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Differential effects of protein kinase C-eta on apoptosis versus senescence

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ABSTRACT

Protein kinase C-eta (PKCη) is considered an anti-apoptotic kinase, which promotes cell survival and chemoresistance in several cancers, including breast cancer. We have recently shown that PKCη positively regulates the anti-apoptotic protein Mcl-1 in breast cancer cells, and depletion of PKCη induced proteasomal degradation of Mcl-1. We therefore examined if depletion of PKCη would enhance cellular sensitivity to chemotherapeutic agents. Silencing of PKCη by siRNA attenuated apoptosis induced by doxorubicin and paclitaxel in both MCF-7 and T47D breast cancer cells. While silencing of Mcl-1 caused a substantial increase in apoptosis induced by doxorubicin, the combined knockdown of PKCη and Mcl-1 was less effective. Depletion of PKCη also caused an increase in the abundance of the cell cycle inhibitor p27 and a decrease in the clonogenic survival of MCF-7 and T47D cells. PKCη knockdown was associated with an increase in senescence-associated β-galactosidase (SA-β-gal) activity but this increase was attenuated by knockdown of p27. The suppression of doxorubicin-induced apoptosis by PKCη knockdown was partially relieved when p27 was depleted. Since loss of proliferative capacity during senescence could cause resistance to chemotherapeutic drugs, our results suggest that PKCη knockdown inhibits apoptosis by inducing p27-mediated senescence.

1. Introduction

Protein kinase C (PKC) plays critical roles in signal transduction and cell regulation [1,2]. PKC constitutes a multi-gene family classified into conventional (α, βI, βII, γ), novel (δ, ε, η, θ) and atypical (ξ, ι) isozymes [3]. Tumor promoting phorbol esters (e.g., TPA) are potent activators of conventional and novel PKCs and can substitute for the physiologic stimulator diacylglycerol (DAG) [3]. Persistent treatment with these phorbol esters leads to degradation or downregulation of conventional and novel PKCs [3].

PKCη is a unique member of the novel PKC isozyme family [3,4]. This is the only novel PKC that either resists downregulation or is up-regulated by phorbol esters [3,4], but it has been implicated in both tumor promotion and tumor suppression [3]. PKCη has been associated with several cancers, including renal cell carcinoma [5], glioblastoma [6], non-small cell carcinoma [7], acute myeloid leukemia [8] and breast cancer [9,10]. However, mice lacking PKCη were more susceptible to tumor formation in a two-stage carcinogenesis model [11] and it is downregulated in hepatocellular carcinoma [12]. Thus, the role of PKCη in cancer remains controversial.

Cellular senescence or loss of proliferative capacity of cells has been associated with both tumor promotion and tumor suppression [13,14]. Senescence can cause tumor suppression by inducing permanent cell cycle arrest and recruiting immune cells to clear senescent cells [13,14]. However, acquisition of senescence-associated secretory phenotype (SASP), which results in the secretion of growth factors, pro-inflammatory cytokines, chemokines and matrix remodeling enzymes, could also promote tumor under certain cellular contexts [13,15]. In addition, loss of proliferative capacity of senescent cells can cause resistance to cell death by apoptosis [13,14].

PKCη is considered an anti-apoptotic kinase since overexpression of PKCη was shown to inhibit apoptosis and contribute to chemoresistance in several cancers [3,16]. We have recently shown that PKCη positively regulates the anti-apoptotic Bcl-2 family protein Mcl-1 by preventing its degradation via the proteasomal pathway [17]. Therefore, we speculated that depletion of PKCη would enhance cell death by apoptosis. However, knockdown of PKCη caused resistance to chemotherapeutic drug-induced apoptosis and this could be explained by the induction of cellular senescence.

Abbreviations: DAG, diacylglycerol; MAPK, mitogen-activated protein kinase; Mcl-1, Myeloid cell leukemia-1; PARP, Poly (ADP-ribose) polymerase; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; SA-β-gal, senescence-associated β-galactosidase; SASP, senescence-associated secretory phenotype

