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Protein Kinase C δ Plays a Key Role in Cellular Senescence Programs of Human Normal Diploid Cells

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In the present study, we clarified that transforming growth factor β (TGF-β) induces cellular senescence in human normal diploid cells, TIG-1, and identified protein kinase Cs (PKCs) as downstream mediators of TGF-β-induced cellular senescence. Among PKCs, we showed that PKC-δ induced cellular senescence in TIG-1 cells and was activated in replicatively and prematurely senescent TIG-1 cells. The causative role of PKC-δ in cellular senescence programs was demonstrated using a kinase negative PKC-δ and small interfering RNA against PKC-δ. Furthermore, PKC-δ was shown to function in human telomerase reverse transcriptase (hTERT) gene repression. These results indicate that PKC-δ plays a key role in cellular senescence programs, and suggest that the induction of senescence and hTERT repression are coordinately regulated by PKC-δ.

Key words: hTERT, PKC-δ, senescence, TGF-β, TIG-1.

Abbreviations: BrdU, bromodeoxyuridine; FBS, fetal bovine serum; hTERT, human telomerase reverse transcriptase; PKC-δ KN, kinase-negative type PKC-δ; moi, multiplicity of infection; PKC-δ CF, PKC-δ catalytic fragment; PDL, population doubling level; PKC, protein kinase C; SA-β-Gal, senescence-associated β-galactosidase; siRNA, small interfering RNA; TAK1, TGF-β-activated kinase 1; TGF-β, transforming growth factor β.

INTRODUCTION

Normal somatic cells undergo a defined number of population doublings in culture, after which they become morphologically distinct and undergo numerous biochemical alterations. This is termed telomere-dependent senescence or replicative senescence. Another type of senescence called premature senescence that is provoked by DNA damage, chromatin perturbation, oncogenes, stress and other inducers has been reported (*1*). Thus far, numerous studies have been conducted to clarify the signalling molecules and cytokines regulating cellular senescence. Previously, we reported that transforming growth factor β (TGF-β) transmits signals to repress the transcription of the human telomerase reverse transcriptase (hTERT) gene and triggers senescence in cancer cells (*2*). Furthermore, we have recently identified TGF-β-activated kinase 1 (TAK1) that functions in repression of hTERT gene transcription and in the induction of senescence (*3*). Here, we attempted to identify the downstream mediator of TGF-β-induced cellular senescence.

TGF-β is a multifunctional cytokine, and shows various effects including cellular senescence, cell growth and differentiation, extracellular matrix production and chemotaxis (*2*, *4–8*). Especially, low dose of TGF-β induces cellular senescence, however high dose of TGF-β is known to provoke apoptosis. Although the varied biological activities of TGF-β have been well documented, the mechanisms through which their multiple effects are transduced remain partially understood. Among them, TGF-β signalling and regulation are known to be mediated by a family of Smad proteins (*9*). However, in addition to Smads, TGF-β has been known to require active protein kinase C δ (PKC-δ) to activate its target genes in various cells (*5*, *6*, *8*, *10–12*). PKC-δ, a ubiquitously expressed kinase involved in various cellular signalling pathways, has been implicated in diverse cell functions (*13*). Based on the importance of PKC-δ in TGF-β signalling, we determined whether PKC-δ functions in TGF-β-induced cellular senescence.

PKC-δ has a multifunctional role in various processes, including growth inhibition, differentiation, apoptosis and tumour suppression. In particular, the involvement of PKC-δ in cellular senescence has recently been indicated (*14*, *15*). The activities of PKC-δ and its catalytic fragment were augmented in replicatively senescent cells, which were believed to lead to inactivation of serum response factor (*15*). Furthermore, elevated levels of reactive oxygen species induced in senescent cells were reported to activate PKC-δ, which leads to the blocking of cytokinesis in senescent cells (*14*). Here, we attempted to clarify the indispensable role of PKC-δ in replicative and premature senescence programs, and

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further evaluated the function of PKC-δ in the regulation of telomerase, a key determinant of replicative senescence.

