

# Protein kinase C $\zeta$ regulates survivin expression and inhibits apoptosis in colon cancer

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**Abstract.** The phosphatidylinositol 3-kinase pathway transduces cell survival signals in different malignancies. Protein kinase C  $\zeta$  (PKC $\zeta$ ) is one of the molecules involved in this pathway. In this study, we investigated the role of PKC $\zeta$  in apoptosis. Short interfering RNA against PKC $\zeta$  (siPKC $\zeta$ ) sensitized HCT116 and SW480 colon cancer cells to TRAIL-induced apoptosis. Among anti-apoptotic proteins, survivin protein and mRNA expression levels decreased after siPKC $\zeta$  transfection while protein half-life did not change. The expression levels of survivin and PKC $\zeta$  were correlated in 18 colon cancer specimens ( $r=0.72$ ,  $P=3.01 \times 10^{-4}$ ). Chemosensitivity to 5-FU was enhanced by siPKC $\zeta$  in HCT116 and SW480 cells. These results indicate that PKC $\zeta$  regulates survivin expression levels and inhibits apoptosis in colon cancer cells. This study provides a rationale for targeting PKC $\zeta$  in combination with chemotherapy for colon cancer treatment.

## Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway transduces cell survival signal in different malignant cells (1,2). The pathway is directly activated by genetic mutations of PI3K or alterations in proteins regulating this pathway. Activating missense mutations of the PI3K catalytic subunit, *PIK3CA*, are reported in 4.6-26.7, 8.3-40.0, 13.6-31.6, 35.6, 1.3-4.2, 6.0-12.1 and 4.3-25.0% of brain, breast, colon, liver, lung, ovary and stomach cancer cases, respectively (3). Phosphatase and tensin homologue (PTEN) antagonizes PI3K signaling by dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>). A germline mutation of *PTEN* leads to Cowden disease characterized by a predisposition to breast and thyroid cancer (4). *KRAS* is frequently mutated in various types of cancer, including colon, lung and pancreatic cancer. PI3K has been shown to be an essen-

tial target molecule in *KRAS*-dependent carcinogenesis *in vitro* and *in vivo* (5). Receptor tyrosine kinases reside upstream of the PI3K pathway: i) epidermal growth factor receptor (EGFR) mutations are reported in 7.6-30.6% of non-small cell lung cancer (6), ii) ERBB2 is overexpressed in 10-34% of breast cancer (7), and iii) BCR-ABL1 chimeric protein is produced and activates the PI3K pathway in chronic myeloid leukemia (8).

PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to produce PIP<sub>3</sub> that activates downstream kinases including phosphoinositide dependent protein kinase 1 (PDK1) and AKT, which is a downstream target of PDK1 (9). Atypical protein kinase C  $\zeta$  (PKC $\zeta$ ) is another kinase in the PI3K pathway. It is a member of protein kinase C family and is activated by PDK1 (10,11), K-RAS (12), and superoxide (13).

Reportedly, overexpression of PKC $\zeta$  is associated with poor prognosis in patients with soft tissue sarcoma (14), cervical cancer (15), and prostate cancer (16). Experimentally, activation of PKC $\zeta$  has been shown to confer aggressive phenotypes to cancer cells through different mechanisms, including promotion of cell proliferation (17,18), migration (13,19) and transactivation of hypoxia-inducible factors (20).

In this study, we show that PKC $\zeta$  inhibits apoptotic stimuli by regulating survivin expression level in colon cancer cells.

## Materials and methods

**Reagents.** Anti-PKC $\zeta$  (sc-216), anti-actin (sc-8432), anti-Bcl-2 (sc-7382), and anti-Bcl-X<sub>L</sub> (sc-8392) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cIAP1 (AF818), anti-cIAP2 (AF817), anti-survivin (AF886), and anti-XIAP (MAB822) antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Anti-phospho-PKC $\zeta$  (NB100-80026) antibody was purchased from Novus Biologicals (Littleton, CO, USA). Anti-Ran antibody was purchased from BD Pharmingen (San Jose, CA, USA). Cycloheximide and 5-fluorouracil (5-FU) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

**Cell culture.** The human colon cancer cell lines HCT116 and SW480 that present the constitutively active PI3K pathway were used in this study (21). SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich)

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supplemented with 10% fetal bovine serum (FBS; Hyclone Thermo Fischer Scientific, Logan, UT, USA) in humidified 5% CO<sub>2</sub> at 37°C. HCT116 cells were cultured in the same condition except that McCoy's 5A medium (Gibco Life Technologies, Gaithersburg, MD, USA) was used instead of DMEM.

*Purification of soluble recombinant human TRAIL (rhTRAIL).* His6-tagged TRAIL expression plasmid (pQE-hTR) that expresses the extracellular portion of human TRAIL was kindly provided by Dr W.S. El-Deiry (Penn State Hershey Cancer Institute, PA, USA). It was transformed into DH10B (Takara Bio Inc., Tokyo, Japan). Briefly, rhTRAIL was induced by 0.5 mM Isopropyl-1-thio<sup>®</sup>-d-galactopyranoside, and purified using Ni-NTA agarose beads (Qiagen, Valencia, CA, USA) as previously described (22).