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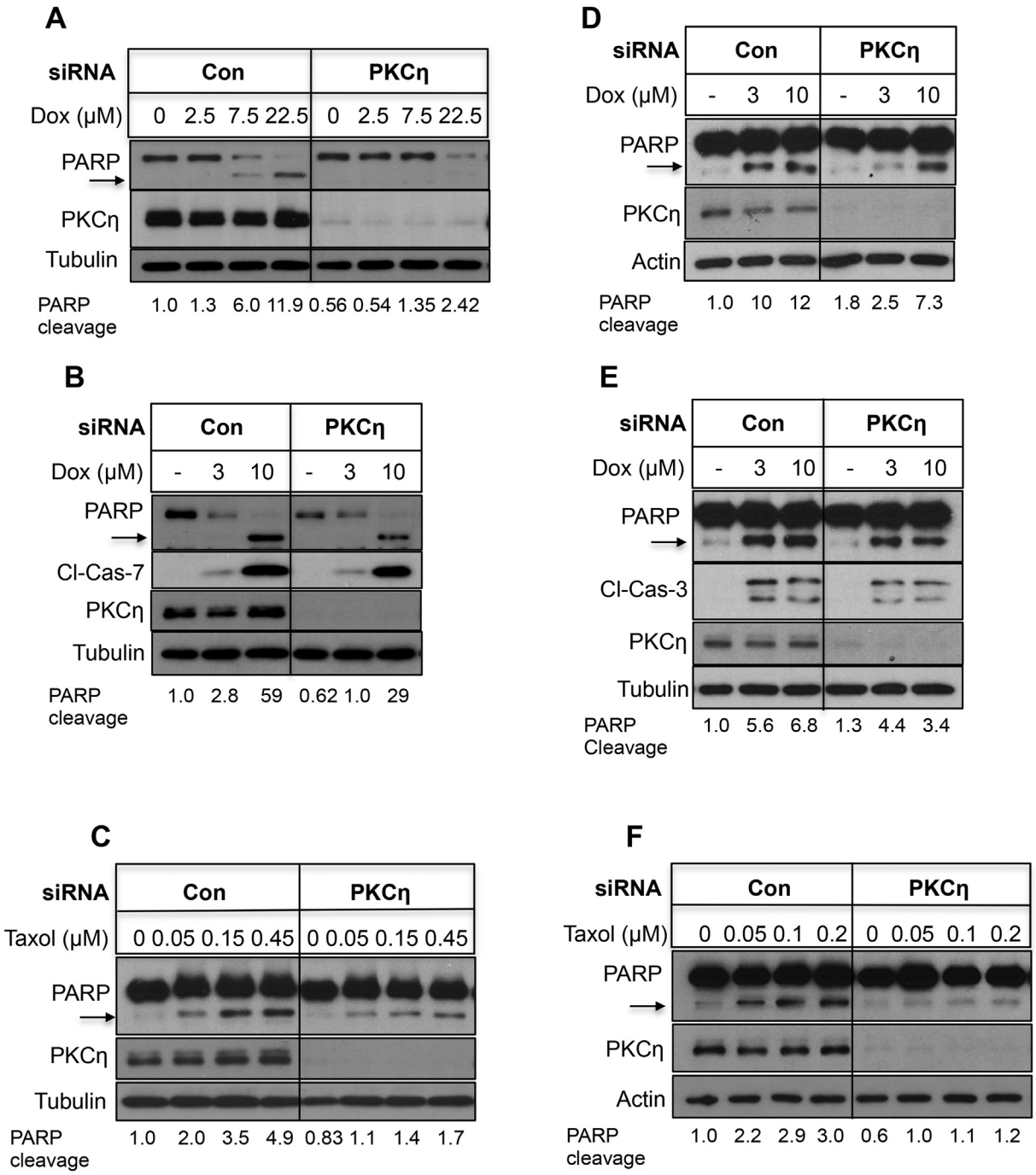

Fig. 1. Effect of PKCη knockdown on apoptosis. MCF-7 (A, B & C) or T47D (D, E & F) cells transfected with control non-targeting or PKCη siRNA were treated with different concentrations of doxorubicin (Dox) (A, B, D & E) or paclitaxel (Taxol) (C & F). Western blot analyses were performed with indicated antibodies. Actin or tubulin was used as a loading control. The arrow indicates cleaved PARP. The cleavage of PARP was quantified using ImageJ software and normalized to the control (cells transfected with control siRNA without any doxorubicin treatment).

2. Materials and methods

2.1. Materials

Polyclonal antibody to PKCη and monoclonal antibody to Mcl-1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies against p21 and p27 were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal antibody against PARP was obtained from Pharmingen (San Diego, CA). Monoclonal antibodies against actin and tubulin were obtained from Sigma (St. Louis, MO). Horseradish-peroxidase-conjugated donkey anti-rabbit and goat anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Polyvinylidene difluoride transfer membrane was from Thermo Fisher Scientific (Waltham, MA) and enhanced chemiluminescence detection kit was from Perkin-Elmer (Shelton, CT). Protease inhibitor and phosphatase inhibitor cocktails were purchased from Calbiochem/EMD-Millipore (Bedford, MA). Control non-targeting and target-specific siRNAs were obtained from Dharmacon (Lafayette, CO) and Qiagen (Germantown, MD). Lipofectamine RNAiMax transfection reagent was obtained from Invitrogen (Carlsbad, CA). Senescence β-galactosidase (SA-β-Gal) staining kit was obtained from Cell Signaling Technology (Danvers,

