

Phosphoinositide-3-Kinase Signaling Controls S-Phase Kinase-Associated Protein 2 Transcription via E2F1 in Pancreatic Ductal Adenocarcinoma Cells

Maximilian Reichert, Dieter Saur, Rainer Hamacher, Roland M. Schmid, and Günter Schneider

Department of Internal Medicine, Technical University of Munich, Munich, Germany

Abstract

The phosphoinositide-3-kinase (PI3K)/AKT signaling pathway controls fundamental processes of cancer cell biology like proliferation and cell survival. The PI3K/AKT pathway is activated in pancreatic ductal adenocarcinoma (PDAC) cells. The molecular mechanisms linking PI3K signaling to the cell cycle machinery in PDAC cells are not investigated in detail. Using the PI3K inhibitor Ly294002 as well as small interfering RNA targeting AKT1 expression, we show that PI3K controls the proliferation and G₁ phase progression of PDAC cells. Gene profiling revealed several important regulators of G₁-S phase progression controlled by PI3K signaling like *p21^{Cip1}*, S-phase kinase-associated protein 2 (*SKP2*), *CDC25a*, *cyclin A*, *cyclin D2*, *CDK2*, and *cyclin E*. We show that the F-box protein *SKP2*, an oncogene up-regulated in PDAC, is transcriptionally regulated by the PI3K/AKT1 pathway in PDAC cells. At the molecular level, the control of the *SKP2* gene by PI3K is due to the regulation of E2F1 binding to the proximal *SKP2* gene promoter. The complex and profound connection of PI3K/AKT1 signaling to the cell cycle qualifies this pathway as a suitable target for therapeutic intervention in PDAC. [Cancer Res 2007;67(9):4149–56]

Introduction

The retinoblastoma protein RB is a well-known regulator of G₁-S phase progression (1). Negative regulation of the cell cycle by RB is due to the ability of RB to bind the transcription factor E2F and repress transcription required for S phase progression. Control of RB activity occurs by the concerted action of G₁ phase cyclins, cyclin-dependent kinases (CDK), and cyclin-dependent kinase inhibitors like p21^{Cip1} and p27^{Kip1}. A functionally inactivated RB characterizes pancreatic ductal adenocarcinoma (PDAC) cells (2).

The E2F family of transcription factor consists of eight members (E2F1–8). They modulate important cellular responses including cell cycle progression, apoptosis, and DNA damage response. Furthermore, the E2F family contributes to carcinogenesis of many human tumors (3). Less is known concerning the function of the E2F transcription factor family in PDAC cells. E2F1 is up-regulated in PDAC cells, and positive correlation with

proliferation markers suggests effector function in G₁-S phase progression (4).

Overexpression of the oncogene S-phase kinase-associated protein 2 (*SKP2*) has been detected in a large number of human cancers, including prostate cancer, oral squamous cell cancer, breast cancer, lymphoma, small cell lung cancer, and colorectal carcinoma (5). High-level *SKP2* expression was observed in PDAC and is an independent predictor of patient outcome (6). At the molecular level, the F-box protein *SKP2* functions as a receptor component of the SCF ubiquitin ligase complex, resulting in ubiquitination and degradation of several important cell cycle regulators like p27^{Kip1}, p57^{Kip1}, p21^{Cip1}, p130, Cdt1, E2F1, hOrc1p, c-myc, and Foxo1 (5). Although *SKP2* is controlled at the transcriptional level by phosphoinositide-3-kinase (PI3K) signaling, the molecular mechanisms linking this pathway to the *SKP2* gene were not investigated in detail (7, 8).

The PI3K pathway is crucial to many aspects of cell growth and survival (9). Although no mutations in *AKT1* and *PI3K* genes have been reported in PDAC cells, the PI3K/AKT pathway is constitutively active (10). Although the mode of activation of the PI3K pathway in PDAC cells is not entirely clear, the contribution of the tumor suppressor phosphatase and tensin homologue deleted in chromosome 10 (*PTEN*) has been shown (11). In addition, a receptor tyrosin kinase is involved in PI3K activation because the insulin receptor substrate 1 (*IRS-1*) was shown to be an essential mediator of PI3K activation in quiescent PDAC cells (12). In PDAC cells, PI3K/AKT signaling regulates chemotherapeutic resistance and proliferation (13–17). Although molecular mechanisms linking PI3K signaling to the cell cycle are not fully understood in PDAC cells, the control of the G₁-S phase progression was shown to depend on PI3K signaling (14, 18). The transcription factors c-myc and NF-κB were shown to be downstream targets of the PI3K pathway in PDAC (11, 19, 20).

Here, we show that PI3K/AKT signaling regulates a complex genetic program needed for G₁ phase progression of PDAC cells. Transcription of *SKP2*, an important regulator of cell cycle progression, is regulated by PI3K via control of E2F1 binding to the proximal *SKP2* gene promoter.

Materials and Methods

Cell culture and reagents. The pancreatic cancer cell lines MiaPaCa2 and DanG, the colon cancer cell lines HCT116 and SW480, and HeLa cells were cultivated in DMEM or RPMI supplemented with 10% FCS and 1% (w/v) penicillin/streptomycin (Invitrogen). Ly294002 (EMD Biosciences) was dissolved in DMSO and stored at –20°C. Double-stranded small interfering RNAs (siRNA) were transfected at a final concentration of 200 nmol/L using OligofectAMINE (Invitrogen) according to the manufacturer's protocol. siRNAs were purchased from Ambion. Target sequences of the used siRNAs were control siRNA 5'-AACAGTCGCGTTTGGACTGG-3' and AKT1 siRNA 5'-AAGCTGGAGAACCTCATGCTG-3'.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Günter Schneider, Technical University of Munich, Klinikum rechts der Isar, II. Department of Internal Medicine, Ismaninger Street 22, 81675 Munich, Germany. Phone: 49-8941402458; Fax: 49-8941404910; E-mail: guenter.schneider@lrz.tum.de.

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Statistical methods. All data were obtained from at least three independent experiments done in duplicate, and the results are presented as mean and SE. To show statistical significance, Student's *t* test was used. *P* values are indicated in the figure legends.

Quantitative reverse transcription-PCR. Total RNA was isolated from pancreatic carcinoma cell lines using the RNeasy kit (Qiagen) following the manufacturer's instructions. Quantitative mRNA analyses were done using real-time PCR analysis (TaqMan, PE Applied Biosystems) as previously described (21). Semiquantitative reverse transcription-PCRs (RT-PCR) were done as previously described (22). Primer sequences are available upon request.