*Sub-G1 analysis.* After treatments, the cells were collected and fixed with 70% ethanol at 4°C. The cells were resuspended in phosphate-citrate buffer. After centrifugation, the pellets were treated with RNase A, stained with propidium iodide (PI), and analyzed by flow cytometry (FACSCanto, BD Bioscience, San Jose, CA, USA).

*Western blotting.* Western blotting was carried out using standard methods. Briefly, cells were sonicated in lysis buffer [20 mM Tris-HCl (pH 8.0), 135 mM NaCl, 5 mM EDTA and 1% NP-40] with a protease inhibitor cocktail (Sigma-Aldrich) and Halt phosphatase inhibitor cocktail (Pierce, Rockford, IL, USA). The samples were separated on a Tris-glycine gel (Life Technologies) under denaturing conditions. Proteins were electro-blotted onto a nitrocellulose membrane. After incubation with antibodies, the membranes were washed, incubated with the appropriate secondary antibody, washed again, and the proteins were detected using ECL Prime Western Blotting Detection kit (GE Healthcare, Piscataway, NJ, USA). The blots were visualized using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA).

*Measurement of caspase-3 and -7 activities.* Caspase-3 and -7 activities were measured using Caspase-Glo 3/7 assay (Promega, Madison, WI, USA). Briefly, 5,000 cells/100  $\mu$ l of conditioned medium were seeded into each well of 96-well plates and the cells were treated using the conditions described in each figure. Assay reagent (100  $\mu$ l) was added to each well, and the cells were incubated for 1 h at room temperature (RT). The luminescence signal was measured using a Veritas<sup>™</sup> Microplate Luminometer (Promega).

*Transduction of short interfering RNA (siRNA).* siRNAs were transfected into cells by electroporation using Amaxa Cell Line Nucleofector Kit V (Lonza, Gaithersburg, MD, USA) and Nucleofector II (Lonza). The programs D-032 and L-024 were used for HCT116 and SW480, respectively, according to the manufacturer's instructions. The sequence 5'-CUACG GCAUGUGCAAGGA-3' was used to silence PKC $\zeta$ . A non-silencing control siRNA (siNSC) was purchased from Applied Biosystems (Foster City, CA, USA).

*Quantification of BIRC5 (survivin) mRNA.* Expression of BIRC5 (survivin) mRNA was determined by quantitative

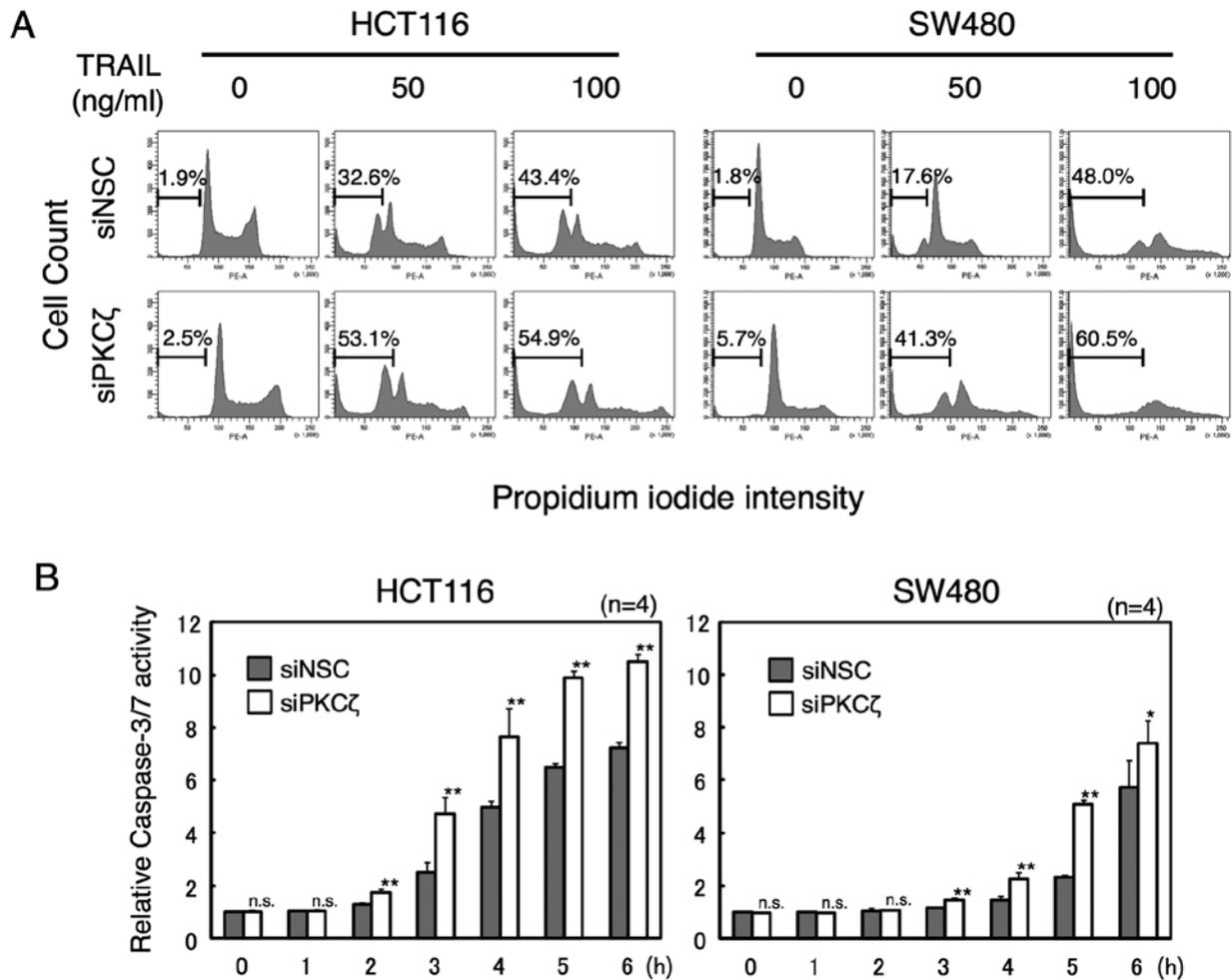
reverse-transcriptase polymerase chain-reaction (RT-PCR) using the ABI PRISM 7700 sequence-detector system (Applied Biosystems). Total RNA was isolated using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions. The cDNA was reverse-transcribed by TaqMan reverse transcription reagents (Applied Biosystems). The gene-specific primers and fluorescent hybridization probes for BIRC5a were as follows: 5'-AAGAAGTGGCCCTTCTTGGA-3', 5'-CAACCGGACGAATGCTTTT-3' and 5'-(FAM)CCAGATGACGACCCCATAGAGGAACA (TAMRA)-3'. These were used as forward primer, reverse primer, and the TaqMan probe, respectively. For the internal control, GAPDH was quantified using primers and TaqMan probe purchased from Applied Biosystems.