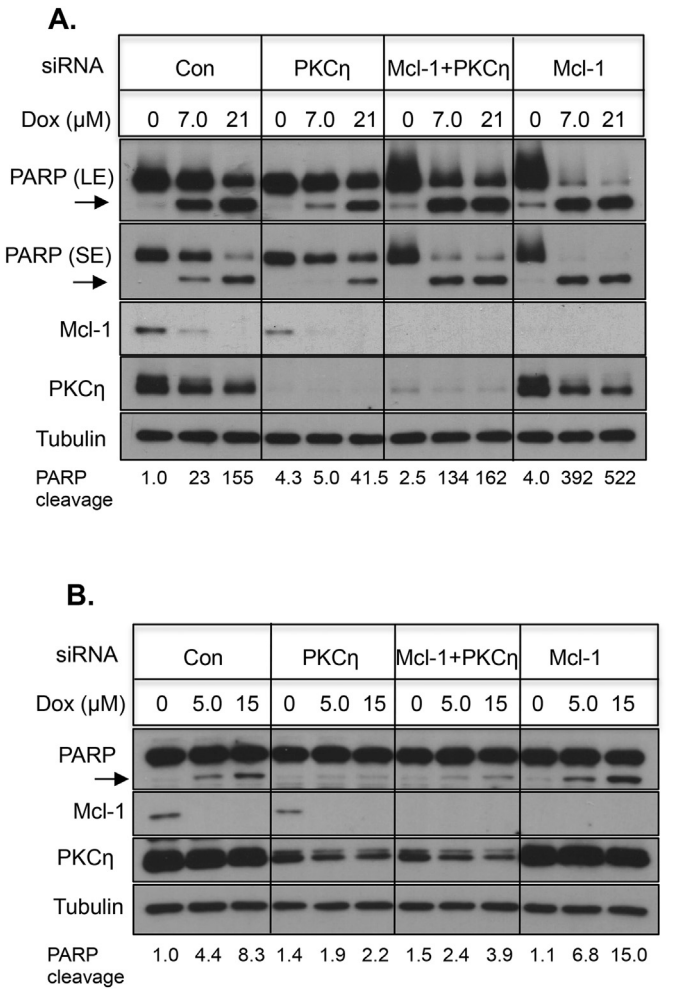

Fig. 2. Effect of Mcl-1 depletion on PKCη knockdown-induced apoptosis. MCF-7 cells (A) or T47D cells (B) were transfected with control, PKCη, Mcl-1 or both PKCη and Mcl-1 siRNA and then treated with different concentrations of doxorubicin. Western blot analyses were performed with indicated antibodies. Tubulin was used as a loading control. LE, Long exposure; SE, Short exposure. The cleavage of PARP was quantified using ImageJ software and normalized to the control.

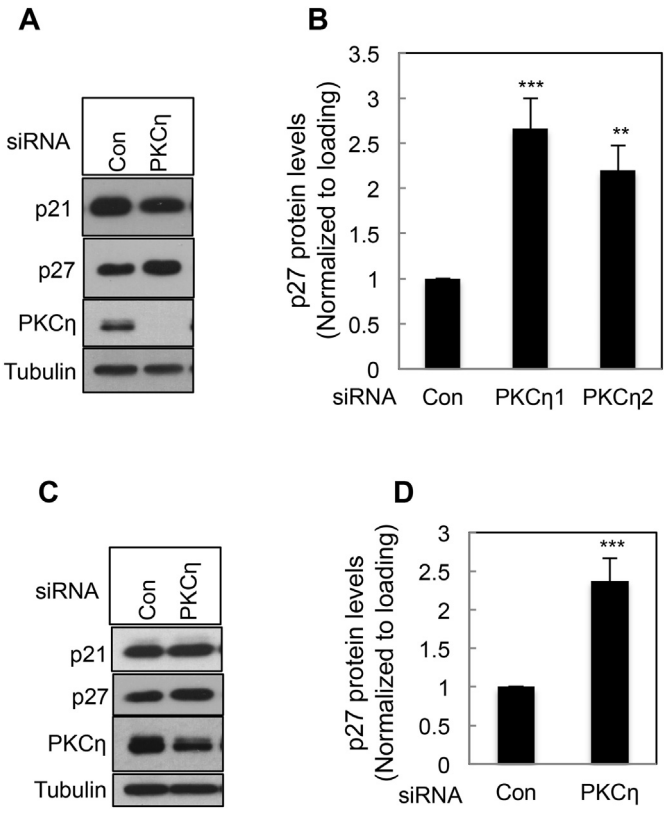

Fig. 3. Effect of PKCη knockdown on cell cycle inhibitors. MCF-7 (A, & B) or T47D (C & D) cells were transfected with control or PKCη siRNAs and Western blot analyses were performed with indicated antibodies. Tubulin was used as a loading control. Each bar represents mean ± S.E. ***, $p \leq 0.0005$; **, $p \leq 0.005$.