Total cell lysates and Western blot. Whole cell lysates were prepared, and Western blots were done as recently described (21, 22). The following antibodies were used: E2F1, cyclin E, CDC25a, p21^{Cip1} (Santa Cruz Biotechnology), SKP2 (Zymed), cyclin A, cyclin D2, CDK2 (BD Biosciences), cyclin D1 (Merck Chemicals Ltd.), and β -actin (Sigma-Aldrich). One representative Western blot out of at least three independent experiments is shown.

Bromodeoxyuridine-incorporation assay and cell cycle analysis. Bromodeoxyuridine (BrdUrd) incorporation was measured using the colorimetric BrdUrd assay according to the manufacturer's instructions (Roche Applied Science). For cell cycle analysis, cells were washed twice in PBS and redissolved in propidium iodide (PI) staining buffer containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μ g/mL PI. After 1 h of incubation at 4°C, flow cytometry was done using a BD Biosciences FACScan. The distribution of cells in different cell cycle stages (G₁-S/G₂ + M) was determined according to their DNA content.

Transfection, plasmids, and luciferase assay. Transfections of reporter genes (500 ng per well) were done using FuGene6 (Roche Applied Science) according to the manufacturer's protocol in 12-well plates. For cotransfections, the DNA amount was kept constant using empty pcDNA3 vector (Invitrogen). After the indicated time points, the cells were incubated in lysis buffer (Promega) for 15 min, harvested, and cleared by centrifugation for 15 min. Lysates were normalized for protein content. Luciferase activity was determined in a LB 9501 luminometer (Berthold) using a luciferase assay system (Promega). At least three independent transfection experiments were done in triplicate. The 5' regulatory region of the human SKP2 gene was amplified by PCR using the following primers: SKP2 forward 5'-CCCGCTCGAGCCCCCTTCCTCTCCACTGTTTC-3' and SKP2 reverse 5'-CCCGCTCGAGCCATTCATGCTCTCCCTTTTTGCAATC-3'. The resulting fragment was cloned into pCR4Blunt-TOPO (Invitrogen). *Sma*I digestion produces a 1,217-bp fragment, which was blunt-ended cloned into pGL3basic (Promega), opened by *Sma*I digestion. This reporter gene construct was called SKP2-975/+224Luc and was further digested with *Nhe*I/*Pf*FI. Religation results in the SKP2-273/+244Luc reporter gene construct. The E2F1 expression vector pCMV-E2F1 was a kind gift of Dr. B. Eymin, and the pE2F1-TA-Luc reporter was a kind gift of Dr. B. Yung. The pGL3 promoter was used as a control (Promega). All plasmids were verified by sequencing.

Gene expression profiling. DanG cells were treated with 25 μ mol/L Ly294002 or left as an untreated control. Duplicates of total RNA were prepared using RNeasy Kit (Qiagen). Labeled cRNA was produced and hybridized onto the Affymetrix GeneChip Human Genome U133 Plus 2.0 set according to Affymetrix standard protocols. Expression data were analyzed using Microarray Suite 5.0. Genes significantly regulated by Ly294002 treatment (>2.5- or <2.5-fold change in expression) with known function in the regulation of G₁-S phase of the cell cycle are presented in Table 1.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were done as recently described (22). An equal amount of chromatin (50–100 μ g) was used for each precipitation. The antibodies used were as follows: E2F1, RNA-polymerase II (Santa Cruz Biotechnology), anti-acetyl-histone H3, anti-acetyl-histone H4 (Upstate), and β -actin (Sigma-Aldrich) as a control. One-twentieth of the precipitated chromatin was used for each PCR reaction. To ensure linearity, 28 to 38 cycles were done, and one representative result out of at least three independent experiments is shown. Sequences of the promoter-specific primers are SKP2-95 5'-CTCCCCGCTACCCCGTGG-3', SKP+135 5'-CAGACCCG-

Table 1.

G₁ to S phase regulators, significantly up-regulated after Ly294002 treatment

Gene symbol	RefSeq Transcript ID
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p21 ^{Cip1}	NM_000389
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G₁ to S phase regulators, significantly down-regulated after Ly294002 treatment

Gene symbol	RefSeq Transcript ID
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SKP2	NM_005983/NM_032637
CDC25a	NM_001789/NM_201567
Cyclin A	NM_001237
Cyclin D2	NM_001759
CDK2	NM_001790/NM_052827
Cyclin E	NM_004702/NM_057735/NM_057749

TAAGCCTAGCAACG-3', glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-AGCTCAGCCTCAAGACCTT-3', GAPDH reverse 5'-AAGAAGATGCGGCTGACTGT-3'.

Results

Inhibition of PI3K signaling impairs proliferation and G₁ phase progression of PDAC cells. To confirm the impact of the PI3K pathway on the proliferation of PDAC cells, we inhibited PI3K using Ly294002 and measured BrdUrd incorporation in the cell lines MiaPaCa2 and DanG. As shown in Fig. 1A, BrdUrd incorporation is reduced 24 h after the addition of Ly294002 to 48% in MiaPaCa2 cells and to 35% in DanG cells compared with untreated control, respectively. The reduced proliferation was due to an accumulation of the cells in the G₁ phase of the cell cycle. The fraction of MiaPaCa2 cells in G₁ was increased from 43.3% to 73.5% 24 h after the addition of Ly294002, accompanied by a decrease of cells in the S (19.6% to 5.6%) and G₂-M (26% to 14.2%) phases (Fig. 1B). In DanG cells, the fraction of cells in the G₁ phase was increased from 41.4% to 55.1% by Ly294002 treatment. Simultaneously, we observed a decrease of cells in S (24.5% to 14.5%) and G₂-M (22.4% to 16.4%) phases after the addition of Ly294002 to DanG cells (Fig. 1B).

These findings suggest that PI3K controls proliferation and G₁ phase progression of PDAC cells.

PI3K signaling controls the expression of important G₁-S phase regulators in PDAC cells. To find genes contributing to the observed G₁ phase arrest after PI3K inhibition in PDAC cells, we did RNA microarray analysis in DanG cells. We focused on genes that were significantly regulated (<2.5- or >2.5-fold change after Ly294002 treatment) and are known to contribute to G₁-S phase progression. The only gene found to be up-regulated after Ly294002 treatment in DanG cells satisfying these criteria was the p21^{CIP1} gene (Table 1). Down-regulated genes after Ly294002 treatment are listed in Table 1. To verify the microarray data, Western blots were done. As shown in Fig. 2A, the protein expression levels of SKP2, CDC25a, cyclin A, CDK2, and cyclin E were indeed decreased after the treatment of MiaPaCa2 and DanG cells with Ly294002. Cyclin D2 expression was decreased in DanG cells by Ly294002 treatment, whereas

cyclin D2 protein was not detected in MiaPaCa2 cells. The p21^{Cip1} protein was up-regulated in MiaPaCa2 and DanG by Ly294002 treatment (Fig. 2A). Furthermore, cyclin D1 protein abundance was decreased by Ly294002 treatment in MiaPaCa2 and DanG cells (Fig. 2A).

