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# Regulation of tumor suppressor PDCD4 by novel protein kinase C isoforms

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ABSTRACT

Transforming growth factor-β1 (TGF-β1) induces apoptosis in normal hepatocytes and hepatoma cells. PDCD4 is involved in TGF-β1-induced apoptosis via the Smad pathway. The tumor promoter 12-O-tetradecanoylphorbor-13-acetate (TPA), a protein kinase C stimulator, inhibits TGF-β1-induced apoptosis. However, the mechanisms of TPA action on PDCD4 expression remain to be elucidated. Therefore. the regulatory mechanism of PDCD4 expression by PKC was investigated. The treatment of the human hepatoma cell line, Huh7 with TPA suppressed PDCD4 protein expression and TGF-β1 failed to increase the PDCD4 protein expression. PKC inhibitors Ro-31-8425 or bisindolylmaleimide-1-hydrocholoride (pan-PKC inhibitors) and rottlerin (PKCδ inhibitor), but not Go6976 (PKCα inhibitor), enhanced the induction of PDCD4 protein by TGF-β1. Furthermore, siRNA-mediated knockdown of PKCδ and ε, but not PKCα, augmented the TGF-β1-stimulated PDCD4 protein expression. However, TPA or pan-PKC inhibitor did not alter the PDCD4 mRNA expression either under basal- and TGF-β1-treated conditions. The down-regulation of PDCD4 by TPA was restored by treatment with the proteasome inhibitor MG132. These data suggest that two isoforms of PKCs are involved in the regulation of the PDCD4 protein expression related to the proteasomal degradation pathway.



## 1. Introduction

The programmed cell death 4 (PDCD4) gene shows an increased expression when apoptosis is induced. It was first reported as MA3, a gene associated with apoptosis in mice [1]. It was also identified as a gene whose expression is inhibited by topoisomerase inhibitor [2], as well as a gene involved in the cell cycle in human [3,4], and the gene was mapped at 10q24 [5]. The overexpression of this gene in cancer cells has been demonstrated to induce apoptosis [6]. Azzoni et al. [7] reported the expression of this gene to be regulated by interleukins. Subsequently, it has similarly been identified as a cancer-related gene in chickens [8,9] and rats [10]. In addition, this gene has also been reported to inhibit skin carcinogenesis and is thought to be a tumor suppressor [11–14].

PDCD4 has a nuclear localization signal (NLS) at both the N- and C-termini, and in the central region contains two MA3 domains homologous to the M1 domain of the protein synthesis initiation factor eIF4G. PDCD4 inhibits the cap-dependent translation through the binding of eIF4A at the MA3 domain, and inhibits transcription of certain genes through the inhibition of AP-1, a heterodimeric transcription factor that promotes cell proliferation [12,15,16]. TGF-β1, which is known to induce apoptosis in primary hepatocytes and hepatocellular carcinoma (HCC) cells, increases PDCD4 expression and PDCD4 accumulates in the nucleus during apoptosis [6]. The ability to avoid apoptosis is one of the important cellular mechanisms in carcinogenesis [17]. The phorbol ester,12-O-tetradecanoylphorbor-13-acetate (TPA) is a protein kinase C (PKC) stimulator and a well-established tumor promoter. Phorbol esters and growth factors that stimulate PKCs are known to antagonize TGF-β1-induced apoptosis and Smad signaling [18–20], however, the mechanisms by which TPA suppress TGF-β1-induced apoptosis has not been fully elucidated. The present study demonstrates that TPA reduces basal and TGF-β1-induced PDCD4 protein expression in a PKC isoform-specific manner.

## 2. Materials and methods

### 2.1. Cells and reagents

The human hepatoma cell line Huh7 was obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). The cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum in 5% $CO^2$ at 37 °C. TGF-β1 and epidermal growth factor (EGF) were purchased from R&D systems (Minneapolis, MN, USA),

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12-O-tetradecanoylphorbor-13-acetate (TPA) from Sigma-Aldrich. The PKC inhibitors Ro-31-8425, bisindolylmaleimide-1-hydrocholoride (Bis-1), Go6976, and rottlerin, the PI3K/Akt inhibitor LY294002, the mTOR inhibitor rapamycin, and the proteasome inhibitor MG132 were from Calbiochem (San Diego, CA, USA).

### 2.2. Cell proliferation assay

$1\times10^4$ cells/well were plated onto a 24-well plate with 0.5 ml of culture medium. TPA was added 24 h after cell placement, and 30 min later the medium was replaced with one containing TGF-β1 at the indicated concentration and the cells were cultured for an additional 48 h. The cells were then rinsed twice with phosphate-buffered saline (PBS) and incubated with 0.5 ml of DMEM containing 50 μl Premix WST-1 (Takara, Shiga, Japan) for 2 h according to the manufacturer's protocol. The absorbance of formazan products at 450 nm was measured with a CS-9300 microplate reader (Shimadzu, Tokyo, Japan).

### 2.3. DNA ladder

Cells grown on 90 mm dishes ($2\times10^6$ cells/dish) were treated with or without TPA in the presence or absence of TGF-β1 in DMEM containing 10% FBS for 48 h. Fragmented DNA was extracted and separated on a 2% agarose gel as previously described [6]. DNA ladders were visualized with Et-Br staining under UV light.

### 2.4. Western blotting

The protein expression of PDCD4 was investigated by Western blotting. The cells cultured under various conditions were collected and lysed by sonication with SDS buffer containing 50 mM Tris (pH 6.8), 2.3% SDS and 1 mM PMSF. The cell debris was eliminated by centrifugation at 10,000 rpm for 10 min and the supernatant was collected. After measuring the protein concentration using a protein assay kit (Bio-Rad, Hercules, CA), 30 or 40 μg of protein was mixed with buffer, separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Bio-Rad), and blocked with 0.1% Tween and 1% skim milk in PBS overnight. The membranes were incubated with anti-PDCD4 antibody [6] in PBS with 0.1% Tween, 1% skim milk for 1 hour. Anti-human β-actin antibody was used as a control. The membranes were washed five times with 0.1% Tween 20 in PBS and stained with horseradish peroxidase-conjugated secondary antibodies. All immunoblots were detected by the enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, England) according to the manufacturer's instructions. Anti-PKCα, PKCδ, and PKCε antibodies were obtained from Santa-Cruz Biotechnology (Santa Cruz, CA), anti-Akt, phopho-Akt (Ser473), S6 kinase, phosphor-S6 kinase (T389), Erk(p44/42), phosphor-Erk (Thr202/Tyr204) from Cell Signaling Technology (Beverly, MA) and β-actin from Sigma (St. Louis, MO). All experiments were independently repeated at least thrice.

### 2.5. Real-time PCR

Total RNA of cells was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Real-time PCR was performed by the TaqMan Gene Expression Assay system (Applied Biosystems, Foster City, CA) using exons 2 and 4 of PDCD4 (No HS00205438-m1) and GAPDH (No HS99999905-m1) as a control according to the manufacturer's protocol.