MA).

2.2. Cell culture

MCF-7 and T47D cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were kept in a humidified incubator at 37 °C with 95% air and 5% CO_2.

2.3. Transfection

Cells were transfected with 10 nM control non-targeting or target-specific siRNAs using Lipofectamine RNAiMax transfection reagent by reverse transfection protocol according to manufacturer's instructions. 48 h following siRNA transfection, cells were treated as indicated in the text and processed for Western blot analysis. Unless otherwise mentioned, cells were transfected with SMARTpool siRNA. The extent of gene knockdown was determined by Western blot analysis.

2.4. Clonogenic cell survival assay

Cells transfected with or without control non-targeting or target-specific siRNAs were cultured until there were at least 50 cells per colony. At the end of the incubation, cells were washed with PBS, fixed with methanol and incubated with 0.025% crystal violet solution in methanol for 15 min. Colonies were counted using ImageJ software (NIH) and the plate was photographed using the BioChemi System (BioImaging System, UVP, Upland, CA).

2.5. Western blot analysis

Cells were lysed in extraction buffer containing 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EGTA, 1 mM EDTA, 1.0% Nonidet-40, 10 mM β-glycerophosphate, protease inhibitor cocktail and phosphatase inhibitor cocktail. Equivalent amounts of total proteins (10–25 μg) were electrophoresed by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane. The blots were visualized using the enhanced chemiluminescence detection reagents and the manufacturer's protocol. The blots were probed with actin or tubulin to control for equal loading.

2.6. Senescence β-galactosidase assay

Cells were transfected with or without control non-targeting, PKCη or p27 siRNA. SA-β-gal activity was determined 4 to 5 days following transfection using SA-β-gal staining kit from Cell Signaling Technology and manufacturer's protocol. Cells were visualized under a Nikon TMS inverted microscope for the development of blue color and pictures were taken at different fields. For each experiment, a minimum of 1000 cells was counted in an unbiased fashion, and the percentage of SA-β-gal-positive cells was determined.

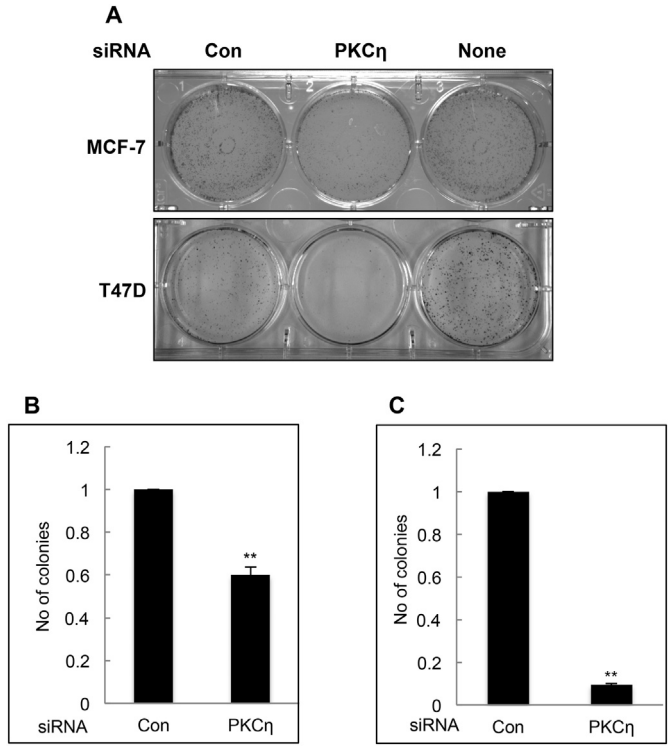

Fig. 4. Effect of PKCη knockdown on clonogenic cell survival. A, MCF-7 or T47D cells were transfected with or without control or PKCη siRNA, and clonogenic assay was performed as described under "Materials and Methods." The bar graph represents average colonies ± S.E. of experiments performed with MCF-7 (B) or T47D (C) cells. **, $p \leq 0.005$.

