AKT Activation by Pdc4 Knockdown Up-Regulates Cyclin D1 Expression and Promotes Cell Proliferation

Genes & Cancer
2(8) 818–828
© The Author(s) 2011
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1947601911431082
http://ganc.sagepub.com

Xiaoling Guo^{1,2,*}, Wenjuan Li^{1,*}, Qing Wang¹, and Hsin-Sheng Yang^{1,3}

Submitted 13-Sep-2011; accepted 02-Nov-2011

Abstract

Programmed cell death 4 (Pdc4), a novel tumor suppressor, inhibits neoplastic transformation and tumor invasion. In this study, the authors found that knockdown of Pdc4 promoted cell proliferation and up-regulated cyclin D1 expression. Previously, the authors demonstrated that Pdc4 knockdown activated NF- κ B-dependent transcription. Mutations of NF- κ B binding sites on the cyclin D1 promoter attenuated the cyclin D1 promoter activity induced by Pdc4 knockdown. In addition, knockdown of NF- κ B/I κ B kinase (IKK) α or IKK β , the kinase regulating NF- κ B activation, inhibited cyclin D1 promoter activity and cyclin D1 expression, indicating that up-regulation of cyclin D1 by Pdc4 knockdown is contributed, at least in part, by NF- κ B activation. To investigate the mechanism of how Pdc4 knockdown activates NF- κ B, the authors found that the levels of AKT phosphorylation and AKT kinase activity were increased in the Pdc4 knockdown cells. Conversely, ectopic expression of Pdc4 inhibited AKT phosphorylation and cyclin D1 expression, suggesting that Pdc4 regulates AKT activity and cyclin D1 expression. Furthermore, knockdown of AKT in the Pdc4 knockdown cells inhibited IKK phosphorylation, NF- κ B activation, cyclin D1 promoter activity, and cyclin D1 expression as well as cell proliferation. Taken together, these findings suggest that activation of NF- κ B by Pdc4 knockdown through AKT contributes to the elevated expression of cyclin D1, thus providing new insights into how loss of Pdc4 expression promotes tumor development.

Keywords

Pdc4, cyclin D1, AKT, NF- κ B, IKK

Introduction

Programmed cell death 4 (Pdc4) is a novel tumor suppressor that is frequently down-regulated in several types of cancers. In colon cancer tissues, the expression of Pdc4 is continuously down-regulated in the order of normal-adenoma-carcinoma.¹ Overexpression of *pdc4* cDNA inhibits 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced transformation in mouse JB6 tumor promotion susceptible cells² and tumor phenotype in JB6 transform cells.³ Recently, Pdc4 has been shown to suppress tumor cell invasion and intravasation in colon tumor RKO cells^{4,5} and prostaglandin E₂-induced invasion in breast cancer MCF7 cells.⁶ Knockdown of Pdc4 expression promotes invasion in colon HT29 and GEO cells^{7,8} as well as breast cancer MCF7 and D47T cells.⁹ In addition to cell culture systems, Pdc4 transgenic mice that overexpress Pdc4 in the epidermis showed significant reduction in 7,12-dimethylbenz(a)anthracene (DMBA)/TPA-induced skin papilloma formation and carcinoma incidence.¹⁰ Conversely, knockout of Pdc4 in mice resulted in induction of lymphomas with frequent metastasis¹¹ and an increase in DMBA/TPA-induced skin papilloma formation and carcinoma incidence.¹² These findings suggest that Pdc4 suppresses carcinogenesis at both promotion and progression stages. Most importantly, a higher Pdc4 protein level correlates with a good prognosis in lung,¹³ colon,¹ and ovarian¹⁴ cancer patients.

Recently, we showed that aerosol delivery of *pdc4* cDNA into a murine lung inhibits cell proliferation and enhances apoptosis in K-ras null mice and AP-1 reporter mice.^{15,16} Similarly, ectopic expression of Pdc4 in ovarian cancer cell lines inhibits cell proliferation and cell cycle progression and induces apoptosis.¹⁷ Moreover, immunohistochemical studies showed that expression of Pdc4 is inversely correlated with the proliferation marker, Ki-67, in the human tissues of intraductal papillary mucinous carcinoma.¹⁸ All of these findings suggest that Pdc4 is involved in the regulation of cell cycle progression and cell proliferation. However, the mechanisms involved in these processes are still unclear.

AKT (also known as protein kinase B) is one of the most frequently activated protein kinases in human cancer.¹⁹ AKT mediates a large spectrum of cellular functions

¹Graduate Center for Toxicology, College of Medicine, University of Kentucky, Lexington, KY, USA

²Shandong University, Jinan, Shandong Province, China

³Markey Cancer Center, University of Kentucky, Lexington, KY, USA

*These authors contributed equally to this work.

Corresponding Author:

Hsin-Sheng Yang, Graduate Center for Toxicology and Markey Cancer Center, College of Medicine, University of Kentucky, 306 HSRB, 800 Rose Street, Lexington, KY 40536
Email: hyang3@uky.edu

including cell proliferation, survival, and apoptosis by phosphorylation of its substrates at serine/threonine residues residing in the RXRXXS/T motif.²⁰ Cyclin D1 is one of the mediators for AKT in regulating cell proliferation. Phosphorylation of cyclin D1 at Thr286 by glycogen synthase kinase 3 β (GSK3 β) leads to ubiquitin-mediated degradation.²¹ AKT-dependent phosphorylation inhibits GSK3 β catalytic activity, thus resulting in stabilization of cyclin D1. In addition, AKT can regulate cyclin D1 expression through the NF- κ B/I κ B kinase (IKK) pathway.²² The NF- κ B family comprises 5 members named p105/p50 (NF- κ B1), p100/p52 (NF- κ B2), p65 (RelA), c-Rel, and RelB, which form homodimers or heterodimers.²³ NF- κ B is mainly activated through IKK.²⁴ In canonical pathways, I κ Bs bind with NF- κ B complexes in the cytoplasm. Phosphorylation of I κ Bs by the IKK α /IKK β complex results in degradation of I κ B, leading to nuclear translocation of various NF- κ B complexes. In noncanonical pathways, activation of the RelB/p52 NF- κ B complex is through processing of p100, a precursor of p52, binding with RelB in the cytoplasm. Upon phosphorylation by IKK α , p100 is processed by proteasome degradation to generate p52, and then the RelB/p52 complex translocates into the nucleus. After translocation into the nucleus, the NF- κ B complex activates the expression of NF- κ B target genes including cyclin D1.²⁴

In this study, we demonstrated that knockdown of Pdc4 promotes cell proliferation and up-regulates cyclin D1 expression, which is regulated, at least in part, through AKT-activated NF- κ B.