### 2.6. Transfection of Huh7 cells with siRNAs

siRNAs against PKCα and δ isoforms (validated siRNA AM51331) are purchased from Ambion (Austin, TX, USA). PKCε-siRNA (HP validated, S100587784) and Allstar negative control siRNA 1027281 were from Qiagen (Heiden, Germany). The cells were transfected with siRNA using Lipofectamine RNAi max (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the cells were seeded on 35 or 60 mm dish and cultured in DMEM supplemented with 10% FCS at 37 °C. After culturing for 2 to 3 days, the medium was changed to antibiotics-free DMEM containing 10% FCS before adding OptiMEM containing Lipofectamine and siRNA mixture and incubated at 37 °C for 24 or 48 h.

## 3. Results

### 3.1. TPA inhibits TGF-β1-induced growth suppression and PDCD4 expression

The effects of TPA on TGF-β1-induced growth suppression were examined in Huh7 cells. As shown in Fig. 1A, TPA antagonized TGF-β1-induced growth suppression in Huh7 cells, confirming previous reports [18]. In addition, the cells treated with TPA showed a decreased DNA ladder formation (Fig. 1B). TGF-β1 induces PDCD4 expression and apoptosis in Huh7 cells [6]. Therefore, the effect of TPA on PDCD4 expression was examined in Huh7 cells. The addition of 10, 100, 500, and 1000 nM TPA suppressed PDCD4 protein expression in a dose-dependent fashion (Fig. 2A). TPA also inhibited PDCD4 protein expression induced by TGF-β1 as shown in Fig. 2B.

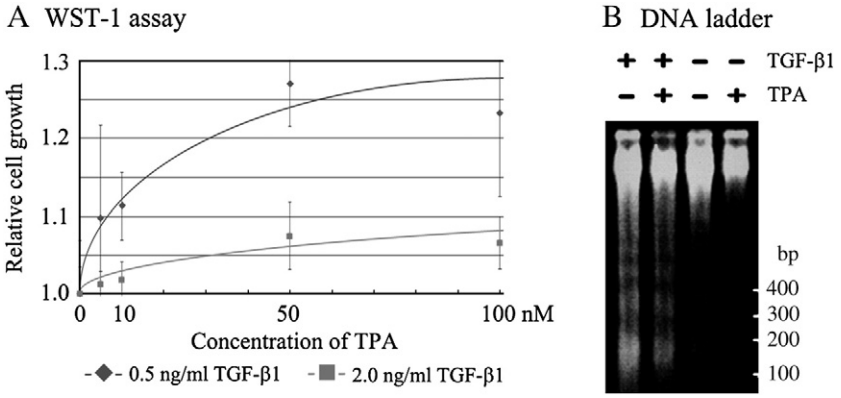


**Fig. 1.** TPA restored TGF-β1-induced cell growth inhibition and apoptosis in Huh7 cells. (A) TPA was added to Huh7 cell culture at the indicated concentration for 30 min, followed by treatment with 0.5 or 2.0 ng/ml of TGF-β1 for 48 h. Cell growth was determined by a WST-1 assay. The values were expressed as the ratio of OD with TPA and without TPA obtained from three independent experiments. (B) Huh7 cells grown on 90 mm dishes (2×106 cells/dish) were treated for 48 h with or without TPA (50 nM) in the presence and absence of TGF-β1 (4 ng/ml), and fragmented DNA was separated on an agarose gel under UV.

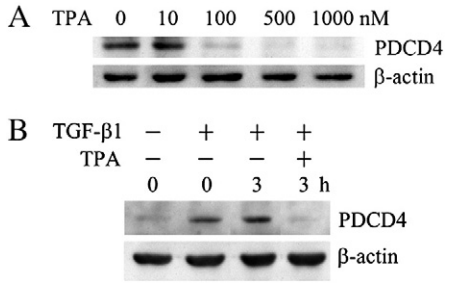


**Fig. 2.** (A) Down-regulation of PDCD4 protein level by TPA. Huh7 cells were cultured with TPA at various concentrations of TPA for 24 h and observed by a Western blot analysis with anti-PDCD4 antibody. (B) TGF-β1-induced PDCD4 protein expression was suppressed by TPA. Huh7 cells were treated with 3 ng/ml of TGF-β1 for 17 h, and thereafter the cells were treated with or without 100 nM of TPA for 3 h in a serum-free medium and then were analyzed by Western blotting.

### 3.2. PI3K-Akt-mTOR pathway inhibition increased PDCD4 expression

PDCD4 is phosphorylated by S6K1 activated via the mitogen-activated PI3K-Akt-mTOR signaling pathway and degraded in the ubiquitin–proteasome system [21]. That pathway was inhibited with the PI3K/Akt inhibitor LY294002 or the mTOR inhibitor rapamycin to determine whether the TPA-induced PDCD4 suppression occurred through the PI3K-Akt-mTOR signaling pathway. We previously have observed that EFG down-regulated PDCD4 levels in inhibiting the TGF-β1-induced apoptosis of Huh7 cells and that EGF-induced suppression of PDCD4 was inhibited by the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin (unpublished data). Since it is well established that mitogens including EGF stimulates PI3K-Akt-mTOR pathway [reviewed in ref. 22], we used EGF to stimulate PI3K-Akt-mTOR pathway of Huh7 cells.

As shown in Fig. 3, neither LY294002 (Fig. 3A) nor rapamycin (Fig. 3B) reversed the TPA-induced PDCD4 suppression, while the inhibitors did reversed the PDCD4 suppression induced by the growth factor EGF. The results showing involvement of the Akt–mTOR–S6K pathway are in accordance with previous reports [13,23,24]. These results also indicate that PKC regulates the PDCD4 levels at least through a pathway other than the Akt–mTOR–S6K pathway. Because the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin were not able to reverse TPA-induced suppression of PDCD4, the phosphorylation of Akt and S6K was investigated to examine if TPA is able to activate the signaling pathway. As shown in Fig. 3C, the phosphorylation of both kinases was slightly stimulated by TPA treatment although the stimulation was weaker than that by EGF. Phosphorylation of Akt (p-Akt) was suppressed by the PI3K inhibitor LY294002 and phosphorylation of S6K (p-S6K) by LY294002 and rapamycin but not by the pan-PKC inhibitor Ro-31-8425. These results indicate that TPA may activate the PI3K–Akt–mTOR–S6K signaling pathway but it is minor in Huh7 cells.