MATERIALS AND METHODS

Cell Culture and Reagents—HEK293 cells (JCRB9068; HSRRB, Osaka, Japan) were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS); TIG-1 cells (Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan) in MEM medium (Nissui, Tokyo, Japan) supplemented with 10% FBS at 37°C in 5% CO_2. Where indicated, TGF-β was added to the culture medium at a final concentration of 10 ng ml^{-1}. The PKC inhibitors Ro-31-8220 (Calbiochem, San Diego, CA) was dissolved in Me_2SO. Where indicated, TIG-1 cells were pretreated with appropriate concentrations of inhibitors and then treated with TGF-β every day in the presence of an inhibitor. Bistratene A was purchased from Sigma (St Louis, MO).

Senescence-Associated β-Galactosidase (SA-β-Gal) Assay—A SA-β-Gal assay was performed according to the method described by Dimri *et al*. (*2*, *16*). Staining was performed at 37°C for 12 h.

Recombinant Adenovirus and Transduction—Recombinant adenoviral vectors were constructed to express PKC-δ, PKC-δ-KN and c-Ha-*ras* by using the Adeno-X Expression System (TaKaRa, Shiga, Japan) according to the manufacturer's protocol (*3*). Briefly, the DNA fragments for PKC-δ and PKC-δ-KN were prepared by PCR by using δ-PKC-GFP and pTB701/HA-PKC-δ-KN (generous gifts from N. Saito and Y. Ono, Kobe University) as templates (*17*). A DNA fragment of the human activated c-Ha-*ras* oncogene was prepared from pCMV-*ras* (*18*). Following DNA sequencing, the fragments were cloned into a shuttle vector (pShuttle; TaKaRa) and next into an adenoviral vector (pAdeno-X; TaKaRa). Adenoviruses (Ad-PKC-δ, Ad-PKC-δ-KN and Ad-ras) were propagated in HEK293 cells and prepared by cycles of freeze thawing. The cells underwent adenoviral transduction for 3 days after a 1 h infection at a multiplicity of infection (moi) of 50–100.

Bromodeoxyuridine (BrdU) Incorporation and DNA-Content Analysis—Subconfluent cultures were metabolically labelled with BrdU (10 μM, 6 h), trypsinized, and fixed with 70% ethanol. After permeabilization with 2 N HCl containing 0.5% Triton X-100, the cells were neutralized with 0.1 M $Na_2B_4O_7$. Incorporated BrdU was exposed by DNase treatment. The cells were stained with FITC-labelled anti-BrdU antibody (BD Biosciences, San Jose, CA) and propidium iodide and subjected to flow cytometry (EPICS XL; Beckman Coulter, Miami, FL).

Western Blotting—Proteins were separated using 10% SDS–PAGE and transferred to a Hybond P membrane (GE Healthcare, Amersham Place, UK). The membrane was probed with anti-PKC-δ rabbit polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA). Horseradish peroxidase-labelled anti-rabbit IgG antibody (GE Healthcare) was used as the secondary antibody. The proteins were detected using the ECL-Plus Western blotting detection system (GE Healthcare) and visualized with an LAS-1000 Luminoimage analyzer (Fujifilm, Tokyo, Japan).

In Vitro Kinase Assay—Preparation of cell lysates and immunoprecipitaiton with the anti-PKC-δ antibody (Cell Signaling, Beverly, MA) was according to the protocol provided by the manufacturer. Immune complexes were precipitated with ImmunoPure Immobilized Protein A/G (Pierce, Rockford, IL), washed three times with wash buffer [20 mM HEPES (pH 7.4), 500 mM NaCl, 10 mM $MgCl_2$] and once with rinse buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$], and suspended in rinse buffer as described elsewhere (*19*). The kinase reaction was performed in the presence of 1× kinase buffer (Cell Signaling), 5 μCi of [γ-^{33}P]ATP (GE Healthcare) and 1 μg of histone H1 (Stressgen, British Columbia, Canada) at 30°C for 10 min. Reaction mixtures were subjected to 12% SDS–PAGE. Bands were detected by using the Image analyser FLA5000 (Fujifilm).