This observation suggests that the G₁ arrest after the inhibition of PI3K is due to a profound alteration of the expression of positive and negative cell cycle-controlling genes.

PI3K signaling controls SKP2 transcription. Because the PI3K signaling pathway regulates *SKP2* transcription and the molecular mechanism linking PI3K signaling to the *SKP2* gene is unclear, we investigated *SKP2* regulation more precisely (7, 8). To confirm the results of the gene profiling experiments, we investigated *SKP2* mRNA expression after Ly294002 treatment in MiaPaCa2 and DanG cells using real-time RT-PCR. Here, we observed a distinct down-regulation of *SKP2* mRNA to 52.9% in MiaPaCa2 and to 28.9% in DanG cells 24 h after the treatment with Ly294002 compared with untreated control (Fig. 2B). In contrast, the *cyclin D1* mRNA expression is not reduced after PI3K inhibition in MiaPaCa2 and DanG cells (Fig. 2B). To show transcriptional control of *SKP2* after the inhibition of PI3K, we did ChIP assays. As shown in Fig. 2C, binding of the RNA-polymerase II to the proximal *SKP2* gene promoter is reduced after the treatment of MiaPaCa2 and DanG cells with Ly294002. As a control, no change of RNA-polymerase II binding to the proximal *GAPDH* promoter was observed after the inhibition of PI3K in both cell lines (Fig. 2C). Furthermore, we found a reduction of acetylated histone H3 and H4 binding to the *SKP2* gene after the treatment of MiaPaCa2 and DanG cells with Ly294002. Again, no alterations of histone acetylation were detected at the *GAPDH* promoter (Fig. 2C). These

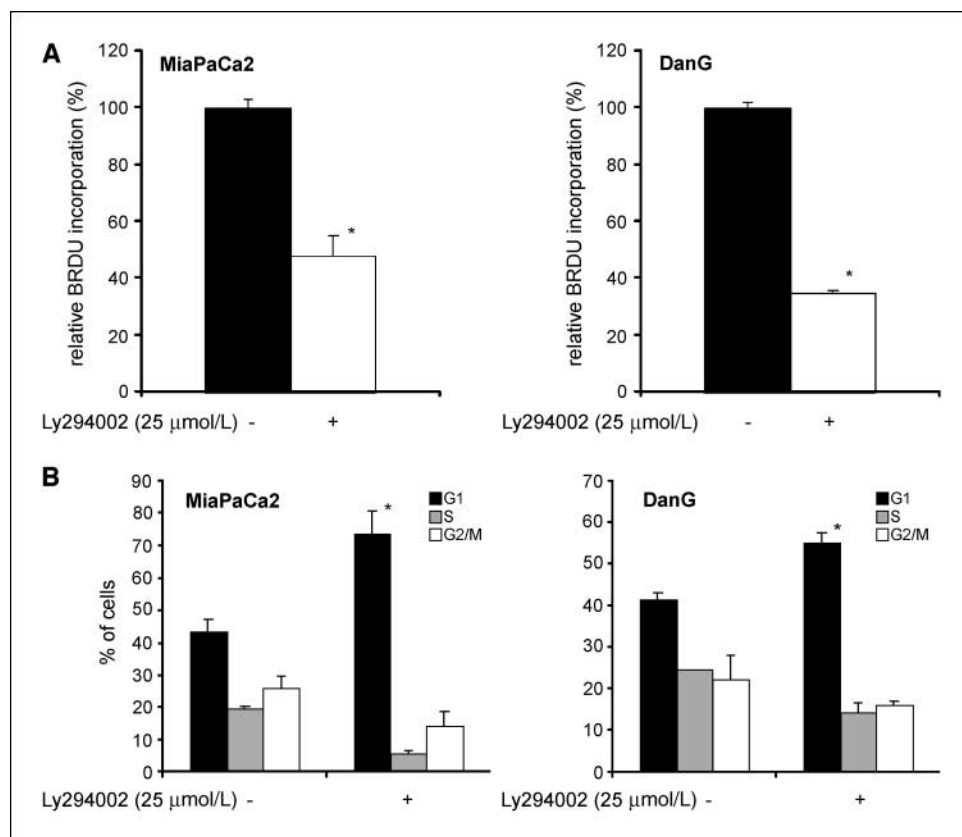
data show that a PI3K-dependent pathway controls *SKP2* transcription in PDAC cells.

AKT1 controls SKP2 mRNA and protein abundance. To confirm the results obtained with Ly294002, we used RNA interference with an AKT1-specific siRNA. Figure 3A shows the knockdown of AKT1 48 h after the transfection of an AKT1-specific siRNA into MiaPaCa2 and DanG cells. A distinct reduction of *SKP2* protein abundance in AKT1 siRNA-treated MiaPaCa2 and DanG cells was observed (Fig. 3A). In addition, the *SKP2* mRNA is reduced to 37.8% in MiaPaCa2 cells and to 31.5% in DanG cells 48 h after the transfection of the AKT1-specific siRNA (Fig. 3B). In line with the findings obtained with Ly294002, we observed no significant regulation of the cyclin D1 mRNA after the transfection of the AKT1 siRNA into MiaPaCa2 and DanG cells. BrdUrd incorporation was reduced after the transfection of the AKT1 siRNA in MiaPaCa2 and DanG cells (data not shown), and the fraction of cells in the G₁ phase was increased from 42% to 62% in MiaPaCa2 and from 45% to 61% in DanG cells 48 h after the transfection of the AKT1-specific siRNA (Fig. 3C). Furthermore, we found reduced binding of RNA-polymerase II to the proximal *SKP2* gene promoter after the transfection of the AKT1-specific siRNA (data not shown).

Together, these data confirm the results obtained with Ly294002 and show that a PI3K/AKT1 pathway controls transcription of the *SKP2* gene in PDAC cells.

PI3K/AKT1 signaling regulates binding of E2F1 to the SKP2 gene promoter. Recently, *SKP2* has been shown to be regulated by E2F1 (23). The proximal human *SKP2* promoter is characterized by three E2F binding sites. To test the contribution of E2F1 toward the transcriptional regulation of *SKP2* after PI3K inhibition, we did

Figure 1. Inhibition of PI3K impairs proliferation and G₁ phase progression of PDAC cells. **A**, BrdUrd incorporation assay of MiaPaCa2 and DanG cells 24 h after the addition of 25 μ mol/L Ly294002 compared with untreated control cells. *, $P < 0.001$. **B**, fluorescence-activated cell sorting (FACS) analysis of cell cycle distribution. MiaPaCa2 and DanG cells were treated with 25 μ mol/L Ly294002 for 24 h, stained with PI, and analyzed by FACS. The fraction of the cells in G₁, S and G₂-M phases is indicated. *, $P < 0.001$ versus controls.