### 3.3. PKC inhibitors increase PDCD4 expression

PKC has numerous isoforms (α, β, γ, δ, ε, η, θ, ζ, ι/τ) and α, β, δ, ε, ζ, and ι have been variably detected in hepatoma cells [25,26]. RT-PCR confirmed that PKC isoforms including PKC α, δ, ε, ι and ζ were expressed in Huh7 cells (data not shown). Of those expressed in Huh7 cells, PKC α, δ, and ε are activated by TPA [27] and we confirmed that

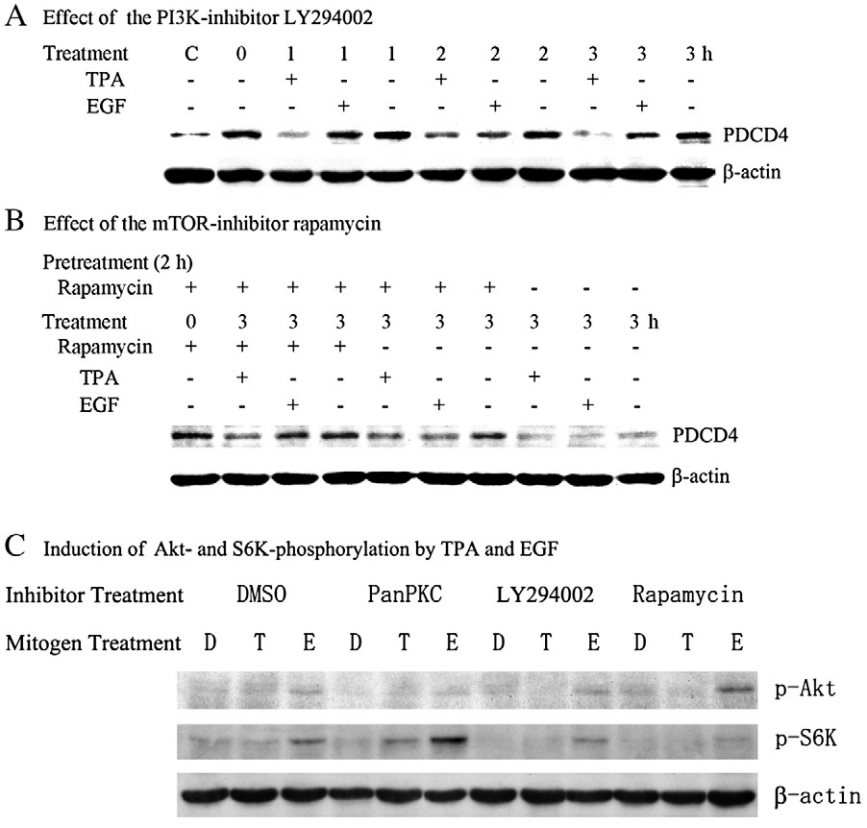


**Fig. 3.** PI3K and mTOR inhibitors did not reverse TPA-induced PDCD4 suppression. (A) After 2 h treatment with 3 μM of LY294002, a PI3K inhibitor, Huh7 cells were further cultured for the time indicated in the figure with the serum-free DMEM containing 3 μM of LY294002 and 50 nM of TPA, 20 ng/ml of EGF or DMSO as a control. C, a control without LY204002 treatment. (B) After 2 h pretreatment with 0.1 nM of rapamycin or DMSO, Huh7 cells were further cultured for 3 h in a serum-free medium containing 50 nM of TPA, 20 ng/ml of EGF or DMSO in the presence or absence of 0.1 nM of rapamycin as indicated in figure. The cell extracts were analyzed by Western blotting using anti-PDCD4 antibody. (C) Induction of Akt and S6K-phosphorylation by TPA and EGF. Huh 7 cells were incubated for 4.5 h with a serum-free DMEM medium containing DMSO as a control, Ro-31-8425, a pan-PKC inhibitor (100 nM), LY294002 (3 μM) or rapamycin (0.1 nM) and then induced with DMSO as a control (D), 50 nM TPA (T) or 20 ng/ml EGF (E) incubating further for 30 min.

by the phosphorylation of PKC isoforms are induced by TPA (data not shown). Huh7 cells were treated with several PKC inhibitors including Bis-1 or Ro-31-8425 (pan PKC inhibitors), Go6976 (PKCα inhibitor), and rottlerin (PKCδ inhibitor) to determine which isoforms are involved in TPA-induced suppression of PDCD4. PDCD4 expression was slightly enhanced in cells treated with Bis-1 prior to TGF-β1 addition, in comparison to cells treated with TGF-β1 alone (Fig. 4A), and PDCD4 expression induced by TGF-β1 was strongly increased in the presence of rottlerin than in its absence (Fig. 4B). While treatment with rottlerin and Ro-31-8425 stimulated an increased expression of PDCD4 protein, the treatment of Huh7 cells with Go6976 for 24 h did not change the PDCD4 protein level (Fig. 4C). These results indicated that PKCδ, but not PKCα, might therefore be involved in the TPA-induced down-regulation of PDCD4.

### 3.4. Knockdown of PKC δ and ε isoforms increased PDCD4 expression

Each PKC isoform was knocked down using siRNA against PKCα, δ and ε to confirm the PKC isoform(s) involved in TPA-mediated suppression of PDCD4. The changes in the PKC isoforms and PDCD4 protein levels were analyzed during siRNA treatment. The transfection of cells with control siRNA did not change the protein levels of PKC isoforms and PDCD4. PKCα siRNA transfection time dependently caused a specific decrease of PKCα protein but the PDCD4 protein levels were not changed (Fig. 5A). PKCδ siRNA decreased the expression of PKCδ protein, and PDCD4 protein levels were up-regulated in association with the decrease of PKCδ protein (Fig. 5A). Huh7 cells were transfected with siRNAs against PKCε at the different concentrations (8 and 16 nM). PKCε siRNA decreased PKCε protein in a dose-dependent manner and PDCD4 protein levels were increased in association with the decrease of PKCε protein levels (Fig. 5B).

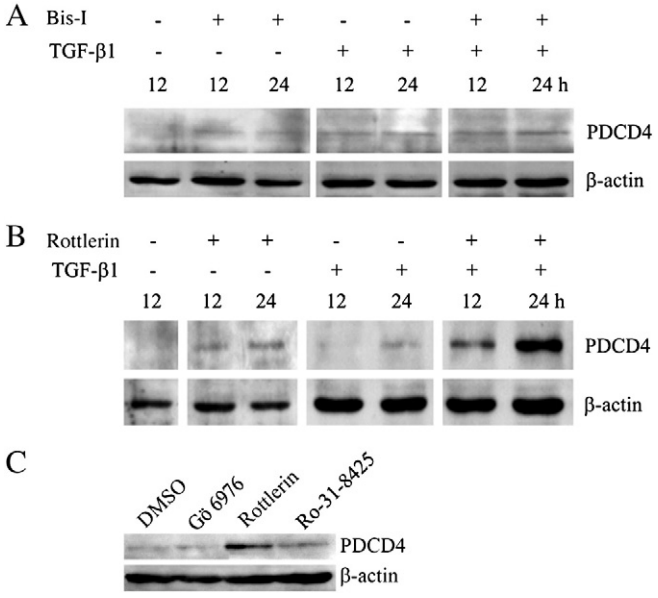


**Fig. 4.** The effects of PKC inhibitors on PDCD4 protein levels. (A) The effects of pan-PKC inhibitor, bisindolylmaleinmide-1-hydrochloride (Bis-1). Huh7 cells were cultured with or without 10 μM of Bis-1 under the absence or presence of 5 ng/ml of TGF-β1 for 12 to 24 h, and then were analyzed by Western blotting for PDCD4. (B) Effects of PKCδ-specific inhibitor, rottlerin. Cells were pre-treated with or without 10 μM rottlerin for 2 h. After the removal of rottlerin, cells were cultured with or without 5 ng/ml of TGF-β1 for 12 to 24 h, followed by a Western blot analysis for PDCD4. (C) Effects of PKC-inhibitors on PDCD4 protein levels. The cells were treated with 5 nM of Go6976 (PKCα inhibitor), 10 μM of rottlerin (PKCδ inhibitor), and 100 nM of Ro-31-8425 (pan-PKC inhibitor) for 2 h and further treated for 24 h without inhibitors, followed by a Western blot analysis for PDCD4.