Results

Knockdown of Pdc4 expression promotes cell proliferation. To investigate the roles of Pdc4 in cell proliferation and survival, we stably knocked down Pdc4 expression in HT29 cells using a lentivirus-mediated system as described previously.⁷ The rate of cell proliferation in Pdc4 knockdown HT29 cells (HT29-shPdc4) and control cells (HT29-shLacZ) was subsequently analyzed using XTT analysis. As shown in Figure 1A, knocking down Pdc4 expression in HT29 cells promotes proliferation. The growth rate of HT29-shPdc4 cells at day 4 and day 5 was approximately 30% to 40% faster than that of HT29-shLacZ cells. Next, we determined whether the increased proliferation in Pdc4 knockdown cells is due to the reduced cell apoptosis. Cells were stained with annexin V and propidium iodide and measured using fluorescence-activated cell sorting (FACS) analysis. Knockdown of Pdc4 did not change apoptosis as compared to control cells (2.73 v. 2.67) (Fig. 1B). In addition, the population of viable cells was similar in HT29-shPdc4 and HT29-shLacZ cells (87.51 v. 89.03) (Fig. 1B). Last, to examine the effects of Pdc4 knockdown on cell cycle progression, cell cycle distribution was examined

using FACS analysis. Compared to HT29-shLacZ cells, HT29-shPdc4 cells showed a reduction in the percentage of cells in the G1 phase (39.19% v. 30.95%) and an increase in the percentage of cells in the G2/M phase (8.85% v. 17.02%) (Fig. 1C). Because the apoptosis assays (Fig. 1B) indicated that the populations of viable and apoptotic cells were similar in HT29-shLacZ and HT29-shPdc4 cells, the increase in cell number in the G2/M phase of the HT29-shPdc4 cells is not likely due to cell growth arrest. It is possible that a fast G1 phase progression of Pdc4 knockdown cells results in an accumulation of cells in the G2/M phase. Pdc4 knockdown did not affect the percentage of cells in the S phase and sub-G1 phase. These results demonstrated that knockdown of Pdc4 increases the rate of cell proliferation but does not decrease the rate of cell death.

Knockdown of Pdc4 up-regulates cyclin D1 expression. The results of cell cycle distribution analysis (Fig. 1C) suggested that knockdown of Pdc4 shortened the G1 phase progression. It has been known that overexpression of cyclin D1 in cultured cells shortens the G1 phase and increases cell proliferation.²⁵ Cyclin D1 promotes G1 phase progression through forming active complexes with cyclin-dependent kinase (CDK) 4 and 6, resulting in the activation of genes for G1-S phase transition.²⁶ To test whether knockdown of Pdc4 up-regulates cyclin D1 expression, the protein and mRNA levels of cyclin D1 in both HT29-shLacZ and HT29-shPdc4 cells were examined. As shown in Figure 2A, the cyclin D1 protein level in HT29-shPdc4 cells was about 21-fold higher than that in HT29-shLacZ cells. Similarly, the cyclin D1 mRNA level was increased approximately 7-fold in HT29-shPdc4 cells compared to that in HT29-shLacZ cells (Fig. 2B). These results indicate that knockdown of Pdc4 induces cyclin D1 expression.

NF- κ B regulates cyclin D1 expression in Pdc4 knockdown cells. The NF- κ B complex has been known to play an essential role in the regulation of cyclin D1 expression.²⁶ Previously, we have demonstrated that knockdown of Pdc4 activates NF- κ B-dependent transcription.⁷ To examine whether Pdc4 knockdown activated NF- κ B enhances cyclin D1 expression, we transfected cyclin D1 promoter luciferase reporter plasmid (D1) into HT29-shLacZ and HT29-shPdc4 cells. The luciferase activity was approximately 32-fold higher in HT29-shPdc4 cells than that in HT29-shLacZ cells (Fig. 3A, D1), indicating that the promoter activity of cyclin D1 is dramatically activated by Pdc4 knockdown. The D1 construct comprises a region of the cyclin D1 promoter from -920 bp to +106 bp (relative to the transcription start site) fused with a luciferase reporter, which contains 3 potential NF- κ B binding sites located at -858 bp, -749 bp, and -39 bp.²⁷ To test whether NF- κ B binding sites mediate the activation of cyclin D1

Figure 1. Knockdown of Pcd4 promotes cell proliferation. **(A)** Knockdown of Pcd4 promotes cell proliferation. HT29-shLacZ or HT29-shPcd4 cells were seeded onto 96-well plates at day 0 (3×10^3 cells/well). The rate of cell growth was measured from day 1 to day 5. The value recorded at day 1 is designated as 100%. Results are expressed as mean $\pm$ standard deviation ($n = 4$). The asterisk indicates a significant difference by 1-way ANOVA ($P < 0.01$). **(B)** Pcd4 knockdown does not affect cell apoptosis. Cells were stained with FITC-annexin V and propidium iodide (PI) and analyzed by flow cytometry. The number in each quadrant represents the percentage of total cells. **(C)** Knockdown of Pcd4 shortens the G1 phase. Cells were treated with 40 μ g/mL PI and analyzed using flow cytometry.