ChIP assays. As shown in Fig. 4A, E2F1 binding to the *SKP2* gene promoter is reduced 24 h after the treatment of MiaPaCa2 (lanes 1 and 2) and DanG cells (lanes 3 and 4) with Ly294002. To determine whether reduced binding of E2F1 to the *SKP2* gene is due to impaired E2F1 protein abundance, we did Western blots. As shown in Fig. 4B, reduced expression of E2F1 protein 24 h after the treatment of MiaPaCa2 and DanG cells with Ly294002 was observed. To detect direct regulation of the *SKP2* promoter by E2F1 in PDAC cells, we did luciferase reporter gene assays. Whereas the pGL3 promoter control vector was not activated by cotransfection of an E2F1 expression plasmid in MiaPaCa2 and DanG cells, the *SKP2* -273/+244 reporter gene construct was dose-dependently activated by cotransfection of E2F1 in both cell lines (Fig. 4C). Furthermore, we measured E2F-dependent transcriptional activity in PDAC cells using an E2F reporter gene construct. Here, we observed down-regulation of E2F-dependent transcriptional activity to 64% compared with untreated controls in MiaPaCa2 cells and to 2% compared with untreated controls in DanG cells 24 h after Ly294002 treatment (Fig. 4D). In line with the down-regulation of E2F-dependent transcriptional activity, we observed reduced *SKP2* promoter activity after Ly294002 treatment. A decrease of *SKP2* promoter activity to 49% compared with untreated controls was observed in MiaPaCa2 cells 24 h after Ly294002 treatment (Fig. 4D). In DanG, we found a decrease of *SKP2* promoter activity to 3% compared with untreated controls

24 h after Ly294002 treatment (Fig. 4D). To confirm the results of PI3K inhibition, we again did AKT1 siRNA transfection. As shown in Fig. 5A, we observed impaired binding of E2F1 to the proximal *SKP2* gene promoter 48 h after the transfection of the AKT1-specific siRNA into MiaPaCa2 (lanes 1 and 2) and DanG cells (lanes 3 and 4). Furthermore, E2F1 protein levels were reduced after transfection of the AKT1-specific siRNA into MiaPaCa2 and DanG cells (Fig. 5B).

These data show that *SKP2* is an E2F1 target gene in PDAC cells, and that PI3K/AKT1 signaling regulates E2F1 binding to the *SKP2* gene promoter due to the control of E2F1 protein expression. Furthermore, decreased E2F1 expression leads to impaired E2F-dependent transcriptional activity and decreased *SKP2* promoter activity in PDAC cells.

Discussion

Although PI3K/AKT pathway is known to be activated in PDAC cells and linked to proliferation, PI3K/AKT-mediated G_1 phase progression is not completely understood. We now show that the PI3K/AKT pathway controls G_1 phase progression of PDAC cells by regulating a complex genetic program, involving several key G_1 phase-controlling genes like *p21^{Cip1}*, *SKP2*, *CDC25a*, *cyclin A*, *cyclin D2*, *CDK2*, and *cyclin E*. Among these genes, *SKP2* shows the most profound down-regulation after PI3K inhibition in gene expression

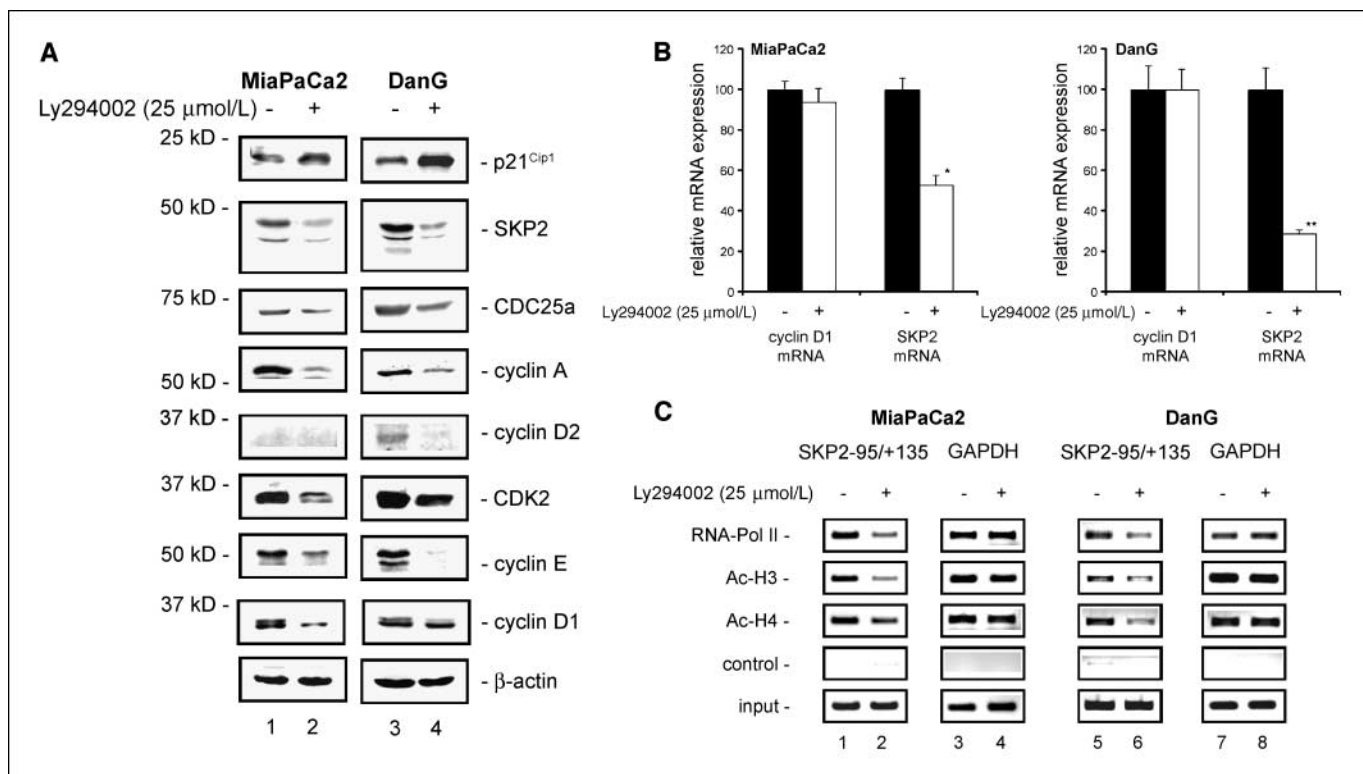
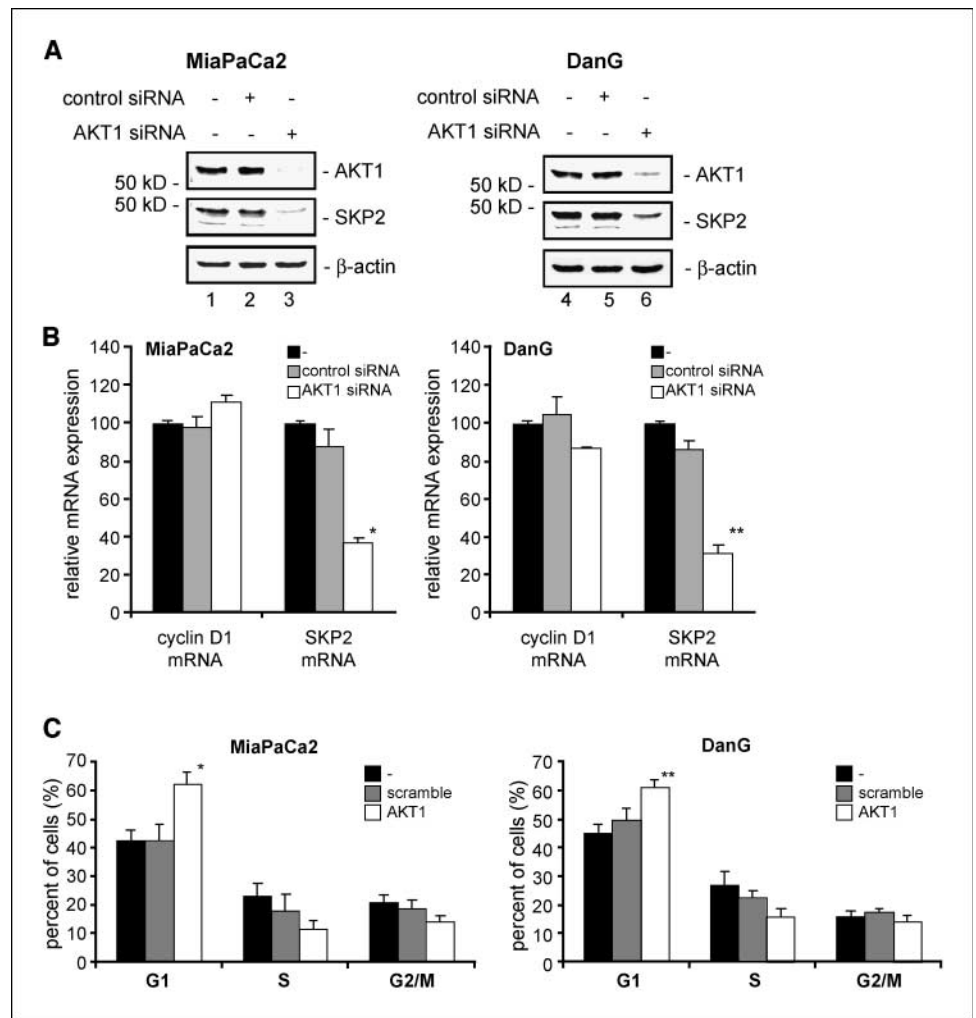