Small Interfering (siRNA) Treatment—Cells were transfected with 10 nM siRNA (the siRNA sequence for targeting PKC-δ were sense, 5′-AUUCGACACGCUAA AGGUCAGUGCC-3′; Invitrogen) by using Lipofect AMINE 2000 (Invitrogen). Non-silencing siRNA (sense, 5′-UUCUCCGAACGUGUCACGUTT-3′; Invitrogen) was utilized as the negative control. At 6 and 24 h after transfection, the culture medium was changed and fresh medium was supplied.

Quantitative Real-Time PCR—RNA was prepared using the RNeasy RNA isolation kit (Qiagen, Hilden, Germany) and digested with DNase on-column using RNase-free DNase (Qiagen). cDNA was prepared using M-MLV reverse transcriptase RNase H$^-$ (Promega, Madison, WI) according to the manufacturer's protocol. Quantitative PCR was performed using SYBR Premix EX *Taq* (TaKaRa) and the Thermal Cycler Dice Real Time System TP-800 (TaKaRa). PCR amplification began with a 10-s denaturation step at 95°C, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 20 s, nd extension at 72°C for 20 s. The samples were analysed in triplicate, and the hTERT level was normalized to the corresponding β-actin level. The PCR primer sequences used were as follows: hTERT top primer CGTACAGGTTTCACGCATGTG and bottom primer AT GACGCGCAGGAAAAATG; human β-actin top primer TGGCACCCAGCACAATGAA and bottom primer CTAA GTCATAGTCCGCCTAGAAGCA.

RESULTS

Involvement of PKC in TGF-β-Induced Senescence—We have reported that TGF-β induces premature senescence in human lung adenocarcinoma A549 cells (*2*). Here, we investigated whether TGF-β induces cellular senescence in human normal diploid cells, and next tried to clarify the involvement of PKC family proteins in the TGF-β-induced senescence by using Ro-31-8220 that inhibits all PKC isozymes. As shown in Fig. 1, TGF-β was found to induce cellular senescence in human normal diploid cells, TIG-1. Furthermore, this TGF-β-induced senescence was attenuated by Ro-31-8220 treatment, indicating that TGF-β induces senescence in TIG-1 cells through PKC.

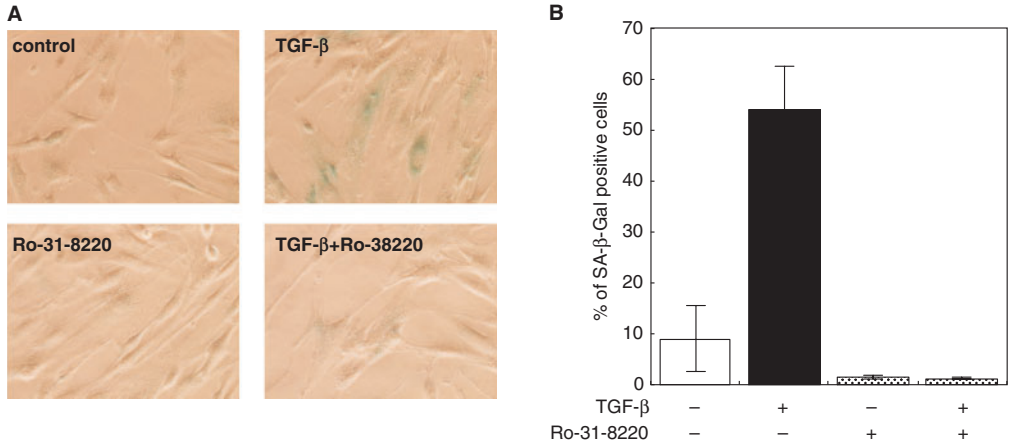

Fig. 1. **Involvement of PKC in TGF-β-induced senescence.** (A) TIG-1 cells were treated with 10 ng/ml of TGF-β in the absence or presence of Ro-31-8220 for 7 days, and assayed for SA-β-Gal activity (magnification: 100×). (B) The number of SA-β-Gal positive cells were counted.