*Determination of half-life of survivin protein.* Protein synthesis of cells was blocked using 100  $\mu$ g/ml cycloheximide, incubated for different times as indicated in each figure, and subjected to western blotting using anti-survivin and anti-actin antibodies. The densitometric units of the blots were measured by the software Quantity One (Bio-Rad Laboratories) and survivin expression level was normalized to that of actin.

*Ubiquitination assay.* siPKC $\zeta$  or siNSC were cotransfected with p3xFLAG-survivin expression vector to cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were treated with 10  $\mu$ M MG132 (Sigma-Aldrich) for 8 h after the transfection, collected and the whole cell lysates were prepared. Ubiquitinated FLAG-tagged survivin was immunoprecipitated using anti-FLAG antibody and they were subjected to immunoblotting analysis using antibodies against ubiquitin or survivin.

*Immunohistochemical staining of tissue sections.* Colorectal cancer tissues were obtained from patients who underwent surgery at Sapporo Medical University Hospital. Written informed consent was obtained before the acquisition of these tissues. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections. Antigen retrieval was performed by boiling the sections at 120°C for 5 min in a microwave oven in preheated 0.01 M sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in ethanol for 10 min. After blocking with 1% non-fat dry milk in PBS (pH 7.4), the sections were incubated with a polyclonal anti-PKC $\zeta$  or anti-survivin antibody for 1 h followed by incubation with biotinylated anti-rabbit IgG (Nichirei Bioscience, Tokyo, Japan) for 30 min. Subsequently, the sections were stained with streptavidin-biotin complex (Nichirei Bioscience), followed by incubation with 3,3'-diaminobenzidine used as the chromogen, and counterstaining with hematoxylin.

*Measurement of cell viability.* The CellTiter-Glo Luminescent Cell Viability assay (Promega) was used according to the manufacturer's instructions. Briefly, 1,000 cells were seeded in each 96-well culture plates. Cells were treated with different conditions as described in each figure, after which the assay reagent was added and incubated for 10 min at RT. The luminescent signal was measured using a Veritas Microplate Luminometer.



**Immunofluorescence staining for p65.** Cells were fixed in ice-cold 50% ethanol for 5 min. The samples were incubated with anti-p65 antibody (D14E2, Cell Signaling Technology) for 24 h at 4°C, washed, and incubated with anti-rabbit IgG Alexa Fluor 488 antibody (1/200 dilution) (Invitrogen, Carlsbad, CA, USA) for 1 h at RT. Then, the nuclei were counterstained with Hoechst 33342 in mounting medium and the fluorescence images were obtained using a Leica AF6000 fluorescence imaging system (Nussloch, Germany).

**Statistical analysis.** Statistical analysis of the data was conducted using Microsoft Excel®. Pearson's correlation coefficient was used to determine the correlation between expression level of PKC $\zeta$  and survivin in colorectal cancer clinical specimens. Statistical significance was evaluated using the Student's t-test or  $\chi^2$  test.