Figure 2. PI3K controls protein abundance of important G_1 -S phase regulators and *SKP2* transcription. **A**, Western blots of *SKP2*, *CDC25a*, cyclin A, cyclin D2, *CDK2*, cyclin E, and cyclin D1 expression in MiaPaCa2 (lanes 1 and 2) and DanG cells (lanes 3 and 4) 24 h after the treatment of cells with 25 μ mol/L Ly294002. The membrane was stripped and probed for β -actin to ensure equal protein loading. **B**, quantitative *cyclin D1* and *SKP2* mRNA expression analysis. Twenty-four hours after the treatment of MiaPaCa2 and DanG cells with 25 μ mol/L Ly294002 (white columns) or vehicle (black columns), total RNA was prepared, and mRNA levels were quantified using real-time PCR analysis and normalized to cyclophilin expression levels. *, $P = 0.002$ versus controls; **, $P < 0.001$ versus controls. **C**, chromatin immunoprecipitation analysis of the *SKP2* gene. Chromatin of MiaPaCa2 and DanG cells treated with 25 μ mol/L Ly294002 or left as an untreated control was immunoprecipitated with a RNA-polymerase II-specific antibody, an anti-acetyl-histone H3, an anti-acetyl-histone H4, or a β -actin antibody as a negative control. Precipitated DNA or 10% of the chromatin input was amplified with gene-specific primers for the *SKP2* or *GAPDH* promoter.

Figure 3. AKT1 knockdown inhibits *SKP2* transcription and G₁ phase progression of PDAC cells. **A**, Western blot analysis of AKT1 and SKP2 protein levels 48 h post-transfection of MiaPaCa2 and DanG cells with an AKT1-specific siRNA. **B**, quantitative *SKP2* and *cyclin D1* mRNA expression analysis. Total RNA was prepared 48 h post-transfection of MiaPaCa2 and DanG cells with an AKT1-specific siRNA. mRNA levels were quantified using real-time PCR analysis and normalized to cyclophilin expression levels. *, $P < 0.001$ versus controls; **, $P = 0.004$ versus control. **C**, FACS analysis of cell cycle distribution. MiaPaCa2 and DanG cells were transfected with an AKT1-specific siRNA. The cells were stained with PI and analyzed by FACS 48 h post-transfection. The fraction of the cells in G₁, S, and G₂-M phases is indicated. *, $P = 0.029$ versus controls; **, $P < 0.001$ versus controls.



experiments (data not shown). In addition, we show that the PI3K/AKT pathway is linked to the *SKP2* gene promoter via E2F1.

The *SKP2* gene is periodically expressed throughout the cell cycle, being low in G₀ phase and mid-G₁ phase. *SKP2* level increases in late G₁ and stays high in S phase (24, 25). Both *SKP2* mRNA and protein levels are regulated. In early G₁ phase, the *SKP2* protein is degraded by the anaphase-promoting complex/cyclosome-containing *cdh1* (APC/C^{cdh1}; refs. 26, 27). Beside *SKP2* protein degradation, *SKP2* transcription is regulated, and the *SKP2* gene promoter is known to link several important signaling pathways and transcription factors like the Notch pathway, the CD28 pathway, the NF- κ B pathway, the forkhead box M1 (Foxm1) transcription factor, and SP1 transcription factor to the cell cycle machinery (22, 28–31). PI3K/AKT signaling is known to control *SKP2* transcription in different cellular systems (7, 8). We now show that in PDAC cells, PI3K/AKT signaling is linked to *SKP2* gene transcription by controlling a cis-acting element in the proximal human *SKP2* gene promoter. We show activation of this promoter region by E2F1 and impaired E2F1 binding to the proximal *SKP2* promoter upon PI3K inhibition. These findings confirm several recent reports. Zhang and Wang characterized the human *SKP2* promoter, showing that the proximal *SKP2* promoter located between +65 and +149 in exon 1 of the *SKP2* gene is essential for trans-activation by E2F1 (23). Furthermore,

hormonal induction of *SKP2* mRNA in 3T3-L1 pre-adipocytes depends on PI3K/AKT signaling, and the PI3K signal is integrated in the proximal murine *SKP2* promoter, harboring an E2F-binding site (32). The stronger reduction of E2F1 protein expression observed after PI3K inhibition in DanG cells might explain the more profound effect on transcription of *SKP2* in this particular cell line.