promoter in Pcd4 knockdown cells, the potential NF- κ B binding sites were mutated and transfected into HT29-shLacZ and HT29-shPcd4 cells. All of the mutated cyclin D1 constructs showed a similar basal level of promoter activity in the HT29-shLacZ cells (Fig. 3A, open bars). The D1-858m and D1-749m constructs, in which the NF- κ B binding site at -858 bp and -749 bp was mutated, respectively, exhibited approximately 30% lower promoter activity than wild-type (D1) in HT29-shPcd4 cells. The D1-39m construct, which the NF- κ B binding site at -39 bp was mutated, showed approximately 50% promoter activity compared to the D1 construct in the HT29-shPcd4 cells. When all 3 potential NF- κ B binding sites were mutated (D1-m3), the D1-m3 construct exhibited a similar promoter activity in HT29-shLacZ and HT29-shPcd4 cells, indicating that mutation of these 3 NF- κ B binding sites abolished the cyclin D1 promoter activity induced by Pcd4 knockdown. These results suggest that NF- κ B binding sites mediate the cyclin D1 promoter activity in the HT29-shPcd4 cells.

IKK α and IKK β , 2 major kinases for activating NF- κ B, phosphorylate I κ B and subsequently lead to translocation of NF- κ B into the nucleus. To determine whether IKK α and/or IKK β play an essential role in activating NF- κ B in the Pcd4 knockdown cells, the phosphorylation status of IKK α / β was first examined in HT29-shLacZ and HT29-shPcd4 cells. The level of phospho-IKK α / β in HT29-shPcd4 cells was approximately 2.2-fold higher than that in HT29-shLacZ cells (Fig. 3B). Interestingly, the total proteins of IKK α and IKK β also showed approximately 1.5- and 3.6-fold higher in HT29-shPcd4 cells than HT29-shLacZ cells, respectively. Next, we determined whether knockdown of IKK α or IKK β reverses the cyclin

Figure 2. Knockdown of Pdc4 up-regulates cyclin D1 expression. **(A)** The protein level of cyclin D1 in HT29-shLacZ and HT29-shPdc4 cells was examined using Western blotting analysis. The ratio of cyclin D1/GAPDH in HT29-shLacZ cells is designated as 1.0. **(B)** The cyclin D1 mRNA levels in HT29-shLacZ and HT29-shPdc4 cells were analyzed with real-time PCR. Three independent experiments were performed with triplicates for each sample every time. Results are expressed as mean $\pm$ standard deviation ($n = 3$). The ratio of cyclin D1/GAPDH in HT29-shLacZ cells is designated as 1. The asterisk indicates a significant difference by 1-way ANOVA ($P < 0.001$).

D1 promoter activity induced by Pdc4 knockdown. HT29-shPdc4 cells were transfected with *IKK α* siRNA or *IKK β* siRNA. Two days after transfection, these cells were further transfected with the cyclin D1 promoter reporter (D1). As shown in Figure 3C, the cyclin D1 promoter activity of cells transfected with *IKK α* siRNA or *IKK β* siRNA was approximately 50% and 65% of that in control cells, respectively. To further confirm that knockdown of IKKs alters cyclin D1 expression, the HT29-shPdc4 cells were transfected with *IKK α* siRNA or *IKK β* siRNA. Four days after transfection, the cyclin D1 protein levels were determined. Transfection of *IKK α* siRNA and *IKK β* siRNA HT29 shPdc4 cells reduced the *IKK α* and *IKK β* protein level to 20% and 10% of that in control cells (control), respectively. In agreement with the results of cyclin D1 promoter activity assays, the cyclin D1 protein in *IKK α* (si-*IKK α*) or *IKK β* (si-*IKK β*) knockdown cells is approximately 40% of that in control cells (Fig. 3D). Taken together, these findings suggest that activation of NF- κ B by IKKs enhances cyclin D1 expression in the Pdc4 knockdown cells.

Knockdown of Pdc4 activates AKT. The AKT signaling pathway plays an important role in regulating cell proliferation, survival, and apoptosis by activating a set of transcription factors including NF- κ B.^{28,29} To test whether knockdown of Pdc4 in colon HT29 cells activates AKT,

the phosphorylation status of AKT in both HT29-shLacZ and HT29-shPdc4 cells was examined. The level of phospho-AKT (Ser473) in HT29-shPdc4 cells is approximately 3.8-fold higher than that in HT29-shLacZ cells (Fig. 4A), whereas the levels of total AKT in both HT29-shPdc4 and HT29-shLacZ cells were similar. Interestingly, the level of phospho-AKT (Thr308) was undetectable in either HT29-shLacZ or HT29-shPdc4 cells (data not shown). This finding raised the question of whether phosphorylation of AKT at Ser473 is sufficient to activate AKT kinase activity. To test this, the AKT kinase assays were performed using phospho-AKT (Ser473) antibody to precipitate the active form of AKT. The kinase activity of antibody-bound AKT was then assayed by adding the AKT substrate, a recombinant GSK3 α/β fusion protein. AKT kinase activity in HT29-shPdc4 cells was approximately 20.2-fold higher than that in HT29-shLacZ cells, whereas the levels of the total AKT in the cell lysates used to immunoprecipitate phospho-AKT were similar (Fig. 4B). These findings suggest that Pdc4 knockdown increases AKT phosphorylation and AKT kinase activity.

To further confirm that Pdc4 regulates AKT activity, Pdc4 was overexpressed in colon LoVo cells using the lentivirus expression system.⁸ The Pdc4-expressing cells (LoVo-Pdc4) exhibited an approximately 3.3-fold higher Pdc4 protein level than the cells transduced with lentivirus-empty vector (LoVo-control) (Fig. 4C). The phosphorylation of AKT at Ser473 was dramatically inhibited by overexpressing Pdc4 (Fig. 4C). In addition, the protein level of cyclin D1 in LoVo-Pdc4 cells was approximately 50% suppressed by Pdc4 (Fig. 4C). These findings collectively suggest that Pdc4 regulates cyclin D1 expression through regulating AKT activation.