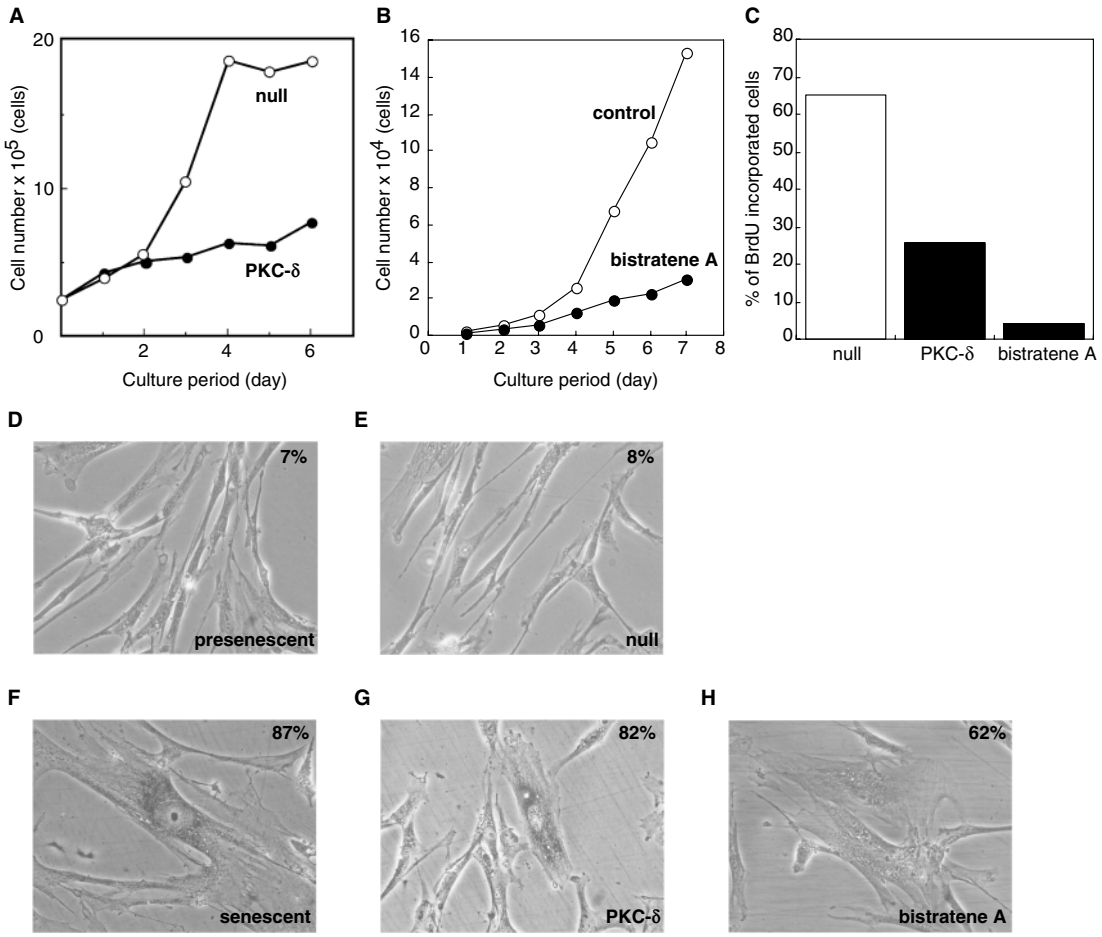

Fig. 2. **PKC-δ induces cellular senescence in human normal diploid cells.** (A) Adenoviral transduction of PKC-δ induced growth inhibition in TIG-1 cells. TIG-1 cells (40 PDL) were transduced with recombinant adenoviruses for PKC-δ (filled circle) or with control adenovirus (null; open circle). After 24 h, the cells were cultured in fresh medium for 6 h, treated with 2.5 nM PMA, and cultured for 7 days. The cell number was counted in triplicate every day. (B) Activation of PKC-δ induced growth inhibition in the TIG-1 cells. The TIG-1 cells (40 PDL) were treated with 100 nM bistratene A for 2 h. Treated (filled circle) and non-treated TIG-1 cells (open circle) were cultured in fresh medium for 7 days. (C) The TIG-1 cells transduced with Ad-PKC-δ or Ad-null, or treated with bistratene A were cultured for 3 days, and used for DNA-content analysis using BrdU. The percentage of BrdU-incorporated cells was determined by flowcytometry. (D–H) SA-β-Gal activity (magnification 200×). SA-β-Gal assays were performed for presenescent TIG-1 cells (40 PDL; D), senescent TIG-1 cells (60 PDL; F), TIG-1 cells (40 PDL) transduced with Ad-null (E) and Ad-PKC-δ (G) and TIG-1 cells (40 PDL) treated with bistratene A (H). The percentage of SA-β-Gal positive cells was counted and indicated at the upper right corner of the photograph.

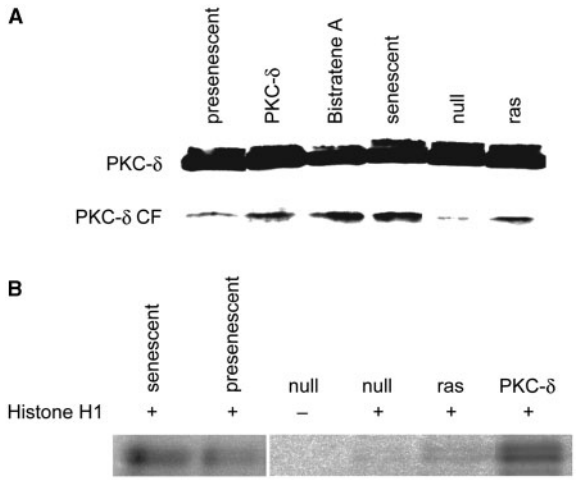