PI3K signaling induces proliferation by regulating several transcription factors. Although Foxo, NF- κ B, MIZ1, p53, AP1, c-Myc, β -catenin, and HIF1 α activities are affected by PI3K signaling, the exact roles of these proteins during PI3K-induced oncogenesis are unknown (9). In addition, interleukin 2 signaling is linked to the cell cycle machinery in T lymphocytes by PI3K-regulated E2F transcriptional activity, and an active form of PI3K promotes E2F activation in lymphoblastoid cell lines (33, 34). In this line, we found decreased E2F1 protein expression after PI3K inhibition in PDAC cell lines, explaining the reduced E2F transcriptional activity observed. Because E2F1 can be degraded by a *SKP2*-dependent pathway, the concurrent down-regulation of both molecules is surprising and points to a disruption of the regulatory feedback loop between E2F1 and *SKP2* in PDAC cells (35). The molecular mechanism of reduced E2F1 protein expression after inhibition of PI3K awaits further experimental investigation.

The role of E2F in regulating the transcription of genes critically involved in G₁-S phase progression as well as the contribution to carcinogenesis is established (3). Interestingly, all cell cycle-controlling genes found to be down-regulated in PDAC cells, after PI3K inhibition (Table 1), are established target genes of E2F or known to harbor E2F binding sites in their 5' regulatory regions (23, 36–40). Therefore, the PI3K/E2F1 pathway might not only explain the regulation of *SKP2*, but also the complex genetic program linking PI3K to the cell cycle in PDAC cells. Although no down-regulation of SKP2 and E2F1 protein abundance and *SKP2* mRNA expression was observed after PI3K inhibition in HeLa cells, the PI3K/E2F1/SKP2 pathway is activated in the colon cancer cell lines HCT116 and SW480 (Supplementary Fig. S1). This

indicates that this pathway is more general, valid, and tumor relevant.

In addition to cell cycle genes regulated at the transcriptional level by PI3K signaling in PDAC cells, we observed down-regulation of cyclin D1 protein expression after inhibition of PI3K without regulation of *cyclin D1* mRNA. Cyclin D1 protein abundance is regulated by proteasomal degradation in a PI3K/AKT/glycogen synthase kinase 3 β (GSK3 β)-dependent pathway (41, 42). Furthermore, a PI3K/AKT/mTOR pathway is known to control cyclin D1 translation (9, 43). Whether reduced cyclin D1 protein expression after the PI3K inhibition is linked to the GSK3 β or the mTOR pathway in PDAC cells is unclear at the moment and awaits further investigation. The stronger down-regulation of cyclin D1 after

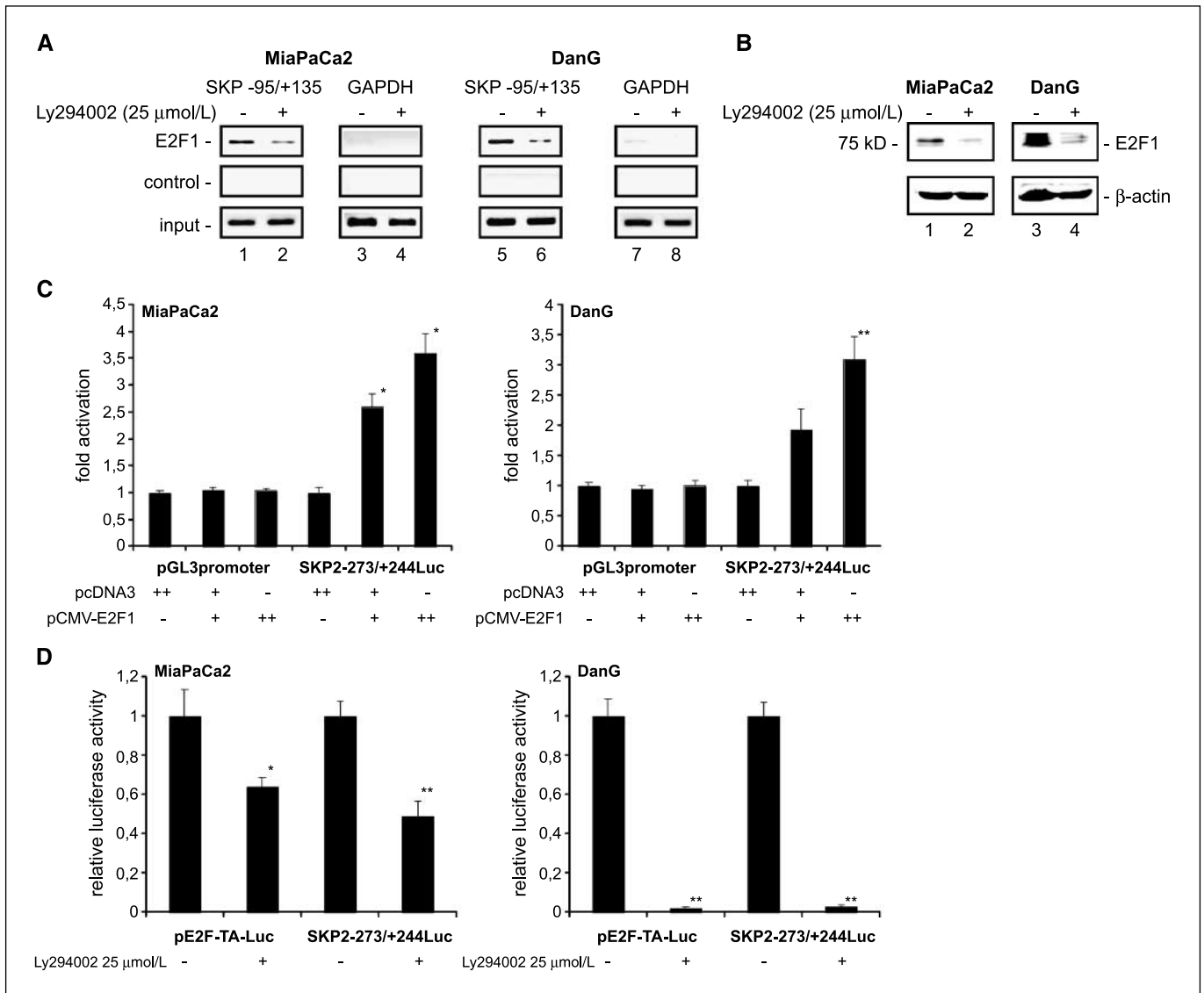
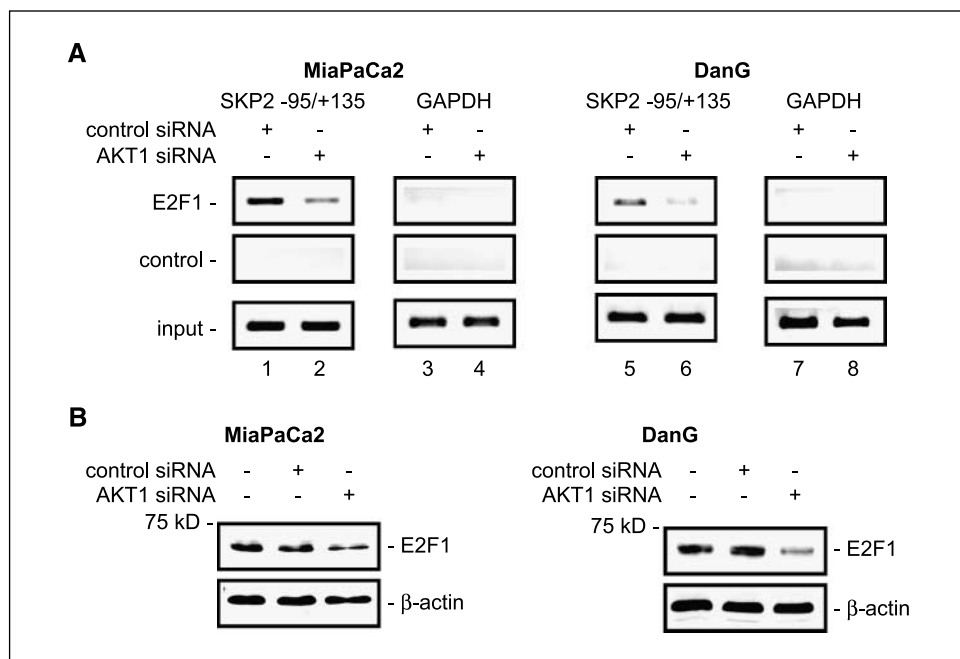