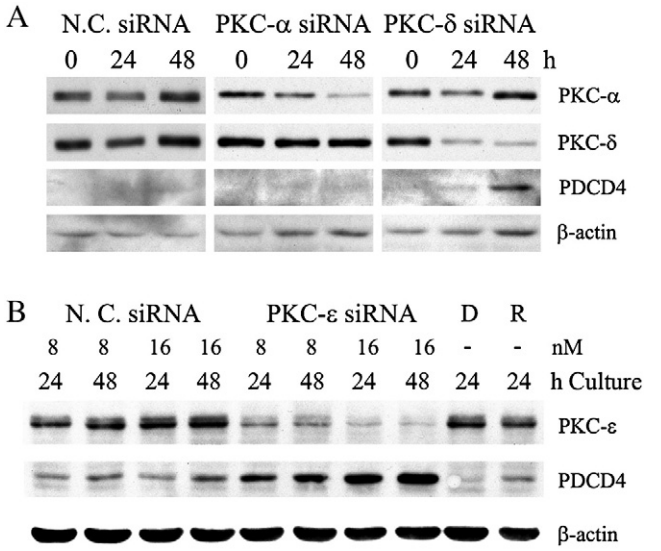


**Fig. 5.** (A) Knock-down of PKCα and PKCδ. Huh7 cells were transfected with 10 nM of siRNA against PKCα, PKCδ or control siRNA for 24 to 48 h followed by a Western blot analysis. (B) Knock down of PKCε. Huh7 cells were transfected with 8 or 16 nM of control and PKCε siRNA and cultured for 24 or 48 h, followed by Western blot analysis. As a control, DMSO (D)- and rottlerin (R) -treated cells for 24 h were indicated.

### 3.5. TPA, PKC inhibitor and PKC-knockdown do not change the expression of PDCD4 mRNA

The effects of TPA and PKC inhibitors on PDCD4 mRNA levels were examined with or without TGF-β1 stimulation of Huh7 cells. Fig. 6A demonstrates that TGF-β1 increased the PDCD4 mRNA expression after 24 h culture as previously shown [6]. However, TPA did not alter the course of PDCD4 mRNA expression irrespective of the presence of TGF-β1. As shown in Fig. 6B, the PDCD4 mRNA levels of PKC-knockdown cells were similar to that of control cells at 24 h after siRNA transfection when PKCδ and ε-knockdown up-regulated the PDCD4 protein levels (Fig. 5). Similarly, PDCD4 mRNA expression was not altered by the pan-PKC inhibitor Ro-31-8425 irrespective of the presence of TGF-β1 (Fig. 6C), thus suggesting that PKCs were not involved in the induction of TGF-β1-mediated PDCD4 mRNA.

### 3.6. The proteasome inhibitor MG132 up-regulates PDCD4 levels

PDCD4 is degraded by proteasome via E3 ubiquitin ligase β-TrCP [21]. Huh7 cells were treated with TPA and/or MG132 to confirm whether the regulation of PDCD4 by PKC was proteasomal degradation-dependent. As shown in Fig. 7A, MG132 restored the expression of PDCD4 protein suppressed by TPA. MG132 again restored the PDCD4 protein levels suppressed by TPA (Fig. 7B), when cells were treated with TGF-β1 for 17 h prior to the addition of TPA and/or MG132, thus suggesting the involvement of PKCs in the PDCD4 degradation through a proteasomal pathway irrespective of TGF-β1.

### 3.7. Knockdown of PKCδ and PKCε inhibited TPA and EGF-induced downregulation of PDCD4

Huh7 cells transfected with PKCδ and PKCε siRNA were treated with TPA or EGF at 24 h after siRNA transfection. PKCε knockdown increased the PDCD4 levels responding to suppression of PKCε, while PKCδ knockdown increased the protein level with a limited level (Fig. 8A). PDCD4 suppression by EGF was dominant and that by TPA is less effective in PKCε knockdown cells, while only EGF but not TPA slightly suppressed PDCD4 expression in PKCδ knockdown cells as shown in Fig. 8A. In the PKC knockdown cells at 24 h after siRNA treatment, phosphorylation of Akt was suppressed in PKCδ

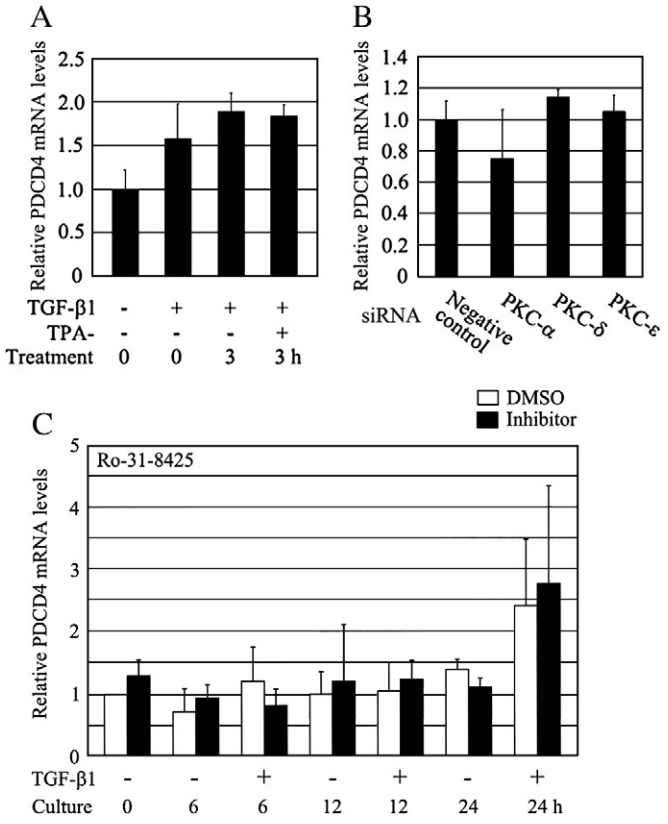


**Fig. 6.** The effects of TPA, PKC inhibitor and PKC-knockdown on the PDCD4 mRNA expression in Huh7 cells. (A) The effects of TPA on the TGF-β1-induced PDCD4 mRNA expression. (B) The effects of PKC-knockdown on the PDCD4 mRNA expression. Cells were transfected with control, PKCα, δ and ε siRNAs and cultured for 24 h. (C) The effects of pan-PKC inhibitor Ro-31-8425 on the expression of PDCD4 mRNA with or without TGF-β1 treatment. All data are expressed as the mean ± SD of three independent experiments.