Fig. 3. **PKC-δ is activated in senescent cells.** (A) Cell lysates from presenescent and senescent TIG-1 cells; those transduced with Ad-PKC-δ, Ad-ras or Ad-null and those treated with bistratene A were separated using 10% SDS–PAGE. The blotted membrane was probed with the anti-PKC-δ antibody. (B) Cell lysates were prepared from the presenescent and senescent TIG-1 cells and those transduced with Ad-PKC-δ, Ad-ras or Ad-null; immunoprecipitation was with the anti-PKC-δ antibody. Immune complexes were applied to an *in vitro* kinase assay using histone H1 as the substrate.

PKC-δ Induces Cellular Senescence in Normal Human Diploid Cells—Next, we focused on PKC-δ for its involvement in cellular senescence programs because PKC-δ is known to function in cell cycle arrest (*14*, *15*). Adenoviral transduction of PKC-δ induced growth inhibition in TIG-1 cells (Fig. 2A and C). Bistratene A, a PKC-δ activator, also strongly inhibited the growth of TIG-1 cells (Fig. 2B and C). Furthermore, PKC-δ transduction and activation caused enlarged and flattened cell morphology and augmented SA-β-Gal activity in TIG-1 cells, as was observed in the senescent TIG-1 cells (Fig. 2D–H). These results indicate that PKC-δ can induce cellular senescence in TIG-1 cells.

PKC-δ is Activated in Senesced Cells—Next, we assessed the activation status of PKC-δ in senescent cells. Here, we prepared two types of senescent cells: replicatively senescent cells by serial passagings and prematurely senescent cells by the introduction of oncogenic ras. Then, we first tested for any changes in the amount of endogenous PKC-δ in senescent cells. Intense bands corresponding to a previously described proteolytic cleavage product comprising the PKC-δ catalytic fragment (PKC-δ CF) were observed in these senescent cells as well as cells transduced with PKC-δ and treated with bistratene A (Fig. 3A).