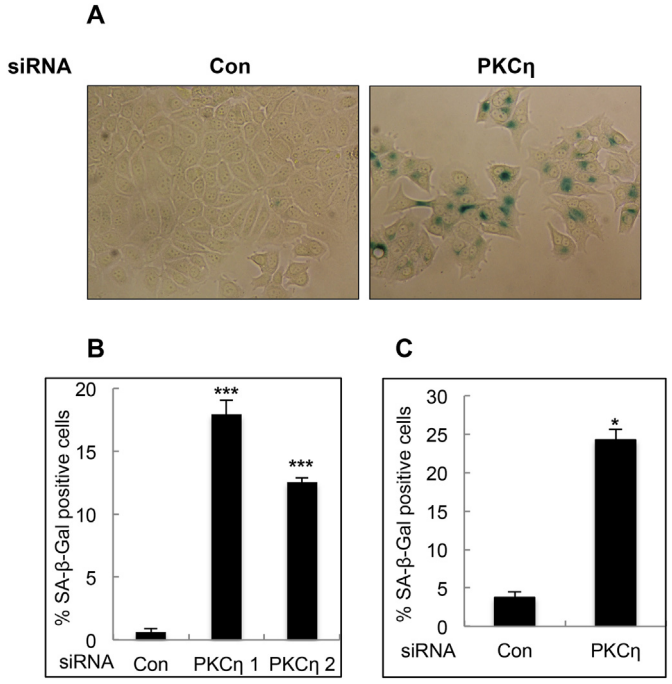

Fig. 5. Effect of PKCη knockdown on senescence. A & B, MCF-7 cells were transfected with either control or PKCη siRNA. Senescence β-gal staining was performed as described under "Materials and Methods." For each individual experiment, at least 1000 cells were counted. B, The bar graph represents mean ± S.E. of results with MCF-7 cells using two different siRNAs. ***, $p \leq 0.0005$; C, SA-β-gal staining was performed with T47D cells transfected with control non-targeting or PKCη siRNA. The bar graph represents mean ± S.E. *, $p \leq 0.05$.

2.7. Statistical analysis

The intensities of immunoreactive proteins were quantified using ImageJ software (National Institutes of Health). Statistical significance was determined by student's *t*-test using GraphPad Prism software. A *p*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Knockdown of PKCη attenuated apoptosis

Overexpression of PKCη was shown to cause resistance to chemotherapeutic drugs [3,16]. We therefore examined if depletion of PKCη would enhance sensitivity of breast cancer cells to chemotherapeutic agents. While treatment of MCF-7 (Fig. 1A and B) and T47D (Fig. 1D and E) cells with doxorubicin enhanced PARP cleavage, depletion of PKCη by two different small interfering RNAs (siRNA) attenuated PARP cleavage by these chemotherapeutic agents. The activation of caspases is essential for apoptosis, and PARP is a substrate for the executioner caspase-3 and caspase-7. During apoptosis, the inactive pro-form of the caspases gets proteolytically cleaved to the active form [18]. Although MCF-7 cells do not express caspase-3, it can induce apoptosis [19] and caspase-7 is activated in response to apoptotic stimuli [20]. The decrease in doxorubicin-induced PARP cleavage by PKCη knockdown was also associated with decrease in cleaved caspase-7 (Cl-Cas-7) in MCF-7 cells (Fig. 1B) and cleaved caspase-3 (Cl-Cas-3) in T47D cells (Fig. 1E), suggesting that depletion of PKCη inhibited rather than enhanced apoptosis. Knockdown of PKCη also decreased cellular sensitivity of MCF-7 (Fig. 1C) and T47D cells (Fig. 1F) to paclitaxel.

3.2. PKCη knockdown counteracted the increase in apoptosis caused by the depletion of Mcl-1

Recently, we showed that PKCη positively regulates the anti-apoptotic Bcl-2 family protein Mcl-1 by protecting it from proteasomal degradation [17]. Therefore, we examined if knockdown of Mcl-1 influences apoptosis in PKCη-deficient cells. Silencing of Mcl-1 alone caused an increase in doxorubicin-induced PARP cleavage in MCF-7 (Fig. 2A) and T47D cells (Fig. 2B). However, combined knockdown of Mcl-1 and PKCη was less effective than Mcl-1 knockdown alone in inducing doxorubicin-induced apoptosis (Fig. 2A & B).

3.3. PKCη knockdown inhibits cell cycle progression

PKCη has been implicated in regulating cell cycle [11,21,22]. We therefore examined the effect of PKCη knockdown on cyclin-dependent kinase (CDK) inhibitors. Knockdown of PKCη caused a modest decrease in p21 in MCF-7 cells (Fig. 3A) but had little effect in T47D cells (Fig. 3C). However, knockdown of PKCη caused a significant increase in p27 in both MCF-7 (Fig. 3A and B) and T47D (Fig. 3C and D). Two different PKCη siRNAs resulted in an increase in p27 in MCF-7 cells (Fig. 3B).