GSK3 β is involved in regulating cyclin D1 expression in HT29-shPdc4 cells. It has been known that AKT also can regulate cyclin D1 expression by inhibiting GSK3 β activity through phosphorylation of GSK3 β , which inhibits proteasome degradation of cyclin D1.³⁰ We have demonstrated that knockdown of Pdc4 activates AKT (Fig. 4). Therefore, it is logical to examine whether inhibition of GSK3 β activity contributes to the stabilization of cyclin D1 in Pdc4 knockdown cells. The level of phospho-GSK3 β was approximately 2.1-fold higher in HT29-shPdc4 cells than that in HT29-shLacZ cells, whereas the total GSK3 β protein level was similar between HT29-shLacZ and HT29-shPdc4 cells (Fig. 5A), suggesting that the activity of GSK3 β was inhibited in the Pdc4 knockdown cells. In addition, we found that treatment with MG132 increased the cyclin D1 protein level approximately 9- and 2-fold in the HT29-shLacZ cells and HT29-Pdc4 cells, respectively (Fig. 5B), indicating that stabilization of cyclin D1 by inhibiting proteasome activity plays a role in up-regulating

Figure 3. Phosphorylation of IKK activates NF- κ B to up-regulate cyclin D1 in Pcdcd4 knockdown cells. **(A)** Mutations of NF- κ B binding sites on the cyclin D1 promoter abolish the Pcdcd4 knockdown-induced cyclin D1 promoter activity. The cyclin D1 promoter reporter constructs (0.2 μ g), wild-type (D1) and NF- κ B binding site-mutated plasmids, were transfected into HT29-shLacZ and HT29-shPcdcd4 cells along with 10 ng of pRL-SV40. After 48 hours, the firefly and Renilla luciferase activities were determined as described previously.⁷ The ratio of firefly luciferase/Renilla luciferase activity in HT29-shLacZ cells transfected with D1 is designated as 1 (D1, open bar). Three independent experiments were performed with 5 replicates for each sample. The represented data are shown and expressed as mean $\pm$ standard deviation ($n = 5$). The asterisk denotes a significant difference as determined by 1-way ANOVA ($P < 0.001$). **(B)** Knockdown of Pcdcd4 increases phosphorylation and expression of IKK α and IKK β . The ratio of target protein/GAPDH in HT29-shLacZ cells is designated as 1.0. **(C)** Knockdown of IKK α or IKK β inhibits cyclin D1 promoter activity. The expression of IKK α (si-IKK α) and IKK β (si-IKK β) was knocked down in the HT29-shPcdcd4 cells by transfection of *ikkα* and *ikkβ* siRNA, respectively. The HT29-shPcdcd4 cells transfected with scramble siRNA were used as the control (control). After 48 hours, 0.2 μ g of the D1 construct was transfected along with 10 ng of pRL-SV40 into si-IKK α , si-IKK β , and control cells. The firefly and Renilla luciferase activities were determined as described in **A**. The ratio of firefly luciferase/Renilla luciferase activity in control cells is designated as 100%. Three independent experiments were performed with 5 replicates for each sample. The represented data are shown and expressed as mean $\pm$ standard deviation ($n = 5$). The asterisk denotes a significant difference as determined by 1-way ANOVA ($P < 0.005$). **(D)** Knockdown of IKK α or IKK β inhibits cyclin D1 expression. Knockdown of IKK α or IKK β is described above. Four days after transfection, cell lysates were prepared, and Western blot assays were performed. The ratio of target protein/GAPDH in control cells is designated as 1.0.

cyclin D1 in both control cells and Pcdcd4 knockdown cells. These results imply that inhibition of GSK3 β activity by Pcdcd4 knockdown contributes to the elevated cyclin D1 level.

Knockdown of AKT reverses cyclin D1 expression, NF- κ B activation, and cell proliferation induced by Pcdcd4 knockdown. To determine the causal role of AKT activation in increasing cell proliferation and cyclin D1 expression in Pcdcd4 knockdown cells, we knocked down AKT expression in HT29-shPcdcd4 cells using *akt* siRNA. The levels of total AKT and phospho-AKT in HT29-shPcdcd4 cells transfected with *akt* siRNA (si-AKT) were about 10% of those in cells transfected with scramble siRNA (control), as shown in Figure 6A. Knockdown of AKT in HT29-shPcdcd4 cells led to the decreased protein level of cyclin D1, phospho-IKK α / β , and phospho-GSK3 β as well as the reduced level of cyclin D1 mRNA (Fig. 6A and 6B), suggesting that AKT contributes to the elevated cyclin D1 expression in HT29-shPcdcd4 cells. However, knockdown of AKT did not alter the total protein levels of IKK α and IKK β (Fig. 6A), suggesting that IKK α and IKK β expressions are not dependent on AKT in HT29-shPcdcd4 cells. In addition, the cyclin D1 promoter activity was also inhibited approximately 50% by AKT knockdown, as shown in Figure 6C. To demonstrate that AKT regulates NF- κ B activation, the NF- κ B-luciferase reporter was transfected into control and si-AKT cells. The NF- κ B-dependent transcription was about 40% lower in si-AKT cells than that in control cells (Fig. 6D), suggesting that AKT knockdown inhibits NF- κ B activation. Last, cell proliferation in control and si-AKT cells was measured using colorimetric XTT analysis. The si-AKT cells showed slower proliferation compared to the control cells (Fig. 6E). The rate of cell proliferation at day 5 and day 6 was approximately 20% to

30% lower in si-AKT cells than that in control cells, indicating that knockdown of AKT reverses the cell proliferation induced by Pcdcd4 knockdown.