knockdown cells but only slightly in PKCε knockdown cells, and not changed in the PKCα knockdown cells compared to negative control cells (Fig. 8B). On the TPA or EGF induction of PKC knockdown cells, the phosphorylation of Akt and S6K was stimulated in PKCε knockdown cells although EGF-induced phosphorylation of the kinases

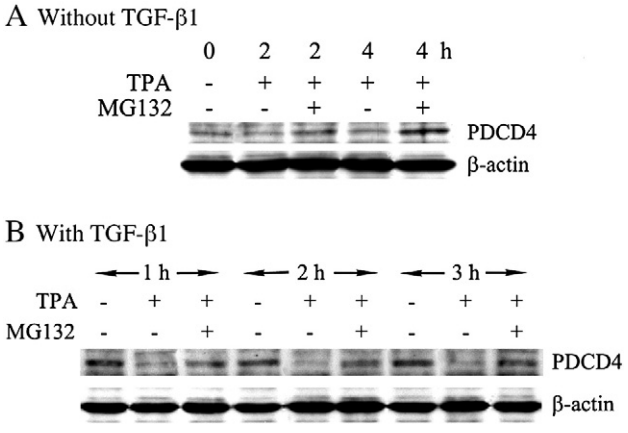


**Fig. 7.** Proteasome inhibitor MG132 inhibits the TPA-induced down-regulation of PDCD4 protein levels. (A) Huh7 cells were treated with 50 nM of TPA in the absence or presence of 20 μM MG132 for the indicated times, followed by a Western blot analysis for PDCD4. (B) Huh7 cells were pretreated with 5 ng/ml of TGF-β1 for 17 h and then TPA and/or MG132 were added. After the addition of 50 nM of TPA and/or 20 μM of MG132, the cells were then cultured for the indicated times in the figure followed by a Western blot analysis for PDCD4.

was more effective than TPA. The mitogen-induced phosphorylation of the kinases was little observed in PKCα and δ knockdown cells at 24 h after siRNA transfection (Fig. 8C). These results indicate that PKCα, δ and ε are involved in Akt–mTOR–S6K signaling pathway although PKCα knockdown did not affect PDCD4 protein level (Fig. 4A).

The phosphorylation of Erk was also suppressed in PKCε and δ knockdown cells as shown in Fig. 8B. Knockdown of PKCδ inhibited the Erk phosphorylation by TPA and EGF while inhibition of Erk phosphorylation in PKCε knockdown cell was only slight. Knockdown of PKCα rather increased the phosphorylation of Erk even at basal level without stimulation of TPA or EGF (Fig. 8C).

### *3.8. TGF-β1 attenuates the phosphorylation of Akt and S6K in Huh7 cells*

Finally, p-Akt and p-S6K levels in TGF-β1 treated cells were investigated. As shown in Fig. 9, TGF-β1 suppressed both p-Akt and p-S6K levels suggesting that TGF-β1 may stimulate PDCD4 expression not only by stimulation of PDCD4 mRNA expression but also by suppressing PDCD4 degradation through the Akt–mTOR–S6K signaling pathway.

## 4. Discussion

TGF-β1 induces PDCD4 via the Smad-dependent pathway and PDCD4 is one of the downstream effectors of TGF-β1 action [6]. TGF-β1 inhibits the proliferation of the epithelial, endothelial and hematopoietic cells and, therefore, acts as a tumor suppressor. However, there is increased expression of TGF-β1 in many types of cancers and the altered response to TGF-β1 is an important step in carcinogenesis including hepatocellular carcinoma. Several factors, such as inflammatory cytokines and fibrotic substances modulate TGF-β signaling and contribute to oncogenesis by modulating inflammation, angiogenesis and invasion [28–31]. PDCD4 acts as a tumor suppressor in several types of cancers and PDCD4 is down-regulated in tumor tissues in comparison to non-tumor tissues in these cancers [6,32–35]. Recently Sheedy et al. [36] reported that PDCD4 regulates inflammatory response after LPS stimulation suggesting the role of PDCD4 in inflammation-related cancers. However, the mechanism by which PDCD4 is suppressed in tumor cells is not fully understood.

PDCD4 protein levels are regulated by posttranscriptional and posttranslational mechanisms. Micro RNAs (miRNAs) are endogenous small non-coding RNAs that regulate the stability or translational efficiency of target messenger RNAs and many miRNAs are aberrantly expressed in human tumors suggesting their crucial roles in proliferation, differentiation, and apoptosis of tumor cells. Among these miRNAs, miR-21 plays key roles in human cancers including liver cancer [37]. Several studies have so far identified PDCD4 as a target of miR-21 and showed that post-transcriptional down-regulation of PDCD4 by miR-21 stimulated transformation, invasion and metastasis [38–40].

The stimulation of cells by growth factors leads to the degradation of PDCD4 protein through phosphorylation of the Ser67 by S6 kinase 1 (S6K1) via the PI3K/Akt/mTOR pathway and ubiquitination by SCF βTRCP ubiquitin ligase [21]. Schmid et al. [13] reported that TPA enhances PDCD4 degradation via activation of the PI3K/Akt/mTOR and MEK signaling pathways. TPA is a potent simulator of PKC and has also been shown to antagonize TGF-β1-induced growth suppression of cells [18]. However, it is not known whether the specific isoforms of PKCs are involved in TPA-mediated regulation of PDCD4 protein. The present study showed that TPA decreased the tumor suppressor PDCD4 protein in a PKC isoform-specific manner. PKC plays important roles in the various aspects of signal transduction pathways that regulate cell proliferation, differentiation, transformation and apoptosis [41]. PKC isoforms are divided into the conventional (cPKCs: α, βI, βII, and γ), novel (nPKCs: δ, ε, η, and θ) and atypical (aPKCs: ζ and