Next, we attempted to determine if PKC-δ activity was altered in senescent cells. Cell lysates from presenescent and senescent cells were immunoprecipitated with the anti-PKC-δ antibody and applied to a kinase reaction for which histone H1 was used as the substrate (*20*). The result demonstrated higher phosphorylation of histone H1 by PKC-δ in senescent cells (Fig. 3B). Together, western blotting and the immunoprecipitation kinase assay demonstrated that PKC-δ activity is increased in both replicatively and prematurely senescent cells, suggesting that PKC-δ plays a key role in cellular senescence programs.

Causative Role of PKC-δ in Senescence Programs—We first investigated the role of PKC-δ in replicatively senescent cells by using kinase negative PKC-δ (PKC-δ-KN). Presenescent [46 population doubling level (PDL)] and senescent cells (55 PDL) were infected with Ad-PKC-δ-KN and cultured for 10 days (Fig. 4A). At the end of culturing, SA-β-Gal activities of these cells were evaluated (Fig. 4B–D). As shown in the results, PKC-δ-KN antagonized senescence phenotypes in senescent cells, as evidenced by resumed growth and attenuated SA-β-Gal activity.

Next, we evaluated the involvement of PKC-δ in prematurely senescent cells by using three siRNAs against PKC-δ. PKC-δ mRNA expression was reduced to less than 10% that of non-treated TIG-1 cells by using these siRNAs; this level of expression continued for 6 days (data not shown). We obtained almost the same results by using these three siRNAs, and then showed here the representative results. Presenescent TIG-1 cells (40 PDL) were transfected with siRNA against PKC-δ or non-silencing siRNA (ns siRNA). After 2 days of culturing, the cells were transduced with Ad-ras and cultured for an additional 5 days (Fig. 4E). At the end of culturing, the SA-β-Gal activities of these cells were evaluated (Fig. 4F–I). In fact, oncogenic ras induced premature senescence in TIG-1 cells as evidenced by growth inhibition and augmented SA-β-Gal activity. However, this oncogenic ras-induced premature senescence was antagonized by reducing the expression of PKC-δ by using siRNA. All these results demonstrate that PKC-δ plays an indispensable role in the induction of senescence programs.

PKC-δ Functions in the Repression of hTERT—Repression of telomerase is a prerequisite for the induction of replicative senescence in normal human somatic cells. Furthermore, we have previously reported that TAK1, which transmits signals to repress the hTERT gene promoter, induces premature senescence in TIG-1 cells (*3*). Thus, these results suggest that the induction of senescence and repression of the hTERT gene are coordinately regulated.

Here, we tried to clarify the functional role of PKC-δ that plays an indispensable role in the induction of cellular senescence in the repression of the hTERT gene. Then, we investigated whether PKC-δ when activated in senescent cells functions in repression of the hTERT gene. The results showed that hTERT repression in senescent TIG-1 cells was relieved following infection with PKC-δ-KN (Fig. 5), demonstrating that PKC-δ functions in the repression of the hTERT gene in senescent cells.

DISCUSSION

Thus far, researchers have reported the following roles of TGF-β in cellular senescence programs: augmented expression of TGF-β in senescent cells (*21*), senescence-inducing ability of TGF-β (*2*, *4*, *22*) and the functional