## Results

**PKC $\zeta$  is anti-apoptotic in colon cancer cells.** To check whether PKC $\zeta$  is anti-apoptotic in colon cancer cells, the effect of siRNA to PKC $\zeta$  (siPKC $\zeta$ ) on colon cancer cell apoptosis was tested. The effectiveness of siPKC $\zeta$  to protein expression was confirmed

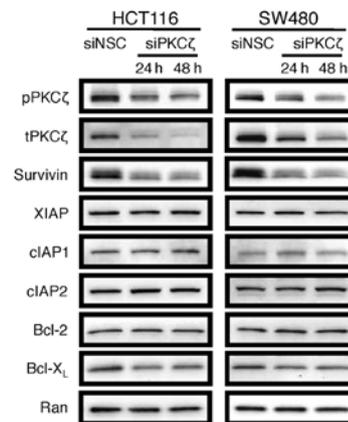


Figure 2. Downregulation of PKC $\zeta$  suppresses survivin protein expression level. HCT116 and SW480 cells were transfected with siPKC $\zeta$  or siNSC and subjected to western blot analysis using antibodies, as indicated.

by immunoblotting (data not shown). Preliminary experiments indicated that TRAIL induced apoptosis in HCT116 and SW480 dose- and time-dependently (data not shown). Sub-G1 analysis (Fig. 1A) and caspase-3/7 assay (Fig. 1B) showed that siPKC $\zeta$

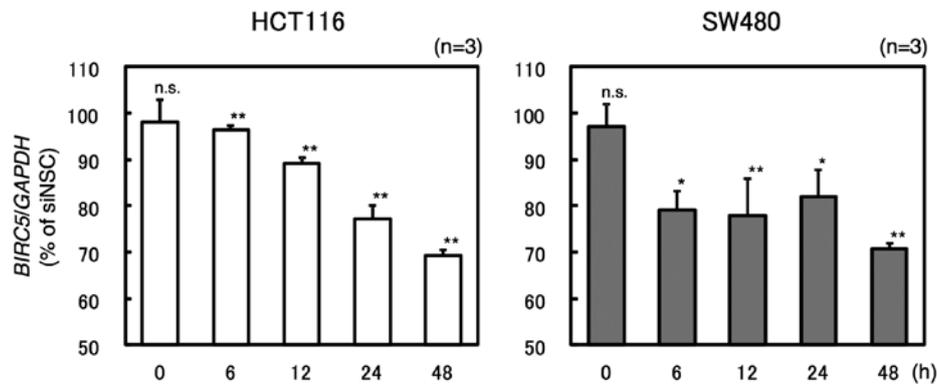


Figure 3. PKC $\zeta$  regulates survivin expression at the transcriptional level. HCT116 and SW480 cells were transfected with siPKC $\zeta$  or siNSC and levels of *BIRC5* (survivin) mRNA was quantified by TaqMan RT-PCR. Level of *BIRC5* mRNAs was normalized relative to that of *GAPDH* transcripts. Error bars denote standard deviation. NS, statistically not significant. \*P<0.05. \*\*P<0.01.