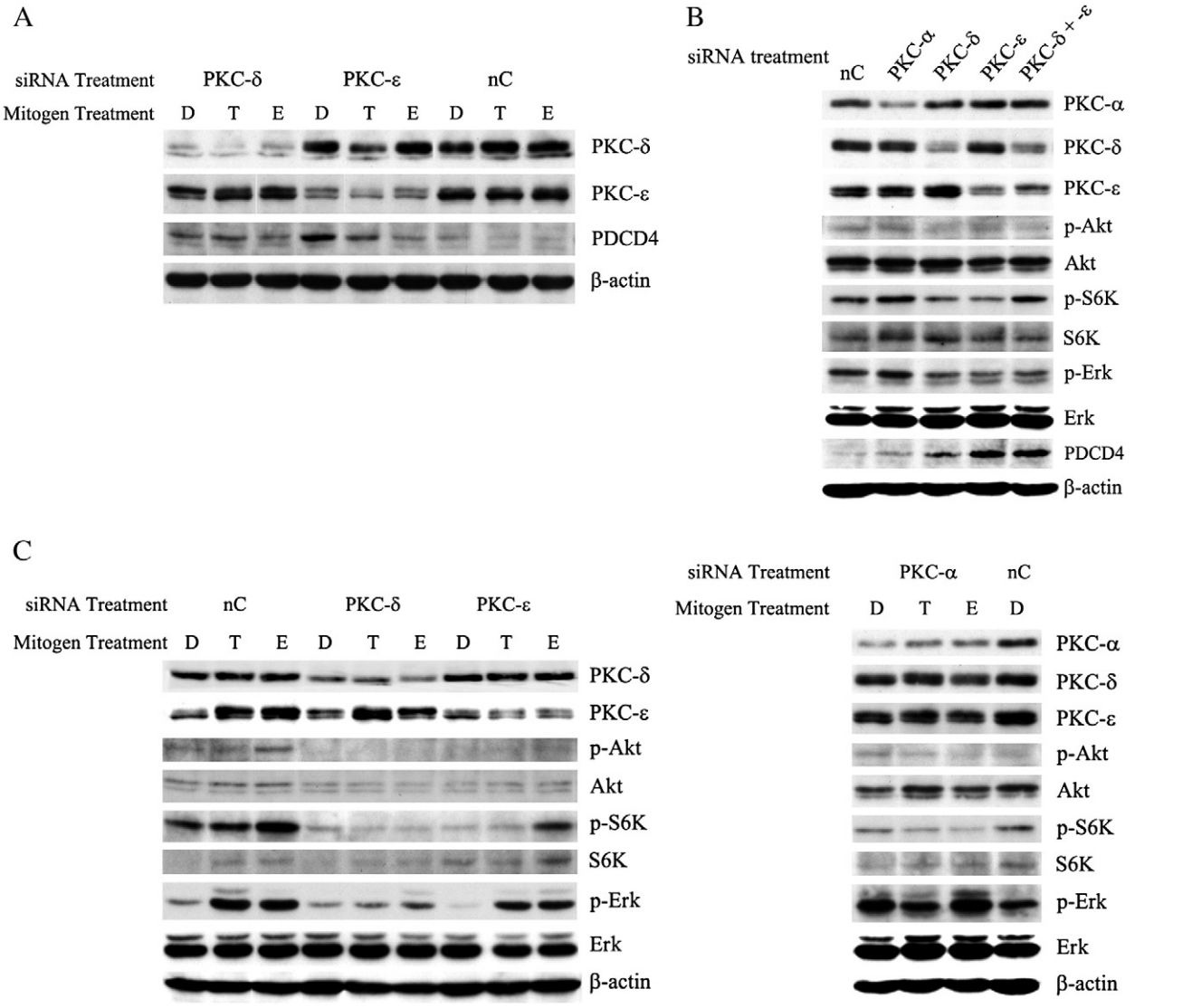


**Fig. 8.** Phosphorylation levels of Akt, S6K and Erk in PKC knockdown cells. Huh 7 cells were transfected with 8 nM PKCα, δ and ε siRNA and cultured for 24 h. (A) TPA and EGF-induced suppression of PDCD4 in PKC-knockdown cells. Huh 7 cells transfected with PKCδ, PKCε and negative control (nC) siRNA were induced with 50 nM of TPA (T), 20 ng/ml of EGF (E) and DMSO as a control (D) in a serum-free DMEM. Cells were examined by Western blotting at 3 h after treatment with mitogen. In the EGF induction same amount of DMSO as the control was added. (B) Phosphorylation levels of Akt, S6K and Erk in PKC-knockdown cells. Huh 7 cells were transfected with siRNA of negative control (nC), PKCα, PKCδ, PKCε and both PKCδ and ε, and examined by Western blotting at 24 h after siRNA transfection. (C) Induction of Akt-, S6K- and Erk-phosphorylation by TPA and EGF. Huh 7 cells transfected with siRNA of negative control (nC), PKCδ, PKCε (left panel) and PKCα (right panel) were treated for 30 min with 50 nM of TPA (T), 20 ng/ml of EGF (E) and DMSO as a control (D) in a serum-free DMEM, and examined with Western blotting. In the EGF induction same amount of DMSO as the control was added.

τ/λ) groups according to the requirement for $Ca^{++}$ and diacylglycerol [42]. Each PKC isoform shows a tissue-specific expression pattern and specific subcellular localization and is thought to play specific roles [43,44]. PKCα, PKCε and PKCδ are dominantly expressed in liver cancer cell lines but expression of PKCβ, PKCγ, PKCη and PKCθ are not. PKCα, a conventional PKC, is overexpressed in some cases of HCC and involved in the development, proliferation and invasion of HCC cells [45–47]. Although the expression of novel PKCs in the liver cancer was reported, the role of the novel PKCs, PKCδ and PKCε in liver cancer remains unclear. PKCδ and PKCε play differential roles in the development and progression of cancer by regulating proliferation, invasion and apoptosis [48]. The current data showed that knockdown of PKCδ or PKCε by siRNA reversed the PDCD4 protein levels after TPA exposure to HCC cells, thus suggesting the requirement of novel PKCs in the down-regulation of PDCD4, most probably via proteasomal degradation. Recent reports have demonstrated the activation of the PI3K/Akt/mTOR pathway to be required to induce the proteasomal degradation of PDCD4. However, the presence of pathways that promote PDCD4 protein degradation other than the PI3K/Akt/mTOR pathway has also suggested [13].

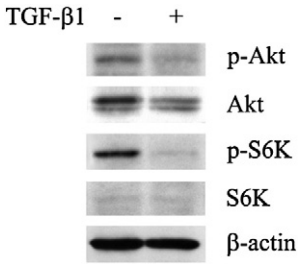


**Fig. 9.** TGF-β1 suppresses phosphorylation of Akt and S6K. Huh 7 cells were cultured for 24 h in the presence and absence of TGF-β1 (4 ng/ml) and analyzed by Western blotting.

We have observed that the PI3K inhibitor LY294002 and mTOR inhibitor rapamycin are not able to reverse the TPA-induced PDCD4

suppression in Huh7 cells in contrast to the results in which LY294002 reversed the TPA-induced PDCD4 suppression in HEK293 cells [13]. Although the pathway other than the PI3K/Akt/mTOR seems to be dominant for the TPA-induced PDCD4 suppression in Huh7 cells, the analyses of phospho-Akt and phospho-S6K indicate that the PI3K–Akt–mTOR–S6K signaling cascade also contributes at least partly in the TPA-induced PDCD4 degradation (Fig. 3C, Fig. 8) in accordance with previous reports [13,23,24]. It is also possible that the pathway other than the PI3K/Akt/mTOR is accelerated by TPA under the presence of TGF-β1 because the phosphorylation of Akt and S6K has been suppressed in TGF-β1-treated Huh7 cells (Fig. 9). Phosphorylation of Erk is stimulated at similar levels by TPA as well as by EGF and is suppressed in the presence of siRNA against PKCδ and ε (Fig. 8B). The suppression of TPA- or EGF-induced Erk phosphorylation by PKCε knockdown was slightly compared to that by PKCδ knockdown (Fig. 8C), suggesting the differential role of PKCδ and ε in the Erk phosphorylation by mitogens. The data also showed the partial association between PDCD4 suppression and Erk phosphorylation, indicating the involvement of Erk signal in the regulation of PDCD4 as recently reported [13]. PDCD4 has a number of putative phosphorylation sites for many types of kinases [10]. It is also possible to speculate that direct phosphorylation of PDCD4 by kinases, such as members of the MAP kinase pathway and PKC family might lead to proteasomal degradation, although this hypothesis requires further study.