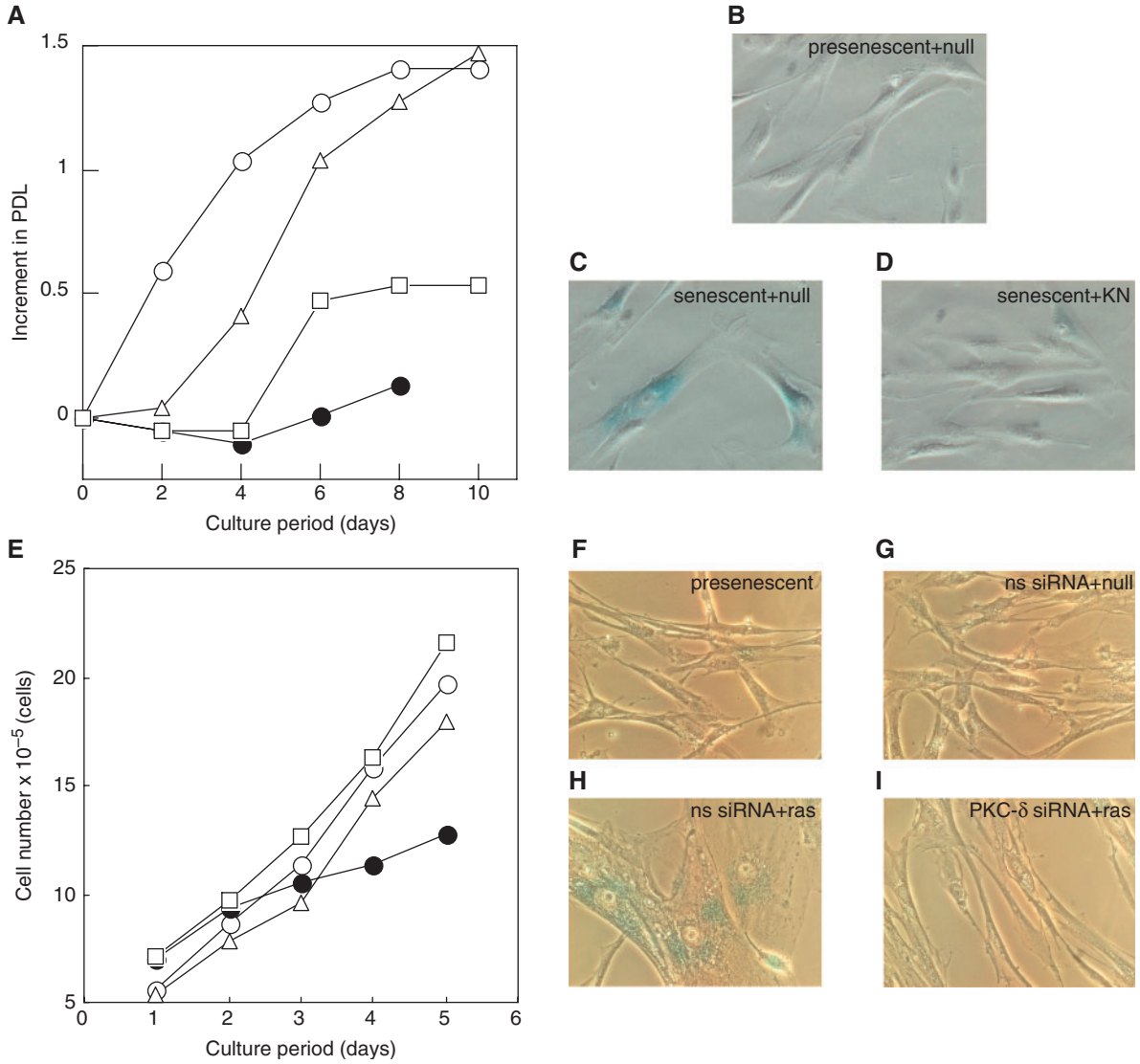

Fig. 4. **Causative role of PKC-δ in senescence programs.** (A) Presenescent (46 PDL) and senescent TIG-1 cells (55 PDL) were infected with PKC-δ-KN and cultured for 10 days, and the number of cells was counted (open circle, presenescent TIG-1 cells; filled circle, senescent TIG-1 cells; open triangle, presenescent TIG-1 cells infected with PKC-δ-KN; open square, senescent TIG-1 cells infected with PKC-δ-KN). (B–D) At the end of culturing, the SA-β-Gal activity of these cells was evaluated (magnification: 200×) (B, prescenescent TIG-1 cells; C, senescent TIG-1 cells; D, senescent TIG-1 cells infected with PKC-δ-KN). (E) Presenescent TIG-1 cells (40 PDL) were transfected with siRNA against PKC-δ or non-silencing siRNA. After 2 days of culture, the cells were seeded at a density of 1.0×10^5 cells/ml. After 24 h, the cells were transduced with Ad-ras or Ad-null. The next day, the cells were reseeded at a concentration of 4×10^4 cells/ml and cultured for 5 days [TIG-1 cells transfected with non-silencing siRNA and transduced with Ad-null (open circle) or Ad-ras (filled circle); the TIG-1 cells transfected with siRNA against PKC-δ and transduced with Ad-null (open triangle) or Ad-ras (open square)]. (F–I) At the end of culturing, the SA-β-Gal activity of these cells was evaluated (magnification 200×) [F, presenescent TIG-1 cells; G, presenecent TIG-1 cells transfected with non-silencing siRNA and transduced with Ad-null (G) or Ad-ras (H); presenescent TIG-1 cells transfected with siRNA against PKC-δ and transduced with Ad-ras (I)].

involvement of TGF-β as a mediator of senescence induced by oxidative stress and UVB (*23*, *24*). In the course of our study to identify a downstream mediator of TGF-β-induced senescence, we found that PKC-δ functions as a critical mediator in senescence programs. Other researchers also