Figure 4. Pdc4 regulates AKT activation. **(A)** Knockdown of Pdc4 increases phosphorylation of AKT. The ratio of AKT (pan)/GAPDH or phospho-AKT (Ser473)/AKT (pan) in HT29-shLacZ cells is designated as 1.0. **(B)** Knockdown of Pdc4 activates AKT kinase activity. The AKT kinase assay was performed as described in Materials and Methods. The ratio of phospho-GSK3/AKT (pan) in HT29-shLacZ cells is designated as 1.0. **(C)** Overexpression of Pdc4 inhibits AKT phosphorylation and cyclin D1 expression. Western blotting analysis was performed with cell lysates from LoVo-control and LoVo-Pdc4 cells. The ratio of target protein/GAPDH in LoVo-control cells is designated as 1.0.

Discussion

Induction of cyclin D1 and its binding to CDK4 and CDK6 is the rate-limiting step during cell cycle progression through G1 phase. The deregulation of this process is a key event in carcinogenesis and tumor progression. Elevation of cyclin D1 expression is frequently found in various types of

human cancers, including breast, lung, and liver cancers.³¹ In human colon cancer, cyclin D1 is elevated in about 30% of human adenocarcinomas and adenomatous polyps.³² How does Pdc4 regulate cyclin D1 expression? Our findings in this study suggest that the increased cyclin D1 expression in Pdc4 knockdown cells is, at least in part, due to the NF- κ B activation that is regulated by IKK α and IKK β through AKT (Fig. 7). Furthermore, it has also been reported that cyclin D1 is a target gene of β -catenin-dependent transcription.³³ Thus, Pdc4 might regulate cyclin D1 transcription via β -catenin-dependent transcription because Pdc4 knockdown activates β -catenin-dependent transcription.^{7,8} In addition to transcriptional regulation, cyclin D1 expression can be regulated at the translational level.³⁴ Pdc4 is a translation inhibitor that preferentially inhibits translation of mRNA with secondary structures at the 5' untranslated region (5'UTR).³⁵⁻³⁷ The mRNAs for numerous growth factors and growth promotion genes, including cyclin D1, usually contain long GC-rich 5'UTR with the potential to form stable secondary structure(s) at the 5' end.³⁸ Therefore, Pdc4 might directly inhibit translation of cyclin D1. Pdc4 is the binding partner of translation initiation factor (eIF) 4A.^{35,36} Inhibiting translation by Pdc4 is believed to inhibit eIF4A's helicase activity.³⁶ Recently, Wedeken *et al.*³⁹ showed that binding to eIF4A is also required for Pdc4 associating with the 48S translation preinitiation complex. In addition, the phosphorylation of 4E binding protein (4EBP) and eIF4B, 2 AKT downstream targets, was increased in

the Pdc4 knockdown cells (unpublished data). Hyperphosphorylation of 4EBP dissociates the cap binding protein eIF4E and results in activation of cap-dependent translation.⁴⁰ Increased phosphorylation of eIF4B has also been shown to enhance translation and neoplastic transformation.^{41,42} Therefore, it is possible that Pdc4 regulates cyclin D1 translation through activation of eIF4E and eIF4B

Figure 5. GSK3 β is involved in the up-regulation of cyclin D1 in Pdc4 knockdown cells. **(A)** Knockdown of Pdc4 increases phosphorylation of GSK3 β . Western blotting analysis was performed with cell lysates from HT29-shLacZ and HT29-shPdc4 cells. The ratio of target protein/GAPDH in HT29-shLacZ cells is designated as 1.0. **(B)** Inhibition of proteasome activity increases cyclin D1 expression. Cells were treated with MG132 (50 μ g/mL) or DMSO for 6 hours. The protein levels of cyclin D1 and GAPDH were determined by Western blot. The ratio of cyclin D1/GAPDH in DMSO-treated HT29-shLacZ and HT29-shPdc4 cells is designated as 1.0.

(Fig. 7). Whether Pdc4 inhibits cyclin D1 translation needs further investigation.

AKT regulates a wide range of oncogenic processes, including cell growth and proliferation through phosphorylation of other protein kinases and transcription factors. AKT contains a central kinase domain with a threonine residue (Thr308) and a C-terminal regulatory site at serine 473 (Ser473). Phosphorylation of both Thr308 and Ser473 is essential for maximal AKT activation.⁴³ In cancer cells, AKT is constitutively activated. Immunohistochemical studies have shown that cancer cells expressing high levels of phospho-AKT (Ser473) were associated with poor prognosis in several types of cancer.²⁹ We report here that knockdown of Pdc4 increases the level of phospho-AKT (Ser473) and overexpression of Pdc4 inhibits the phosphorylation of AKT in colon tumor cell lines (Fig. 4), suggesting that Pdc4 is able to regulate AKT activation. In addition, regulating AKT phosphorylation by Pdc4 has also been observed in neuroendocrine Bon-1 cells.⁴⁴ Allgayer *et al.*¹ showed that the level of Pdc4 and phospho-AKT (Ser473) is inversely correlated in human colon cancer tissues. These findings collectively suggest that the inhibitory role of Pdc4 in human colon cancer development is at least partially achieved by inhibiting AKT activation. On the other hand, it has been reported that AKT is able to phosphorylate Pdc4 at Ser67 and Ser457, and ribosomal protein S6 kinase, a downstream target of AKT, can phosphorylate Pdc4 at Ser67.^{45,46} These findings reveal a feedback mechanism of regulation between Pdc4 and AKT. Because AKT is a critical kinase during cancer

development, agents targeted either upstream or downstream of the AKT signaling pathway for cancer therapy being actively developed. Our findings of Pdc4 regulating AKT activation may provide a novel therapeutic approach for targeting AKT.

We also noted that knockdown of Pdc4 up-regulates IKK α and IKK β protein levels (Fig. 3B) and knockdown of AKT does not alter the expression of IKK α and IKK β (Fig. 6A). These findings imply that Pdc4 but not AKT regulates IKK α and IKK β expression. *In vitro* studies have shown that the stem-loop structures with free energies (ΔG) of between -30 and -70 kcal/mol are sufficient to inhibit translation.⁴⁷ The ΔG values of the partial IKK α and IKK β 5' UTR are -39.7 kcal/mol and -47.3 kcal/mol (as calculated with the mfold program), respectively. Thus, it is possible that Pdc4 regu-

lates the translation of IKK α and IKK β . It is also possible that Pdc4 translationally regulates transcription factors that control IKK α and IKK β expression. The mechanism of how Pdc4 regulates IKK α and IKK β expression needs further investigation.