In conclusion, the current study revealed that the tumor suppressor PDCD4 is regulated in a PKC isoform-specific manner. The results showed the involvement of the novel PKC isoforms, PKCδ and PKCε, in the degradation of PDCD4 protein through proteasomes. As a result, the inhibition of PDCD4 degradation through PKC inhibition might therefore provide a novel strategy for cancer prevention and/or cancer treatment.

## References


[1] K. Shibahara, M. Asano, Y. Ishida, T. Aoki, T. Koike, T. Honjo, Isolation of a novel mouse gene MA-3 that is induced upon programmed cell death, Gene 166 (1996) 297–301.
[2] Y. Onishi, S. Hashimoto, H. Kizaki, Cloning of the TIS gene suppressed by topoisomerase inhibitors, Gene 215 (1998) 453–459.
[3] S. Matsuhashi, H. Yoshinaga, H. Yatsuki, A. Tsugita, K. Hori, Isolation of a novel gene form a human cell line with Pr-28 MAb which recognizes a nuclear antigen involved in the cell cycle, Res. Commun. Biochem. Cell Mol. Biol. 1 (1997) 109–120.
[4] H. Yoshinaga, S. Matsuhashi, C. Fujiyama, Z. Masaki, Novel human PDCD4 (H731) gene expressed in proliferative cells is expressed in the small duct epithelial cells of the breast as revealed by an anti-H731 antibody, Pathol. Int. 49 (1999) 1067–1077.
[5] H. Soejima, O. Miyoshi, H. Yoshinaga, Z. Masaki, I. Ozaki, S. Kajihara, N. Niikawa, S. Matsuhashi, T. Mukai, Assignment of the programmed cell death 4 gene (PDCD4) to human chromosome band 10q24 by in situ hybridization, Cytogenet. Cell Genet. 87 (1999) 113–114.
[6] H. Zhang, I. Ozaki, T. Mizuta, H. Hamajima, T. Yasutake, Y. Eguchi, H. Ideguchi, K. Yamamoto, S. Matsuhashi, Involvement of programmed cell death 4 in transforming growth factor-β1-induced apoptosis in human hepatocellular carcinoma, Oncogene 25 (2006) 6101–6112.
[7] L. Azzoni, O. Zatsepnia, B. Abebe, I.M. Bennett, P. Kanakaraj, B.J. Rerussia, Differential transcriptional regulation of CD161 and a novel gene, 197/15a by IL-2, IL-15 and IL-12 in NK and T cells, J. Immunol. 161 (1998) 3493–3500.
[8] U. Schlichter, O. Burk, S. Worpenberg, K.H. Klempnauer, The chicken Pdcd4 gene is regulated by v-Myb, Oncogene 20 (2001) 231–239.
[9] U. Schlichter, D. Kattmann, H. Appl, J. Miethe, A. Brehmer-Fastnacht, K.H. Klempauner, Identification of the myb-inducible promoter of the chicken Pdcd4 gene, Biochem. Biophys. Acta. 1520 (2001) 99–104.
[10] A. Goke, R. Goke, A. Knolle, H. Trusheim, H. Schmidt, A. Wilmen, R. Carmody, B. Goke, Y.H. Chen, DUG is a novel homologue of translation initiation factor 4G that binds eIF4A, Biochem. Biophys. Res. Commun. 297 (2002) 78–82.
[11] J.L. Cmarik, H. Min, G. Hegamyer, S. Zhang, M. Kulesz-Martin, H. Yoshinaga, S. Matsuhashi, N.H. Colburn, Differentially expressed protein Pdcd4 inhibits tumor promoter-induced neoplastic transformation, Proc. Natl. Acad. Sci. U. S. A. 96 (1996) 14037–14042.
[12] A.P. Jansen, C.E. Camalier, N.H. Colburn, Epidermal expression of the translation inhibitor programmed cell death 4 suppresses tumorigenesis, Cancer Res. 65 (2005) 6034–6041.
[13] T. Schmid, A.P. Jansen, A.R. Baker, G. Hegamyer, J.P. Hagan, N.H. Colburn, Translation inhibitor Pdcd4 is targeted for degradation during tumor promotion, Cancer Res. 68 (2008) 1254–1260.
[14] A. Hilliard, B. Hilliard, S. Zheng, H. Sun, T. Miwa, S. Song, R. Goke, Y.H. Chen, Translational regulation of autoimmune inflammation and lymphoma genesis by programmed cell death 4, J. Immunol. 177 (2006) 8095–8102.
[15] H.S. Yang, J.L. Knies, C. Stark, N.H. Colburn, Pdcd4 suppresses tumor phenotype in JB6 cells by inhibiting AP-1 transactivation, Oncogene 22 (2003) 3712–3720.
[16] H.S. Yang, M.H. Cho, H. Zakowicz, G. Hegamyer, N. Sonenberg, N.H. Colburn, A novel function of the MA-3 domains in transformation and translation suppressor Pdcd4 is essential for its binding to eukaryotic translation initiation factor 4A, Mol. Cell. Biol. 24 (2004) 3894–3906.
[17] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell 100 (2000) 57–70.
[18] T. Motyl, M. Kasterka, K. Grzelkowska, J. Ostrowski, M. Filipecki, E. Malicka, T. Pioszaj, Phorbol ester(12-O-tetradecanoylphorbol-13-acetate) prevents ornithine decarboxylase inhibition and apoptosis in L1210 cells exposed to TGF-β1, Int. J. Biochem. Cell Biol. 28 (1996) 1327–1335.
[19] M.P. De Caestecker, E. Piek, A.B. Roberts, Role of transforming growth factor-β signaling in cancer, J. Natl Cancer Inst. 92 (2000) 1388–1402.
[20] I. Yakymovych, P. ten Dijke, C.H. Heldin, S. Souchelnytskyi, Regulation of Smad signaling by protein kinase C, FASEB J. 15 (2001) 553–555.
[21] N.V. Dorrello, A. Peschiaroli, D. Guardavaccaro, N.H. Colburn, N.E. Sherman, M. Pagano, S6K1- and βTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth, Science 314 (2006) 467–471.
[22] J. Schlessinger, Cell signaling by receptor tyrosine kinases, Cell 103 (2000) 211–225.
[23] B. Ozpolat, U. Akar, M. Steiner, I. Zorrilla-Calancha, M. Tirado-Gometz, N. Colburn, M. Danilenko, S. Kornblau, G.L. Berestein, Programmed cell death-4 tumor suppressor protein contributes to retinoic acid-induced terminal granulocytic differentiation of human myeloid leukemia cell, Mol. Cancer Res. 5 (2007) 95–108.
[24] B. Lankat-Buttgereit, S. Muller, H. Schmidt, K.G. Parhofer, T.M. Gress, R. Goke, Knockdown of pdcd4 results in induction of proprotein convertase 1/3 and potent secretion of chromogranin A and secretogranin II in a neuroendocrine cell line, Biol. Cell 100 (2008) 703–715.
[25] S.L. Hsu, Y.H. Chou, S.C. Yin, J.Y. Liu, Differential effects of phorbol ester on growth and protein kinase C isoenzyme regulation in human hepatoma Hep3B cells, Biochem. J. 333 (1998) 57–64.
[26] T. Wu, Y. Hsieh, Y. Hsieh, J. Liu, Regulation of PKCα decreases cell proliferation, migration, and invasion of human malignant hepatocellular carcinoma, J. Cell. Biochem. 103 (2008) 9–20.
[27] S. Takai, R. Matsushima-Nishiwaki, H. Tokuda, E. Yasuda, H. Toyoda, Y. Kaneoka, A. Yamaguchi, T. Kumada, O. Kozawa, Protein kinase C δ regulates the phosphorylation of heat shock protein 27 in human hepatocellular carcinoma, Life Sci. 81 (2007) 585–591.
[28] J. Seoane, Escaping from the TGFβ anti-proliferative control, Carcinogenesis 27 (2006) 2148–2156.
[29] B. Bierie, H.L. Moses, Tumor microenviroment: TGFbeta: the molecular Jekyll and Hyde of cancer, Nat. Rev. Cancer 6 (2006) 506–520.
[30] K. Matsuzaki, M. Murata, K. Yoshida, G. Sekimoto, Y. Uemura, N. Sakaida, M. Kaibori, Y. Kamiyama, M. Nishizawa, J. Fujisawa, K. Okazaki, T. Seki, Chronic inflammation associated with hepatitis C virus infection perturbs hepatic transforming growth factor β signaling, promoting cirrhosis and hepatocellular carcinoma, Hepatology 46 (2007) 48–57.
[31] H. Zhang, I. Ozaki, T. Mizuta, T. Yoshimura, S. Matsuhashi, Y. Eguchi, T. Yasutake, A. Hisatomi, T. Sakai, K. Yamamoto, Transforming growth factor-β1-induced apoptosis is blocked by β1-integrin-mediated mitogen-activated protein kinase activation in human hepatoma cells, Cancer Sci. 95 (2004) 878–886.
[32] Y. Chen, T. Knosel, G. Kristiansen, A. Pietas, M.E. Garber, S. Matsuhashi, I. Ozaki, I. Petersen, Loss of PDCD4 expression in human lung cancer correlates with tumor progression and prognosis, J. Pathol. 200 (2003) 640–646.
[33] G. Mudduluru, F. Medved, R. Grobholz, C. Jost, A. Gruber, J.H. Leupold, S. Post, A. Jansen, N.H. Colburn, H. Allgayer, Loss of programmed cell death 4 expression marks adenoma-carcinoma transition, correlates inversely with phosphorylated protein kinase B, and is an independent prognostic factor in resected colorectal cancer, Cancer 110 (2007) 1697–1707.
[34] Y.H. Wen, X. Shi, L. Chiriboga, S. Matsuhashi, H. Yee, O. Afonia, Alterations in the expression of PDCD4 in ductal carcinoma of the breast, Oncol. Rep. 18 (2007) 1387–1393.
[35] M. Shiota, H. Izumi, A. Tanimoto, M. Takahashi, N. Miyamoto, E. Kashiwagi, A. Kidani, G. Hirano, D. Masubuchi, Y. Fukunaka, Y. Yasuniwa, S. Naito, S. Nishizawa, Y. Sasaguri, K. Kohno, Programmed cell death protein 4 down-regulated Y-Box binding protein-1 expression via direct interaction with Twist1 to suppress cancer cell growth, Cancer Res. 69 (2009) 3148–3156.
[36] F.J. Sheedy, E. Pallson-McDermott, E.J. Hennessy, C. Martin, J.J. O'Leary, Q. Ruan, D. S. Johnson, Y. Chen Test, article sample title placed here, Nat. Immunol. 11 (2010) 141–147.
[37] R. Visone, C.M. Croce, MiRNAs and Cancer, Am. J. Pathol. 174 (2009) 1131–1138.
[38] L.B. Frankel, N.R. Christoffersen, A. Jacobsen, M. Lindow, A. Krogh, A.H. Lund, Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells, J. Biol. Chem. 283 (2008) 1026–1033.
[39] I.A. Asangani, S.A. Rasheed, D.A. Nikolova, J.H. Leupold, N.H. Colburn, S. Post, H. Allgayer, MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer, Oncogene 27 (2008) 2128–2136.
[40] S. Zhu, H. Wu, F. Wu, D. Nie, S. Sheng, Y.Y. Mo, MicroRNA-21 targets tumor suppressor genes in invasion and metastasis, Cell Res. 18 (2008) 350–359.
[41] A.C. Newton, Protein kinase C: Structure, function, and regulation, J. Biol. Chem. 270 (1995) 28495–28498.
[42] S. Ohno, Y. Nishizuka, Protein kinase C isotypes and their specific functions: Prologue, J. Biochem. 132 (2002) 509–511.