Figure 4. PI3K controls *SKP2* via E2F1. **A**, chromatin immunoprecipitation analysis of the *SKP2* gene. Chromatin of MiaPaCa2 and DanG cells treated with 25 μ mol/L Ly294002 for 24 h or left as an untreated control was immunoprecipitated with an E2F1-specific antibody or a β -actin antibody as a negative control. Precipitated DNA or 10% of the chromatin input was amplified with gene-specific primers for the *SKP2* or *GAPDH* promoter. **B**, Western blots of E2F1 expression in MiaPaCa2 (lanes 1 and 2) and DanG cells (lanes 3 and 4) 24 h after the treatment of cells with 25 μ mol/L Ly294002. The membrane was stripped and probed for β -actin to ensure equal protein loading. **C**, 500 ng of the pGL3 promoter control or the SKP2-273/+244Luc vector was cotransfected with 100 ng (+) or 200 ng (++) pCMV-E2F1 expression vector into MiaPaCa2 and DanG cells. The amount of DNA was kept constant using pcDNA3. Luciferase activity was measured 24 h after the transfection. *, $P < 0.001$ versus controls; **, $P = 0.002$ versus controls. **D**, 500 ng of pE2F-TA-Luc or SKP2-273/+244Luc was transfected into MiaPaCa2 and DanG cells. Cells were treated with 25 μ mol/L Ly294002 or were left as an untreated control 24 h after the transfection. Luciferase activity was measured 24 h after the Ly294002 treatment. *, $P = 0.003$ versus controls; **, $P < 0.001$ versus controls.

Figure 5. AKT1 controls SKP2 via E2F1. **A**, chromatin immunoprecipitation analysis of the *SKP2* gene. Chromatin of MiaPaCa2 and DanG cells transfected with an AKT1-specific siRNA or transfected with a control siRNA was immunoprecipitated with an E2F1-specific antibody or an β -actin antibody as a negative control 48 h after the transfection. Precipitated DNA or 10% of the chromatin input was amplified with gene-specific primers for the *SKP2* or *GAPDH* promoter. **B**, Western blots of E2F1 expression in MiaPaCa2 (lanes 1–3) and DanG cells (lanes 3–5) 48 h after the transfection of the cells with no siRNA, a control, or an AKT1-specific siRNA. The membrane was stripped and probed for β -actin to ensure equal protein loading.



PI3K inhibition in MiaPaCa2 cells might explain the greater cell cycle effect in this cell line.

In summary, our work shows that the PI3K/AKT pathway is connected to several key cell cycle-controlling genes to assure unrestrained G₁-S phase progression and proliferation of PDAC cells. This multilayer interaction of the PI3K/AKT signaling with the cell cycle machinery qualifies PI3K/AKT signaling as a suitable target for therapeutic intervention in PDAC.