Together, the current results demonstrate that knockdown of Pdc4 promotes cell proliferation through AKT activation because knockdown of AKT in the Pdc4 knockdown cells inhibits proliferation and cyclin D1 expression. Activation of AKT by Pdc4 knockdown leads to up-regulation of cyclin D1 expression through NF- κ B activation and GSK3 β phosphorylation. These findings provide new insights into how loss of Pdc4 expression promotes colon tumor development.

Materials and Methods

Cell culture. HT29-shLacZ and HT29-shPdc4 cells were generated as described previously⁷ and grown in McCoy's medium containing 10% FBS, 2 mM L-glutamine, and 500 U/mL penicillin-streptomycin. LoVo-control and LoVo-Pdc4 cells were generated as described previously⁸ and grown in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, and 500 U/mL penicillin-streptomycin. Cells were incubated at 37°C with 5% CO₂ in a humidified incubator.

Cell proliferation assay. Cell proliferation was measured using Cell Proliferation Kit II (XTT) (Roche Applied Science, Indianapolis, IN) as described previously.⁴² Briefly,

Figure 6. Knockdown of AKT inhibits cyclin D1 expression, NF- κ B activation, and cell proliferation. Scramble siRNA (control) and *akt* siRNA (si-AKT) were transfected into HT29-shPdcd4 cells using transfection reagent according to the manufacturer's protocol. **(A)** Western blotting analysis was performed with cell lysates from control and si-AKT cells. The ratio of target protein/GAPDH in control cells is designated as 1.0. **(B)** The mRNA levels of cyclin D1 in the control and si-AKT cells were determined by real-time PCR, as described in Figure 2. Three independent experiments were performed in triplicates for each sample. Results are expressed as mean $\pm$ standard deviation ($n = 3$). The cyclin D1/GAPDH ratio in control cells is designated as 100%. The asterisk indicates a significant difference as determined by 1-way ANOVA ($P < 0.05$). **(C, D)** Knockdown of AKT inhibits cyclin D1 promoter activity and NF- κ B activation. Control and si-AKT cells were transfected with 0.2 μ g of D1 plasmid or NF- κ B luciferase reporter² along with 10 ng of pRL-SV40. After 48 hours, the firefly and Renilla luciferase activities were determined. The ratio of firefly luciferase/Renilla luciferase activity in control cells is designated as 100%. Three independent experiments were performed in 5 replicates for each sample. The represented data are shown and expressed as mean $\pm$ standard deviation. The asterisk denotes a significant difference as determined by 1-way ANOVA ($P < 0.001$). **(E)** Knockdown of AKT inhibits cell proliferation. Control or si-AKT cells were seeded in 96-well plates at day 0 (3×10^3 cells/well). The rate of cell growth was measured from day 1 to day 6, as described in Figure 1. The value recorded on day 1 for control and si-AKT cells is designated as 100%. The asterisk indicates a significant difference by 1-way ANOVA ($P < 0.05$).

3×10^3 cells/well were seeded onto 96-well plates. The sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) labeling mixture was added to the cell culture for 2 hours. The concentration of water-soluble formazan salt was measured using the iMark microplate reader (Bio-Rad Laboratories, Hercules, CA) at 490 nm.

Cell cycle and annexin V apoptosis assay. For cell cycle assays, 1×10^6 cells were washed with PBS twice and then incubated with 70% (v/v) ethanol overnight. After washing with PBS, the cells were suspended in PBS containing 0.1% (v/v) Triton X-100, 40 μ g/mL propidium iodide (Sigma, St. Louis, MO), and 50 μ g/mL RNase A (Sigma) at 37°C for 30 minutes. Apoptosis assays were performed using the annexin V-FITC apoptosis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Analysis was performed using a FACS Calibur cell analyzer (BD Biosciences, San Jose, CA).

Western blot analysis. Aliquots containing 20 to 40 μ g of protein were separated onto SDS-PAGE and transferred to nitrocellulose membranes as described previously.² Subsequently, the membrane was incubated with primary antibodies overnight followed by horseradish peroxidase-linked secondary antibody for 1 hour. The target protein was visualized by chemiluminescence (Pierce, Rockford, IL). The antibodies against cyclin D1, phospho-AKT (Ser473), AKT (pan), phospho-GSK3 β (Ser9), GSK3 β , phospho-IKK α / β (Ser176/180), IKK α and IKK β were purchased from Cell Signaling (1:1,000 dilution) (Danvers, MA). The GAPDH antibody was purchased from Santa Cruz Biotechnology (1:2,000 dilution) (Santa Cruz, CA). The phospho-AKT (Ser473) and AKT (pan) antibodies recognize all 3 AKT isoforms.

Figure 7. The scheme depicting the regulation pathways of cyclin D1 expression by Pdc4 knockdown. See text for details.

Real-time PCR. The total RNA isolation and real-time PCR were performed as described previously.⁷ Briefly, after synthesis of the first-strand cDNA using the Superscript First-Strand Kit (Invitrogen), mRNA levels of *cyclin D1* or *GAPDH* were quantified by real-time PCR in a LightCycler 480 (Roche Applied Science). The PCR cycling was performed at 95°C for 6 minutes followed by 40 cycles of denaturation (95°C for 15 seconds), annealing (61°C for 30 seconds), and extension (72°C for 20 seconds). To determine the specificity of the PCR, the amplified products were subjected to melt-curve analysis using the standard machine method. The target mRNA level was normalized to the internal control, GAPDH, using the formula $\Delta C_T = C_T(\text{target}) - C_T(\text{GAPDH})$ (C_T = threshold cycle). The level of target gene expression in control cells was designated as 100%. The relative expression levels were calculated using the equation of $100 \times 2^{-(\text{average } \Delta C_T(\text{test}) - \text{average } \Delta C_T(\text{control}))}$.⁴⁸ The primers used for amplifying *cyclin D1* and *GAPDH* were purchased from SA Biosciences (Frederick, MD).