To determine if an increase in p27 is associated with decrease in cell proliferation, we performed long-term clonogenic assay (Fig. 4). PKCη knockdown caused a significant decrease in the colony forming ability of both MCF-7 (Fig. 4B) and T47D (Fig. 4C) cells.

3.4. Knockdown of PKCη induced senescence

Since permanent cell cycle arrest or cellular senescence may inhibit cell death by apoptosis [23], we examined the effect of PKCη knockdown on cellular senescence by monitoring senescence-associated β-galactosidase (SA-β-gal) activity. Fig. 5 shows that PKCη knockdown caused a significant increase in SA-β-gal positive cells in both MCF-7 (Fig. 5A and B) and T47D cells (Fig. 5C).

Since p27 is a well-known effector of senescence [24], and PKCη

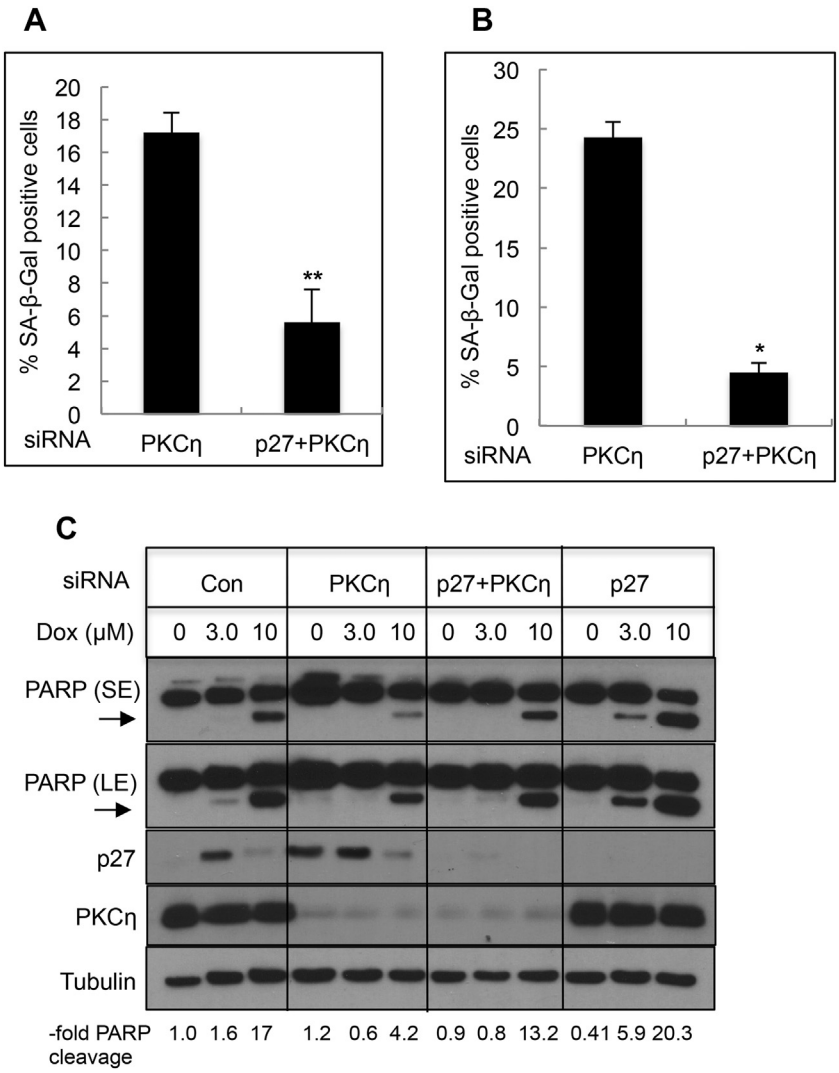

Fig. 6. Effect of p27 depletion on PKCη knockdown-induced senescence and apoptosis. MCF-7 (A & C) or T47D (B) cells were transfected with control, PKCη, p27 or both PKCη and p27 siRNAs as indicated. A & B, SA-β-gal staining was performed as described under "Materials and Methods." The bar graph represents mean ± S.E. **, $p \leq 0.005$; *, $p \leq 0.05$. C, Western blot analysis was performed with indicated antibodies. The results are representative of 3 independent experiments. The cleavage of PARP was quantified using ImageJ software and normalized to the control.

knockdown enhanced p27 in both MCF-7 and T47D cells, we examined if depletion of p27 affects senescence induced by PKCη knockdown. Silencing of p27 alone had little effect on senescence (data not shown) but it caused a significant decrease in SA-β-gal-positive cells following PKCη knockdown in both MCF-7 (6A) and T47D (6B) cells.