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References

- Du W, Pogoriler J. Retinoblastoma family genes. *Oncogene* 2006;25:5190–200.
- Rozenblum E, Schutte M, Goggins M, et al. Tumor-suppressive pathways in pancreatic carcinoma. *Cancer Res* 1997;57:1731–4.
- Tsantoulis PK, Gorgoulis VG. Involvement of E2F transcription factor family in cancer. *Eur J Cancer* 2005; 41:2403–14.
- Yamazaki K, Yajima T, Nagao T, et al. Expression of transcription factor E2F-1 in pancreatic ductal carcinoma: an immunohistochemical study. *Pathol Res Pract* 2003;199:23–8.
- Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* 2006;6:369–81.
- Einama T, Kagata Y, Tsuda H, et al. High-level Skp2 expression in pancreatic ductal adenocarcinoma: correlation with the extent of lymph node metastasis, higher histological grade, and poorer patient outcome. *Pancreas* 2006;32:376–81.
- Mamillapalli R, Gavrilova N, Mihaylova VT, et al. PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2). *Curr Biol* 2001;11:263–7.
- Andreu EJ, Lledo E, Poch E, et al. BCR-ABL induces the expression of Skp2 through the PI3K pathway to promote p27Kip1 degradation and proliferation of chronic myelogenous leukemia cells. *Cancer Res* 2005; 65:3264–72.
- Bader AG, Kang S, Zhao L, Vogt PK. Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* 2005;5:921–9.
- Schlieman MG, Fahy BN, Ramsamooj R, Beckett L, Bold RJ. Incidence, mechanism and prognostic value of activated AKT in pancreas cancer. *Br J Cancer* 2003;89:2110–5.
- Asano T, Yao Y, Zhu J, Li D, Abbruzzese JL, Reddy SA. The PI 3-kinase/Akt signaling pathway is activated due to aberrant Pten expression and targets transcription factors NF- κ B and c-Myc in pancreatic cancer cells. *Oncogene* 2004;23:8571–80.
- Asano T, Yao Y, Shin S, McCubrey J, Abbruzzese JL, Reddy SA. Insulin receptor substrate is a mediator of phosphoinositide 3-kinase activation in quiescent pancreatic cancer cells. *Cancer Res* 2005;65:9164–8.
- Ng SSW, Tsao MS, Chow S, Hedley DW. Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. *Cancer Res* 2000;60:5451–5.
- Perugini RA, McDade TP, Vitimberga FJ, Jr., Callery MP. Pancreatic cancer cell proliferation is phosphatidylinositide 3-kinase dependent. *J Surg Res* 2000;90:39–44.
- Bondar VM, Sweeney-Gotsch B, Andreeff M, Mills GB, McConkey DJ. Inhibition of the phosphatidylinositide 3-kinase-AKT pathway induces apoptosis in pancreatic carcinoma cells *in vitro* and *in vivo*. *Mol Cancer Ther* 2002;1:989–97.
- Arlt A, Gehrz A, Muerkoster S, et al. Role of NF- κ B and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. *Oncogene* 2003;22:3243–51.
- Fahy BN, Schlieman M, Virudachalam S, Bold RJ. AKT inhibition is associated with chemosensitization in the pancreatic cancer cell line MIA-PaCa-2. *Br J Cancer* 2003;89:391–7.
- Takeda A, Osaki M, Adachi K, Honjo S, Ito H. Role of the phosphatidylinositol 3'-kinase-Akt signal pathway in the proliferation of human pancreatic ductal carcinoma cell lines. *Pancreas* 2004;28:353–8.
- Shah SA, Potter MW, Hedeshian MH, Kim RD, Chari RS, Callery MP. PI-3' kinase and NF- κ B cross-signaling in human pancreatic cancer cells. *J Gastrointest Surg* 2001;5:603–12; discussion 12–3.
- Liptay S, Weber CK, Ludwig L, Wagner M, Adler G, Schmid RM. Mitogenic and antiapoptotic role of constitutive NF- κ B/Rel activity in pancreatic cancer. *Int J Cancer* 2003;105:735–46.
- Schneider G, Oswald F, Wahl C, Greten FR, Adler G, Schmid RM. Cyclosporine inhibits growth through the activating transcription factor/cAMP-responsive element-binding protein binding site in the cyclin D1 promoter. *J Biol Chem* 2002;277:43599–607.
- Schneider G, Saur D, Siveke JT, Fritsch R, Greten FR, Schmid RM. IKK α controls p52/RelB at the skp2 gene promoter to regulate G₁- to S-phase progression. *EMBO J* 2006;25:3801–12.
- Zhang L, Wang C. F-box protein Skp2: a novel transcriptional target of E2F. *Oncogene* 2006;25: 2615–27.
- Zhang H, Kobayashi R, Galaktionov K, Beach D. p19Skp1 and p45Skp2 are essential elements of the cyclin A-CDK2 S phase kinase. *Cell* 1995;82:915–25.
- Wirbelauer C, Sutterluty H, Blondel M, et al. The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. *EMBO J* 2000;19:5362–75.
- Bashir T, Dorrello NV, Amador V, Guardavaccaro D,

- Pagano M. Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. *Nature* 2004; 428:190-3.
27. Wei W, Ayad NG, Wan Y, Zhang GJ, Kirschner MW, Kaelin WG, Jr. Degradation of the SCF component Skp2 in cell-cycle phase G₁ by the anaphase-promoting complex. *Nature* 2004;428:194-8.
28. Sarmiento LM, Huang H, Limon A, et al. Notch1 modulates timing of G₁-S progression by inducing SKP2 transcription and p27 Kip1 degradation. *J Exp Med* 2005; 202:157-68.
29. Wang IC, Chen YJ, Hughes D, et al. Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. *Mol Cell Biol* 2005;25: 10875-94.
30. Appleman LJ, Chernova I, Li L, Boussiotis VA. CD28 costimulation mediates transcription of SKP2 and CKS1, the substrate recognition components of SCFSkp2 ubiquitin ligase that leads p27kip1 to degradation. *Cell Cycle* 2006;5:2123-9.
31. Huang YC, Hung WC. 1,25-Dihydroxyvitamin D(3) transcriptionally represses p45(Skp2) expression via the Sp1 sites in human prostate cancer cells. *J Cell Physiol* 2006;209:363-9.
32. Auld CA, Caccia CD, Morrison RF. Hormonal induction of adipogenesis induces Skp2 expression through PI3K and MAPK pathways. *J Cell Biochem* 2006;100:204-16.
33. Brennan P, Babbage JW, Burgering BM, Groner B, Reif K, Cantrell DA. Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity* 1997;7:679-89.
34. Brennan P, Mehl AM, Jones M, Rowe M. Phosphatidylinositol 3-kinase is essential for the proliferation of lymphoblastoid cells. *Oncogene* 2002;21:1263-71.
35. Marti A, Wirbelauer C, Scheffner M, Krek W. Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. *Nat Cell Biol* 1999;1:14-9.
36. Ohtani K, DeGregori J, Nevins JR. Regulation of the cyclin E gene by transcription factor E2F1. *Proc Natl Acad Sci U S A* 1995;92:12146-50.
37. Schulze A, Zerfass K, Spitkovsky D, et al. Cell cycle regulation of the cyclin A gene promoter is mediated by a variant E2F site. *Proc Natl Acad Sci U S A* 1995;92:11264-8.
38. Shiffman D, Brooks EE, Brooks AR, Chan CS, Milner PG. Characterization of the human cyclin-dependent kinase 2 gene. Promoter analysis and gene structure. *J Biol Chem* 1996;271:12199-204.
39. Brooks AR, Shiffman D, Chan CS, Brooks EE, Milner PG. Functional analysis of the human cyclin D2 and cyclin D3 promoters. *J Biol Chem* 1996;271:9090-9.
40. Iavarone A, Massague J. E2F and histone deacetylase mediate transforming growth factor β repression of cdc25A during keratinocyte cell cycle arrest. *Mol Cell Biol* 1999;19:916-22.
41. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3 β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 1998;12: 3499-511.
42. Cohen P, Frame S. The renaissance of GSK3. *Nat Rev Mol Cell Biol* 2001;2:769-76.
43. Muise-Helmericks RC, Grimes HL, Bellacosa A, Malstrom SE, Tschlis PN, Rosen N. Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. *J Biol Chem* 1998;273:29864-72.