Site-specific mutagenesis. The NF-κB binding sites on the *cyclin D1* promoter were mutated using the wild-type cyclin D1 promoter fused with the luciferase construct (D1) as the template. The D1 construct was purchased from Switch-Gear Genomics (Menlo Park, CA), which includes -920 bp to +106 bp of cyclin D1 promoter region (relative to the transcription start site) fused with the luciferase reporter. The site-specific mutagenesis was performed using Quick-Change II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

AKT kinase activity assay. AKT kinase assays were performed using the AKT kinase assay kit (Cell Signaling) according to the manufacturer's protocol. Briefly, cells were lysed in 500 μL lysis buffer and incubated on ice for 1 hour. After centrifugation, an aliquot (500 μg) of supernatant was added to 20 μL of immobilized AKT Ser473 antibody beads and incubated with gentle rocking overnight at 4°C. The beads were then washed with 500 μL of 1x lysis buffer twice and 500 μL of 1x kinase assay buffer twice. The beads were suspended in 50 μL of 1x kinase assay

buffer containing 200 μM of ATP and 2 μg of recombinant GSK-3 fusion protein and incubated for 30 minutes at 30°C. The reaction was terminated by adding 25 μL of 4x sodium dodecyl sulfate sample buffer. The proteins were separated onto a 4% to 12% Bis-Tris polyacrylamide gel (Invitrogen) and immunoblotted using phospho-GSK3α/β (Ser21/9) antibody. The band intensity was quantified using VisionWork LS image acquisition and analysis software (UVP, Upland, CA).

Transient transfection and luciferase activity assays. Cells (3×10^4 cells/well in 24-well plates) were transiently transfected with 0.2 μg of luciferase reporter plasmid along with 10 ng of pRL-SV40 (Promega, Madison, WI) by using TurboFect *in vitro* transfection reagent (Fermentas, Glen Burnie, MD). After 48 hours, the cells were lysed, and the luciferase activity was determined as described previously.⁷ The luciferase activity of each sample is normalized against Renilla luciferase activity for monitoring transfection efficiency.

Knockdown of IKK and AKT. HT29-shPdc4 cells (2×10^5 cells on a 60-mm plate) were transfected with 22 μL of 10 μM *IKKα* siRNA (Santa Cruz Biotechnology), *IKKβ* siRNA (Santa Cruz Biotechnology), *akt* siRNA (Santa Cruz Biotechnology), or scramble siRNA (negative control) (Invitrogen) using INTERFERin transfection reagent (Polyplus-Transfection Inc., New York, NY) according to the manufacturer's protocol. For Western blot assays, cells were collected at day 4 after transfection. For transfection assays, cells were split at day 2 after transfection.

Acknowledgments

The authors gratefully thank Yan Zhang for the technical assistance.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This work was supported by the National Institutes of Health [grant number R01CA129015].

References

- Mudduluru G, Medved F, Grobholz R, *et al.* 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*. 2007;110:1697-707.