3.5. Knockdown of p27 enhanced apoptosis

We then determined if attenuation of PKCη knockdown-induced senescence by p27 depletion has any impact on apoptosis (Fig. 6C). p27 knockdown alone caused an increase in doxorubicin-induced PARP cleavage. While depletion of PKCη caused a decrease in doxorubicin-induced PARP cleavage, combined knockdown of p27 and PKCη restored apoptosis in PKCη-deficient cells. The extent of doxorubicin-induced PARP cleavage was similar to control siRNA-transfected cells (Fig. 6C). These results suggest that knockdown of PKCη can confer resistance to chemotherapeutic drugs by inducing senescence.

To determine if the increase in apoptosis by p27 knockdown was due to reduction in Mcl-1, we examined if silencing of p27 affects Mcl-1 level. Knockdown of p27 in MCF-7 cells either alone or in combination with PKCη knockdown had little effect on Mcl-1 level (Fig. 7A and B). We also examined, if Mcl-1 affects p27 level. Knockdown of Mcl-1 caused a modest increase in p27 level and it did not prevent increase in p27 caused by PKCη deficiency (Fig. 7C and D). These results suggest that PKCη triggers two distinct pathways to regulate apoptosis and senescence.

4. Discussion

PKCη is generally considered an anti-apoptotic kinase [3,16]. We and others have shown that overexpression of PKCη confers resistance to several apoptotic stimuli [3,16]. Thus, it is expected that decrease in PKCη level would enhance apoptosis and restore chemosensitivity. We, however, made an unexpected observation that PKCη knockdown decreased cellular sensitivity to chemotherapeutic agents. We made a novel observation that PKCη knockdown contributed to chemoresistance by inducing cellular senescence.

Cell death by apoptosis is regulated by pro- and anti-apoptotic proteins. We have recently shown that PKCη positively regulates the anti-apoptotic Bcl-2 family protein Mcl-1 by preventing its degradation via the proteasomal pathway [17]. This is consistent with the anti-apoptotic function of PKCη. Although Mcl-1 knockdown caused a substantial increase in apoptosis induced by doxorubicin, depletion of PKCη attenuated the effect of Mcl-1 knockdown.