revealed that PKC-δ is activated and/or mediates several types of cellular senescence, including replicative, oncogene-induced and stress-induced senescences (*14*, *15*). Here, we clarified the indispensable and causative role of PKC-δ in replicative and oncogene-induced senescences via inhibitory experiments of PKC-δ.

Telomerase repression is a prerequisite for provoking replicative senescence in human normal diploid cells. Furthermore, we have revealed that TAK1 can repress the transcription of the hTERT gene, and triggers cellular senescence in TIG-1 cells (*3*). In the present study, we demonstrated that PKC-δ functions in the

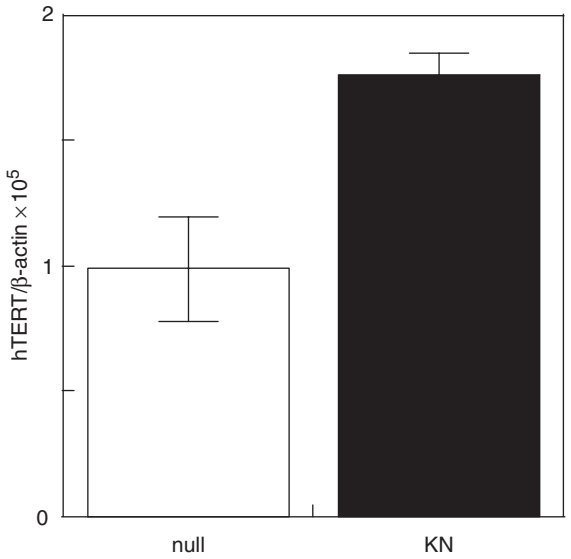

Fig. 5. **PKC-δ functions in the repression of hTERT expression.** Senescent TIG-1 cells (55 PDL) were infected with PKC-δ-KN and cultured for 3 days. hTERT expression levels in these cells were assessed by quantitative real-time PCR in triplicate and normalized to the corresponding β-actin.

repression of transcription of the hTERT gene as well as the induction of cellular senescence, as in the case of TAK1. These results show that particular signals, including TAK1 and PKC-δ, coordinately regulate the repression of the hTERT gene and induction of cellular senescence. These molecules might be key regulators of cell mortality and novel targets for cancer therapy.

Furthermore, transcription of the hTERT gene in human normal diploid cells is reported to be repressed, dependent upon histone deacetylase (*25*). Although we need to clarify the molecular mechanisms of the PKC-δ-induced repression of the hTERT gene in future studies, PKC-δ-mediated repression is believed to be an additional repression mechanism of the hTERT gene in senescent cells.

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CONFLICT OF INTEREST

None declared.

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