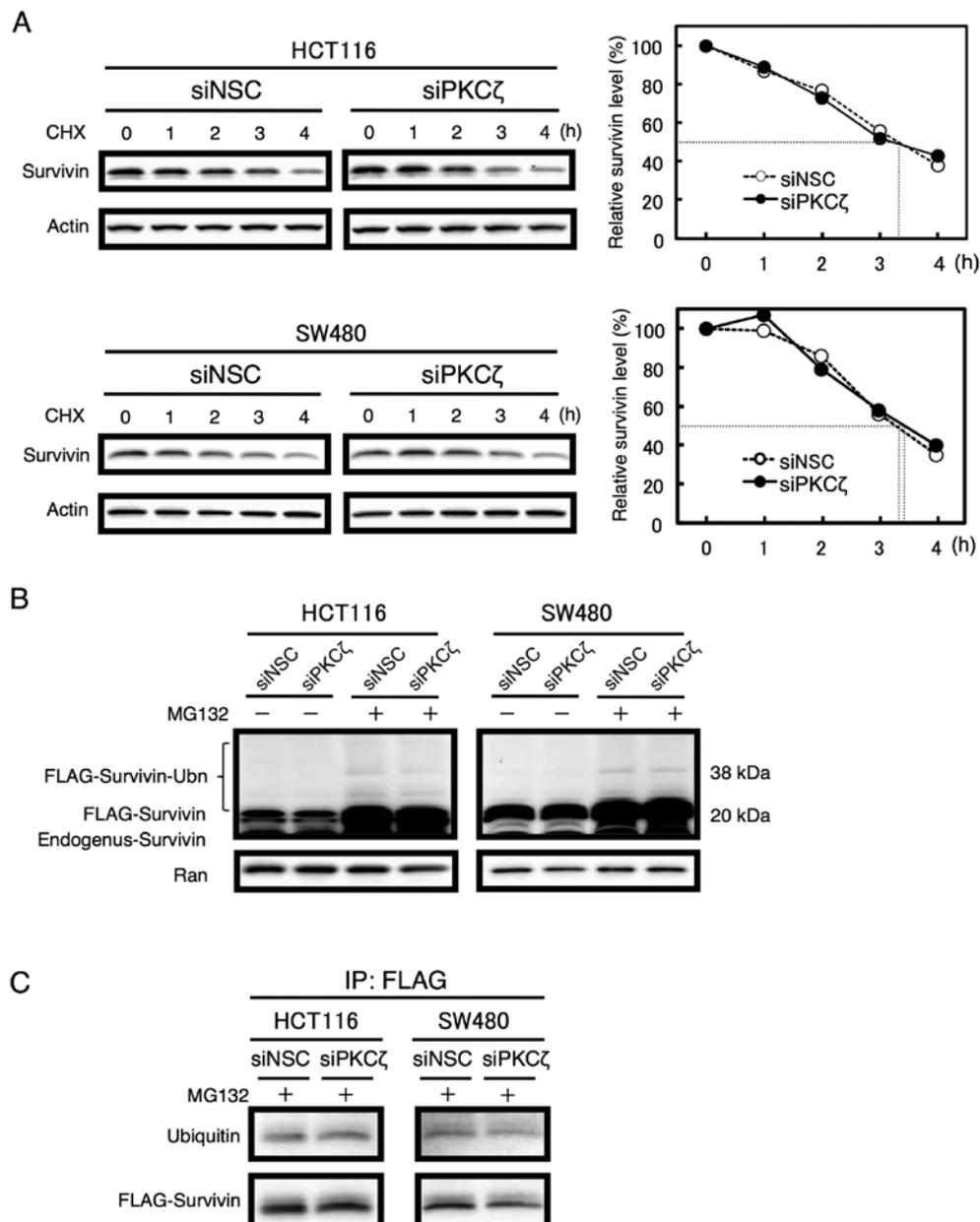


Figure 4. Downregulation of PKC $\zeta$  does not affect the protein half-life of survivin. (A) HCT116 and SW480 cells were transfected with siPKC $\zeta$  or siNSC, incubated for 24 h, after then half-life of survivin protein was monitored as described in Materials and methods. Left, western blotting; right, densitometric units of western blots represented as line graphs. (B) HCT116 and SW480 cells were transfected with p3xFLAG-survivin and subjected to immunoblotting analysis using anti-survivin antibody. (C) Ubiquitination status of survivin protein was analyzed as described in Materials and methods.