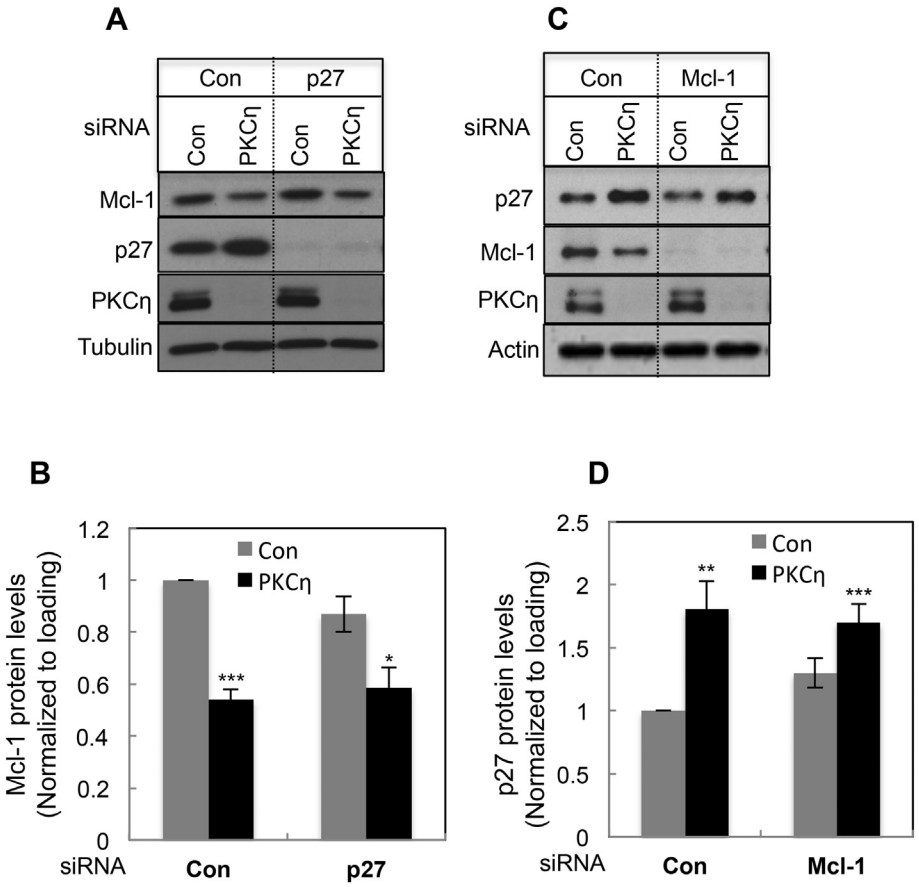

Fig. 7. Effect of p27 depletion on Mcl-1 level and vice versa. MCF-7 cells were transfected with control, PKCη, p27 or both PKCη and p27 siRNAs (A & B) or control, PKCη, Mcl-1 or both PKCη and Mcl-1 siRNAs (C & D). Western blot analyses were performed with indicated antibodies. The bar graph represents mean ± S.E. Grey bar-Cells transfected with control siRNA; Black bar-Cells transfected with PKCη siRNA; ***, $p \leq 0.0005$; **, $p \leq 0.005$; *, $p \leq 0.05$.

Several reports have suggested the involvement of PKCη in regulating cell cycle. PKCη was shown to associate with cyclin E/Cdk2/p21 complex in keratinocytes [22,25]. The expression of cyclin-dependent kinase (CDK) inhibitor p21 was elevated by PKCη overexpression but p27 was not affected [21]. Consistent with these reports we found that knockdown of PKCη resulted in a decrease in p21 in MCF-7 cells but had little effect in T47D cells. We, however, found that knockdown of PKCη caused a significant increase in p27 in both MCF-7 and T47D cells and this was associated with decrease in cell number in a long-term clonogenic assay.

Since most chemotherapeutic drugs kill actively proliferating cells, loss of proliferative capacity of cells during senescence can inhibit cell death by apoptosis [23]. Since cyclin-dependent kinase (CDK) inhibitors are important mediators of senescence [24] and PKCη knockdown decreased cell number in long-term clonogenic assays, we speculated that PKCη deficiency induces senescence. In fact, we found that knockdown of PKCη induced senescence in both MCF-7 and T47D cells.

Cyclin-dependent kinase inhibitors, such as p16, p21 and p27 play key roles in the induction of cellular senescence [24]. p16INK4A is not expressed in MCF-7 and T47D cells [26]. While p53/p21 pathway plays an important role in senescence [24], T47D cells contain mutant p53 [27]. Since PKCη knockdown caused a substantial increase in p27 in both MCF-7 and T47D cells, we examined the possibility that knockdown of p27 is responsible for PKCη knockdown-induced senescence. Depletion of p27 not only inhibited PKCη knockdown-induced senescence but also enhanced doxorubicin-induced apoptosis in PKCη-deficient cells.

Since PKCη regulates the levels of both the anti-apoptotic protein Mcl-1 and the CDK inhibitor p27, we examined the interrelationship between these two proteins. Knockdown of p27 had little effect on Mcl-1, suggesting that the restoration of apoptosis in PKCη-depleted cells was due to inhibition of senescence rather than induction of apoptosis caused by the depletion of anti-apoptotic Mcl-1. On the other hand, knockdown of Mcl-1 caused a modest increase rather than decrease in p27 although Mcl-1 knockdown alone caused a substantial increase in apoptosis. These results suggest that PKCη may utilize two distinct pathways to regulate apoptosis versus senescence. While PKCη may function as an anti-apoptotic protein via the induction of Mcl-1 by PKCη/ERK pathway, depletion of PKCη can counteract the efficacy of chemotherapeutic agents that kill actively proliferating cancer cells by inducing senescence via the p27 pathway. Therefore, caution should be exercised prior to targeting PKCη for cancer therapy. For example, dual targeting of PKCη and p27 may be a better therapeutic approach when used in combination with cytotoxic chemotherapeutic drugs.

There are controversies regarding the role of PKCη in tumor promotion versus tumor suppression [3]. Cellular senescence can cause tumor suppression by inducing permanent cell cycle arrest and recruiting immune cells to clear senescent cells [13,14]. However, senescent cells are metabolically active. Senescence-associated secretory phenotype (SASP), which is associated with the secretion of growth factors, pro-inflammatory cytokines, chemokines and matrix remodeling enzymes, could also facilitate tumor growth under certain cellular environments [13,15]. Depending on the cellular context, PKCη knockdown-induced senescence may promote or suppress tumor, and this may explain the divergent role of PKCη in cancer.

5. Conclusions

We made a novel observation that knockdown of PKCη induces senescence and confers resistance to chemotherapeutic drugs. We further demonstrate that upregulation of p27 was responsible for the induction of senescence caused by PKCη deficiency, and depletion of p27 could restore apoptosis in PKCη-depleted cells.

Conflicts of interest

None.

Funding

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Author contributions

A.B. designed and performed experiments, and prepared the manuscript. D.P. performed experiments and R.B. assisted with some experiments and counted senescent cells.

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