2. Yang HS, Jansen AP, Nair R, *et al.* A novel transformation suppressor, Pdc4, inhibits AP-1 transactivation but not NF-kappaB or ODC transactivation. *Oncogene*. 2001;20:669-76.
3. Yang HS, Knies JL, Stark C, Colburn NH. Pdc4 suppresses tumor phenotype in JB6 cells by inhibiting AP-1 transactivation. *Oncogene*. 2003;22:3712-20.
4. Yang HS, Matthews CP, Clair T, *et al.* Tumorigenesis suppressor Pdc4 down-regulates MAP4K1 expression to suppress colon carcinoma cell invasion. *Mol Cell Biol*. 2006;26:1297-306.
5. Leupold JH, Yang HS, Colburn NH, Asangani I, Post S, Allgayer H. Tumor suppressor Pdc4 inhibits invasion/intravasation and regulates urokinase receptor (u-PAR) gene expression via Sp-transcription factors. *Oncogene*. 2007;26:4550-62.
6. Nieves-Alicea R, Colburn NH, Simeone AM, Tari AM. Programmed cell death 4 inhibits breast cancer cell invasion by increasing tissue inhibitor of metalloproteinases-2 expression. *Breast Cancer Res Treat*. 2009;114:203-9.
7. Wang Q, Sun Z, Yang HS. Downregulation of tumor suppressor Pdc4 promotes invasion and activates both beta-catenin/Tcf and AP-1-dependent transcription in colon carcinoma cells. *Oncogene*. 2008;27:1527-35.
8. Wang Q, Sun ZX, Allgayer H, Yang HS. Downregulation of E-cadherin is an essential event in activating beta-catenin/Tcf-dependent transcription and expression of its target genes in Pdc4 knockdown cells. *Oncogene*. 2010;29:128-38.
9. Santhanam AN, Baker AR, Hegamyer G, Kirschmann DA, Colburn NH. Pdc4 repression of lysyl oxidase inhibits hypoxia-induced breast cancer cell invasion. *Oncogene*. 2010;29:3921-32.
10. Jansen AP, Camalier CE, Colburn NH. Epidermal expression of the translation inhibitor programmed cell death 4 suppresses tumorigenesis. *Cancer Res*. 2005;65:6034-41.
11. Hilliard A, Hilliard B, Zheng SJ, *et al.* Translational regulation of autoimmune inflammation and lymphoma genesis by programmed cell death 4. *J Immunol*. 2006;177:8095-102.
12. Schmid T, Jansen AP, Baker AR, Hegamyer G, Hagan JP, Colburn NH. Translation inhibitor Pdc4 is targeted for degradation during tumor promotion. *Cancer Res*. 2008;68:1254-60.
13. Chen Y, Knosel T, Kristiansen G, *et al.* Loss of PDCD4 expression in human lung cancer correlates with tumour progression and prognosis. *J Pathol*. 2003;200:640-6.
14. Wei NA, Liu SS, Leung TH, *et al.* Loss of programmed cell death 4 (Pdc4) associates with the progression of ovarian cancer. *Mol Cancer*. 2009;8:70.
15. Hwang SK, Jin H, Kwon JT, *et al.* Aerosol-delivered programmed cell death 4 enhanced apoptosis, controlled cell cycle and suppressed AP-1 activity in the lungs of AP-1 luciferase reporter mice. *Gene Ther*. 2007;14:1353-61.
16. Jin H, Kim TH, Hwang SK, *et al.* Aerosol delivery of urocanic acid-modified chitosan/programmed cell death 4 complex regulated apoptosis, cell cycle and angiogenesis in lung of K-ras null mice. *Mol Cancer Ther*. 2006;5:1041-9.
17. Wei ZT, Zhang X, Wang XY, *et al.* PDCD4 inhibits the malignant phenotype of ovarian cancer cells. *Cancer Sci*. 2009;100:1408-13.
18. Hayashi A, Aishima S, Miyasaka Y, *et al.* Pdc4 expression in intraductal papillary mucinous neoplasm of the pancreas: its association with tumor progression and proliferation. *Hum Pathol*. 2010;41:1507-15.
19. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer*. 2002;2:489-501.
20. Lawlor MA, Alessi DR. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci*. 2001;114:2903-10.
21. Guo Y, Yang K, Harwalkar J, *et al.* Phosphorylation of cyclin D1 at Thr 286 during S phase leads to its proteasomal degradation and allows efficient DNA synthesis. *Oncogene*. 2005;24:2599-612.
22. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature*. 1999;401:82-5.
23. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. *Cell*. 2008;132:344-62.
24. Hacker H, Karin M. Regulation and function of IKK and IKK-related kinases. *Sci STKE*. 2006;2006:re13.
25. Quelle DE, Ashmun RA, Shurtleff SA, *et al.* Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev*. 1993;7:1559-71.
26. Witzel II, Koh LF, Perkins ND. Regulation of cyclin D1 gene expression. *Biochem Soc Trans*. 2010;38:217-22.
27. Rocha S, Martin AM, Meek DW, Perkins ND. p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-kappaB subunit with histone deacetylase 1. *Mol Cell Biol*. 2003;23:4713-27.
28. Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle*. 2003;2:339-45.
29. Downward J. PI 3-kinase, Akt and cell survival. *Semin Cell Dev Biol*. 2004;15:177-82.
30. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev*. 1998;12:3499-511.
31. Kim JK, Diehl JA. Nuclear cyclin D1: an oncogenic driver in human cancer. *J Cell Physiol*. 2009;220:292-6.
32. Arber N, Hibshoosh H, Moss SF, *et al.* Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis. *Gastroenterology*. 1996;110:669-74.
33. Shtutman M, Zhurinsky J, Simcha I, *et al.* The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A*. 1999;96:5522-7.
34. Rosenwald IB, Lazaris-Karatzas A, Sonenberg N, Schmidt EV. Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E. *Mol Cell Biol*. 1993;13:7358-63.
35. Yang HS, Cho MH, Zakowicz H, Hegamyer G, Sonenberg N, Colburn NH. A novel function of the MA-3 domains in transformation and translation suppressor Pdc4 is essential for its binding to eukaryotic translation initiation factor 4A. *Mol Cell Biol*. 2004;24:3894-906.
36. Yang HS, Jansen AP, Komar AA, *et al.* The transformation suppressor Pdc4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. *Mol Cell Biol*. 2003;23:26-37.
37. Loh PG, Yang HS, Walsh MA, *et al.* Structural basis for translational inhibition by the tumour suppressor Pdc4. *EMBO J*. 2009;28:274-85.

38. De Benedetti A, Harris AL. eIF4E expression in tumors: its possible role in progression of malignancies. *Int J Biochem Cell Biol.* 1999;31:59-72.
39. Wedeken L, Ohnheiser J, Hirschi B, Wethkamp N, Klempnauer KH. Association of tumor suppressor protein Pdc4 with ribosomes is mediated by protein-protein and protein RNA interactions. *Genes Cancer.* 2010;1:293-301.
40. Gingras AC, Raught B, Sonenberg N. mTOR signaling to translation. *Curr Top Microbiol Immunol.* 2004;279:169-97.
41. Shahbazian D, Roux PP, Mieulet V, *et al.* The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. *EMBO J.* 2006;25:2781-91.
42. Zhang Y, Wang Q, Guo X, Miller R, Guo Y, Yang HS. Activation and up-regulation of translation initiation factor 4B contribute to arsenic-induced transformation. *Mol Carcinog.* 2011;50:528-38.
43. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev.* 1999;13:2905-27.
44. Lankat-Buttgereit B, Muller S, Schmidt H, Parhofer KG, Gress TM, Goke R. Knockdown of Pdc4 results in induction of pro-protein convertase 1/3 and potent secretion of chromogranin A and secretogranin II in a neuroendocrine cell line. *Biol Cell.* 2008;100:703-15.
45. Palamarchuk A, Efanov A, Maximov V, Aqeilan RI, Croce CM, Pekarsky Y. Akt phosphorylates and regulates Pdc4 tumor suppressor protein. *Cancer Res.* 2005;65:11282-6.
46. Dorrello NV, Peschiaroli A, Guardavaccaro D, Colburn NH, Sherman NE, Pagano M. S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science.* 2006;314:467-71.
47. Gray NK, Hentze MW. Regulation of protein synthesis by mRNA structure. *Mol Biol Rep.* 1994;19:195-200.
48. Portereiko MF, Lloyd A, Steffen JG, Punwani JA, Otsuga D, Drews GN. AGL80 is required for central cell and endosperm development in arabidopsis. *Plant Cell.* 2006;18:1862-72.