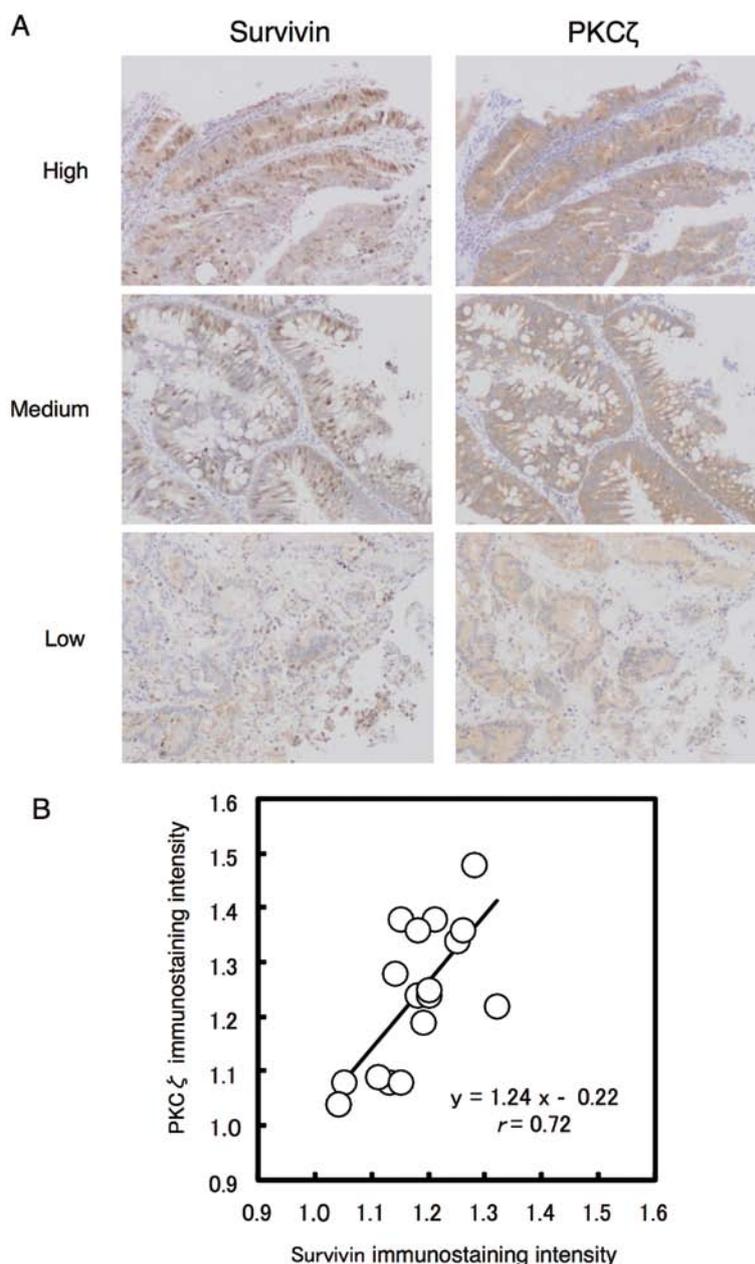


Figure 5. Survivin and PKC $\zeta$  expression levels correlate in colon cancer specimens. (A) Immunostaining for survivin (left) and PKC $\zeta$  (right) in serial sections obtained from colon cancer tissues. Representative specimens after immunostaining for survivin and PKC $\zeta$  are shown. Bright field images of immunostained tissues were captured using a digital camera (original magnification, x100). (B) Relationship between immunostaining intensities of survivin and PKC $\zeta$  in colon cancer specimens. Brightness of immunostaining was analyzed using Photoshop Elements software. Immunostaining intensities of survivin and PKC $\zeta$  in cancer cells were calculated as a brightness ratio relative to non-neoplastic stromal cells.

enhanced TRAIL sensitivity both in HCT116 and SW480 cells. Conversely, overexpression of PKC $\zeta$  conferred TRAIL resistance to HCT116 cells (data not shown). These results show that PKC $\zeta$  is anti-apoptotic in colon cancer cells.

*Survivin is regulated by PKC $\zeta$ .* To determine the molecular mechanism by which PKC $\zeta$  inhibits apoptosis in colon cancer cells, expression levels of anti-apoptotic proteins were analyzed in cells transfected with siPKC $\zeta$  and siNSC. As shown in Fig. 2, among the anti-apoptotic proteins analyzed in this study, survivin was the only protein downregulated by siPKC $\zeta$  in both HCT116 and SW480 cells. siPKC $\zeta$  reduced the expression levels of survivin in HCT116 and SW480 cells

in a time-dependent manner while overexpression of PKC $\zeta$  upregulated survivin expression level in HCT116 cells (data not shown). These results show that expression level of survivin is regulated by PKC $\zeta$  in colon cancer cells.

*PKC $\zeta$  regulates survivin expression at transcriptional level.* Generally, survivin is regulated by two different mechanisms: i) regulation at the transcriptional level or ii) by post-translational modifications. First, mRNA level of *BIRC5* (survivin) was quantified by TaqMan RT-PCR. As shown in Fig. 3, siPKC $\zeta$  reduced *BIRC5* transcript expression level in HCT116 and SW480 cells. Half-life of survivin protein in cells silenced with siPKC $\zeta$  (Fig. 4A) or in HCT116 cells overexpressing

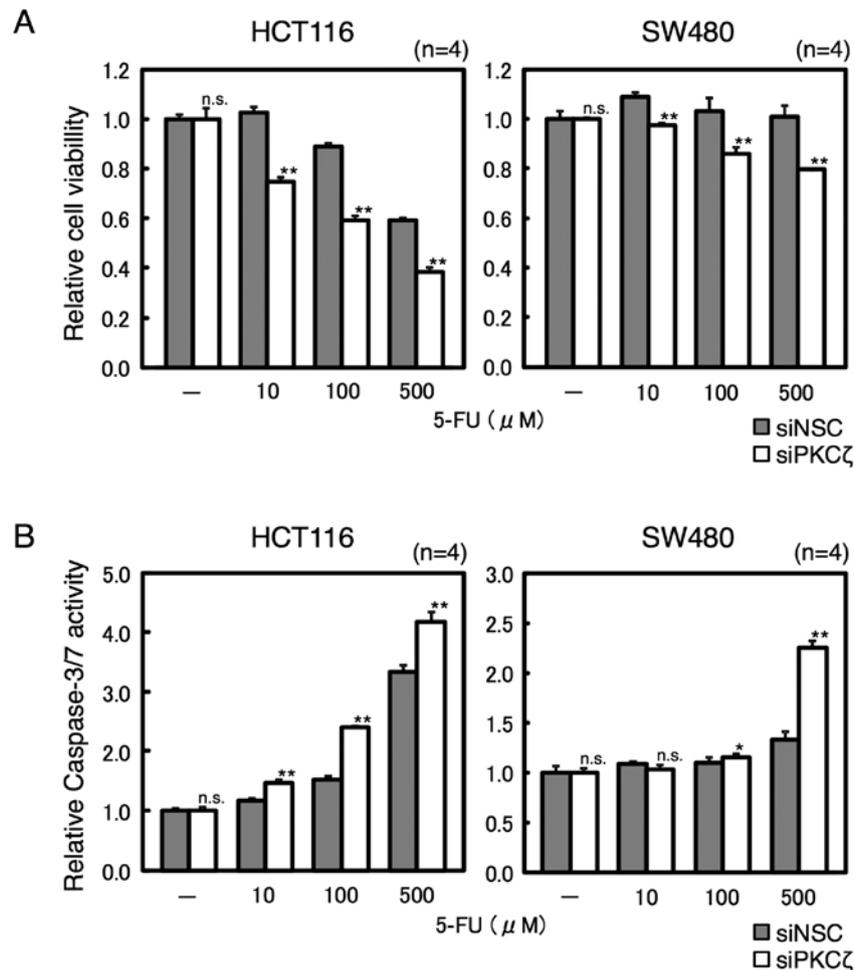


Figure 6. siPKC $\zeta$  sensitizes colon cancer cells to 5-FU. HCT116 and SW480 cells were treated with different concentrations of 5-FU as indicated for 24 h. Cell viability (A) and caspase-3/7 activity (B) were measured as described in Materials and methods. Relative luminescence signals to cells without 5-FU treatment were calculated and shown as bar graphs. Error bars denote standard deviation. NS, statistically not significant. \* $P < 0.05$ . \*\* $P < 0.01$ .

