-1347. doi:10.1007/s10495-007-0066-8
36. Gorgoulis V, Adams PD, Alimonti A, et al. Cellular senescence: defining a path forward. *Cell*. 2019;179(4):813-827. doi:10.1016/j.cell.2019.10.005
37. Nakagawa M, Oliva JL, Kothapalli D, Fournier A, Assoian RK, Kazanietz MG. Phorbol ester-induced G1 phase arrest selectively mediated by protein kinase Cdelta-dependent induction of p21. *J Biol Chem*. 2005;280(40):33926-33934. doi:10.1074/jbc.M505748200
38. Perletti G, Marras E, Dondi D, et al. p21(Waf1/Cip1) and p53 are downstream effectors of protein kinase C delta in tumor suppression and differentiation in human colon cancer cells. *Int J Cancer*. 2005;113(1):42-53. doi:10.1002/ijc.20535
39. Yoshida K, Liu H, Miki Y. Protein kinase C delta regulates Ser46 phosphorylation of p53 tumor suppressor in the apoptotic response to DNA damage. *J Biol Chem*. 2006;281(9):5734-5740. doi:10.1074/jbc.M512074200
40. Liu H, Lu ZG, Miki Y, Yoshida K. Protein kinase C delta induces transcription of the TP53 tumor suppressor gene by controlling death-promoting factor Btf in the apoptotic response to DNA damage. *Mol Cell Biol*. 2007;27(24):8480-8491. doi:10.1128/MCB.01126-07
41. Wu TT, Hsieh YH, Wu CC, Hsieh YS, Huang CY, Liu JY. Overexpression of protein kinase C alpha mRNA in human hepatocellular carcinoma: a potential marker of disease prognosis. *Clin Chim Acta*. 2007;382(1–2):54-58. doi:10.1016/j.cca.2007.03.018
42. McKiernan E, O'Brien K, Grebenchtchikov N, et al. Protein kinase Cdelta expression in breast cancer as measured by real-time PCR, western blotting and ELISA. *Br J Cancer*. 2008;99(10):1644-1650. doi:10.1038/sj.bjc.6604728
43. Allen-Petersen BL, Carter CJ, Ohm AM, Reyland ME. Protein kinase Cδ is required for ErbB2-driven mammary gland tumorigenesis and negatively correlates with prognosis in human breast cancer. *Oncogene*. 2014;33(10):1306-1315. doi:10.1038/onc.2013.59
44. Lee PC, Fang YF, Yamaguchi H, et al. Targeting PKCδ as a therapeutic strategy against heterogeneous mechanisms of EGFR inhibitor resistance in EGFR-mutant lung cancer. *Cancer Cell*. 2018;34(6):954-969.e4. doi:10.1016/j.ccell.2018.11.007
45. Acosta JC, O'Loghlen A, Banito A, et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell*. 2008;133(6):1006-1018. doi:10.1016/j.cell.2008.03.038
46. Coppé JP, Patil CK, Rodier F, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol*. 2008;6(12):2853-2868. doi:10.1371/journal.pbio.0060301
47. Olsen CL, Gardie B, Yaswen P, Stampfer MR. Raf-1-induced growth arrest in human mammary epithelial cells is p16-independent and is overcome in immortal cells during conversion. *Oncogene*. 2002;21(41):6328-6339. doi:10.1038/sj.onc.1205780
48. Tang DG, Tokumoto YM, Apperly JA, Lloyd AC, Raff MC. Lack of replicative senescence in cultured rat oligodendrocyte precursor cells. *Science*. 2001;291(5505):868-871. doi:10.1126/science.1056780

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