[43] M. Goldberg, S.F. Steinberg, Tissue-specific developmental regulation of protein kinase C isoforms, Biochem. Pharmacol. 51 (1996) 1089–1093.
[44] Y. Shirai, N. Saito, Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting, J. Biochem. 132 (2002) 663–668.
[45] G. Perletti, L. Tessitore, E. Sesca, P. Pani, M.U. Dianzani, F. Piccinini, εPKC acts like a marker of progressive malignancy in rat liver, but fails to enhance tumorigenesis in rat hepatoma cells in culture, Biochem. Biophys. Res. Commun. 221 (1996) 688–691.
[46] Y. Lee, S. Hong, M. Lee, M. Kim, J. Jang, Differential expression of protein kinase C isoforms in diethylnitrosamine-initiated rat liver, Cancer Lett. 126 (1998) 17–22.
[47] H. Tsai, Y. Hsieh, S. Kuo, S. Chen, S. Yu, C. Huang, A. Chang, Y. Wang, M. Tsai, Y. Liu, Alteration in the expression of protein kinase C isoforms in human hepatocellular carcinoma, Cancer Lett. 161 (2000) 171–175.
[48] E.M. Griner, M.G. Kazanietz, Protein kinase C and other diacylglycerol effectors in cancer, Nat. Rev. Cancer 7 (2007) 281–294.