PKC $\zeta$  (data not shown) did not differ from that of the control cells. We were unable to find a condition that shows difference in ubiquitination status of survivin in HCT116 cells transduced with siNSC and siPKC $\zeta$  (Fig. 4B and C). These results indicate that PKC $\zeta$  regulates survivin expression at the transcriptional level.

*Correlatin of survivin and PKC $\zeta$  expression levels in colon cancer.* Expression levels of survivin and PKC $\zeta$  were investigated in colon cancer specimens. Immunohistochemical analysis confirmed that survivin and PKC $\zeta$  were expressed in 18 samples examined in this study. Generally, expression levels of these proteins were higher in cancer cells than neighboring normal cells (Fig. 5A). Moreover, expression levels of survivin and PKC $\zeta$  were positively correlated (Fig. 5B,  $r = 0.72$ ,  $P = 3.01 \times 10^{-4}$ ).

*siPKC $\zeta$  sensitizes colon cancer cells to 5-FU.* We next checked whether siPKC $\zeta$  sensitizes colon cancer cells to 5-FU. SW480 cells were relatively more chemo-resistant than HCT116 cells (Fig. 6A). Transfection of siPKC $\zeta$  enhanced caspase-3/7 activity in HCT116 and SW480 cells and sensitized HCT116 and SW480 cells to 5-FU (Fig. 6B).

## Discussion

In this study, we showed that PKC $\zeta$  inhibits apoptosis by regulating survivin expression at the transcriptional level in colon cancer cells. Moreover, expression level of PKC $\zeta$  and survivin correlated in colorectal cancer specimens, and silencing of PKC $\zeta$  sensitized colon cancer cell lines to 5-FU, one of the key drugs in colon cancer chemotherapy (23).

Previously, the group of Quillet-Mary has shown that PKC $\zeta$  is a component of DISC and inhibits Fas ligand-induced apoptosis in leukemic cells (24,25). PKC $\zeta$  is also known to phosphorylate mitochondrial protein Bax to abrogate its pro-apoptotic function (26). PKC $\zeta$  plays an essential role in the activation of the nuclear factor (NF)- $\kappa$ B cell survival pathway (27). The result obtained in this study, upregulation of survivin, is a novel mechanism by which PKC $\zeta$  is inhibiting apoptosis.

Survivin is a multifunctional protein that belongs to the inhibitor of apoptosis protein (IAP) family and confers chemo- and radio-resistance to cancer cells (28,29). Survivin inhibits apoptosis by stabilizing and enhancing anti-apoptotic activity of X-linked IAP, and by sequestering pro-apoptotic mitochondrial protein Smac/DIABLO (30-33). Transcription of *BIRC5* is regulated by several distinct mechanisms. It

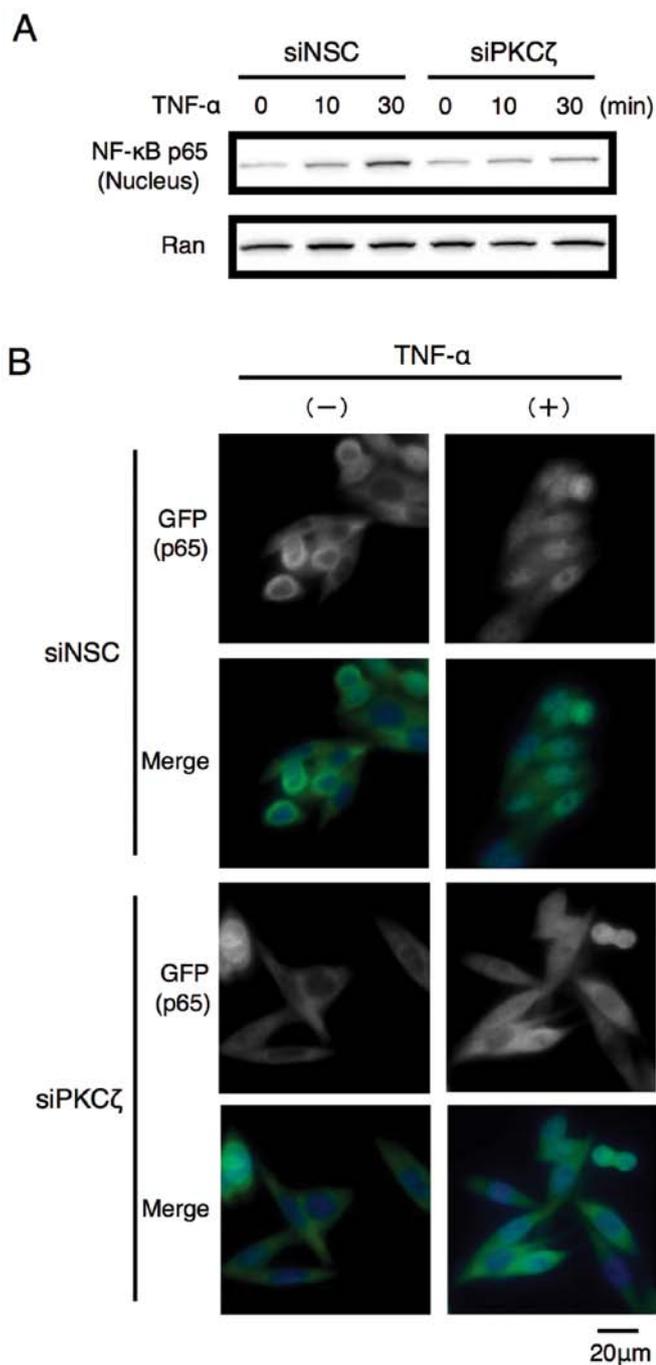


Figure 7. siPKC $\zeta$  reduces activation of NF- $\kappa$ B. (A) SW480 cells transfected with siRNA against siPKC $\zeta$  or siNISC were treated with or without TNF- $\alpha$  (20 ng/ml) for times depicted in the figure. The nuclear extracts were subjected to immunoblotting analysis using anti-p65 antibody. (B) SW480 cells were treated with or without TNF- $\alpha$  (20 ng/ml) for 30 min. The cells were transfected with siRNA against siPKC $\zeta$  or siNISC and they were subjected to immunofluorescence study using anti-p65 antibody. Green, p65. Blue, nuclei.

is transcriptionally upregulated by TCF4, STAT3, PML4 and c-REL, and downregulated by p53 (34). ERK and AKT pathways have been shown to cooperate in the translational regulation of survivin (35). Among these transcription factors and pathways, the NF- $\kappa$ B pathway is activated by PKC $\zeta$  (27,36). Thus, involvement of NF- $\kappa$ B in regulation of *BIRC5a* by PKC $\zeta$  was studied. As shown in Fig. 7, siPKC $\zeta$  reduced

the activation of NF- $\kappa$ B. This result suggests that the NF- $\kappa$ B pathway may be involved in survivin regulation by PKC $\zeta$ .

Proteins are synthesized and degraded equally at steady state. The effect of siRNAs on the level of expression of a protein largely depends on its turnover. Protein with a short half-life elicits dramatic decrease after gene-silencing whereas transient knock-down of a gene presents little impact on the expression level of proteins presenting longer half-life (37). From the results of Fig. 4A and previous studies (38), survivin has a short half-life. siPKC $\zeta$  reduced the expression level of *BIRC5* mRNA to ~70% compared to siNISC in HCT116 and SW480 cells (Fig. 3). However, it decreased survivin protein levels to less than half of that of cells transfected with siNISC (Fig. 2). Results from this gene-silencing experiment indicate that approximately one third of *BIRC5* mRNA is dependent on PKC $\zeta$  and that targeting PKC $\zeta$  is sufficient to reduce survivin expression levels for sensitization of colon cancer cells to apoptotic stimuli (Figs. 1 and 6).

TRAIL belongs to the TNF superfamily and gathers expectations as an anticancer drug because it kills a wide variety of transformed cells while sparing normal cells *in vitro* and *in vivo* (39). Recombinant human TRAIL and agonistic antibodies against its cognate receptors are undergoing clinical trials; they show low toxicity, although, small therapeutic effects have been observed when they are used as a monotherapy (40). Studies in the field are currently focusing on TRAIL-resistance mechanisms and finding TRAIL sensitizers. Results of this study revealed that PKC $\zeta$  may be a potential therapeutic target to overcome TRAIL-resistance in colon cancer.

The PKC family consists of 10 serine/threonine protein kinases, which are divided into three subfamilies based on their dependency on phospholipids and Ca<sup>2+</sup>. PKC isoforms are regulators of cell life and death. Generally, PKC $\alpha$ , PKC $\epsilon$ , PKC $\zeta$  and PKC $\iota/\lambda$  are anti-apoptotic; PKC $\delta$  is pro-apoptotic (41). Atypical PKCs, PKC $\zeta$  and PKC $\iota/\lambda$ , share homology with each other and PKC $\iota/\lambda$  is considered as an oncogene (42). This study leaves two unexplained aspects. The first is the determination of the common and distinct functions of PKC $\zeta$  and PKC $\iota/\lambda$  in the apoptotic pathway. AKT is another anti-apoptotic kinase in the PI3K pathway that is dependent of PIP<sub>3</sub>. Thus, the second unexplained aspect is the possibility of crosstalk between AKT and PKC $\zeta$ . Accumulating evidence led us to understand that there are tuned wirings in the apoptotic pathway (43-45). Revealing the relative contributions as well as crosstalk of these anti-apoptotic proteins to the cell death pathway require further studies.

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