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Krüppel-like Factor 4 Induces *p27^{Kip1}* Expression in and Suppresses the Growth and Metastasis of Human Pancreatic Cancer Cells

Daoyan Wei¹, Masahsi Kanai¹, Zhiliang Jia¹, Xiangdong Le¹, and Keping Xie^{1,2}

¹ Department of Gastrointestinal Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

² Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Abstract

The zinc finger transcription factor Krüppel-like factor 4 (KLF4) has been implicated in both tumor suppression and progression. However, its function in pancreatic cancer has not been well characterized. Here, we show that pancreatic cancer cell lines expressed various levels of KLF4 RNA and protein. Ectopic expression of KLF4 by FG and BxPC-3 pancreatic cancer cells resulted in cell cycle arrest and marked inhibition of cell growth *in vitro* and attenuation of tumor growth and metastasis in an orthotopic mouse model. Overexpression of KLF4 also led to significant induction of *p27^{Kip1}* expression, at both the RNA and protein levels, in a dose- and time-dependent manner, indicating that KLF4 transcriptionally regulates the expression of *p27^{Kip1}*. Chromatin immunoprecipitation assays consistently showed that KLF4 protein physically interacts with the *p27^{Kip1}* promoter. Promoter deletion and point mutation analyses indicated that a region between nucleotides –435 and –60 of the *p27^{Kip1}* promoter and intact of the three KLF4-binding sites within that region were required for the full induction of *p27^{Kip1}* promoter activity by KLF4. Our findings suggest that KLF4 transactivates *p27^{Kip1}* expression and inhibits the growth and metastasis of human pancreatic cancer.

Introduction

Pancreatic cancer is currently the fourth leading cause of cancer-related death in the United States (1). The great majority of pancreatic cancers are adenocarcinomas arising from the ductal cells of the exocrine pancreas. The etiology and pathogenesis of pancreatic adenocarcinoma remain unclear, although heterogeneous genetic alterations have been reported. The most common of these genetic alterations include point mutations of *KRAS*; inactivation of *CDKN2A/p16/MTS1*, *p53*, and *MADH4/SMAD4/DPC4*; and overexpression of several mitogenic growth factors and their tyrosine kinase receptors (2). The phosphatidylinositol 3-kinase/Akt pathway was recently found to be activated in pancreatic cancer owing to aberrant expression of Akt or phosphatase and tensin homologue (3,4). Continued identification of signature gene alterations in pancreatic cancer will provide a conceptual framework to guide

Requests for reprints: Keping Xie, Department of Gastrointestinal Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Unit 426, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-792-2828; Fax: 713-745-1163; E-mail: kepxie@mdanderson.org.

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future analyses of this complex disease and the development of strategies for its early detection and effective treatment.

Krüppel-like factor 4 (KLF4; formerly known as gut-enriched Krüppel-like factor) is a zinc finger transcription factor, and its mRNA is expressed primarily in postmitotic, terminally differentiated epithelial cells in organs such as skin, lungs, and gastrointestinal tract (5,6). In cell culture, KLF4 expression can be increased by serum deprivation, contact inhibition, and DNA damage (7,8). Conversely, reduced expression of KLF4 has been reported in various tumors (9–12), and restoration of KLF4 expression can induce growth arrest in colon cancer cells or apoptosis in bladder or gastric cancer cells and leukemia (12–15). Accumulating clinical evidence also suggests that KLF4 functions as a tumor suppressor; genetic and epigenetic alterations of the *KLF4* gene have been found in gastrointestinal cancers (12,16–18). On the other hand, KLF4 expression is increased in primary breast ductal carcinoma (19) and in oral and dermal squamous cell carcinomas (10,20), suggesting that KLF4 is important in tumor development and progression (21–23). Moreover, whether and how KLF4 functions in pancreatic cancer remains unknown.

In the present study, we investigated the expression and molecular function of KLF4 in human pancreatic cancer cells. Our results showed that KLF4 positively regulates *p27^{Kip1}* expression and inhibits the growth and metastasis of human pancreatic cancer.

Materials and Methods

Cell lines and culture conditions

The human pancreatic adenocarcinoma cell lines Capan-1, AsPC-1, BxPC-3, Hs766T, MiaPaCa-2, and PANC-1 and human embryonic kidney (HEK) 293 cells were purchased from the American Type Culture Collection; FG human pancreatic adenocarcinoma cell line was obtained from Michael P. Vezeridis (Brown Medical School, Providence, RI; ref. 24). All cell lines were maintained in plastic flasks as adherent monolayers in MEM supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution (Flow Laboratories).

Generation of KLF4-expressing vectors

Adenoviral vectors were generated and used as reported previously (12). Retroviral vectors were generated using Retro-X system from Clontech. Briefly, Phoenix ecotropic packaging cells were transfected with the retroviral vector pLXSN-FLAG-KLF4 or the corresponding vector without KLF4 using Lipofectamine 2000 reagent (Invitrogen). Retroviral supernatants were collected, filtered, and used for infecting target cells according to the manufacturer's instruction. The green fluorescent protein (GFP)-tagged vector pEGFP-KLF4 was created by inserting a *HindIII-SalI* fragment encoding human KLF4 into pEGFP-C1 (Clontech); sequences were confirmed by DNA sequence analysis.

Animals

Athymic BALB/c nude mice were purchased from The Jackson Laboratory and housed in laminar flow cabinets under specific pathogen-free conditions according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH. The xenograft experiments were done when the mice were 8 wk old.

Immunocytochemical analysis

FG cells were seeded in Falcon culture slides (Becton Dickinson) at 1×10^5 per well in DMEM supplemented with 10% FBS. Cells were transfected with pEGFP-KLF4, permeabilized in 0.1% NP40, and incubated with a specific anti-p27^{Kip1} serums and then Texas red-labeled secondary antibody. Nuclear staining was accomplished by incubation in a solution containing 10 $\mu\text{g}/\text{mL}$ 4',6-diamidino-2-phenylindole (Sigma-Aldrich). Fluorescence imaging was performed with a Zeiss Axiophoto 2 microscope (Carl Zeiss, Inc.), with Adobe Photoshop 8.0 used to capture the images.

Cell proliferation assay

FG and BxPC-3 cells were seeded at 4×10^5 per well in six-well culture plates and incubated for 12 h at 37°C, after which they were incubated in serum-free medium containing Ad-FLAG-KLF4 or Ad-EGFP at a multiplicity of infection (MOI) of 20 for the FG cells or 15 for the BxPC-3 cells for 2 h at 37°C. Cells were then washed with serum-free medium, replenished with complete DMEM, and incubated for 1 to 4 d at 37°C. Cells were tested for viability with trypan blue exclusion and counted daily with a hemocytometer. To measure cell doubling time, 1×10^4 cells in 1.5 mL complete medium were plated in triplicate in 24-well plates. Cells were trypsinized and then counted daily for 1 wk using a hemocytometer. Cell population doubling time (DT) in hours was determined using the following equation: $DT \text{ (hours)} = 0.693(t - t_0) / \ln(N_t/N_0)$, where t_0 = time at which exponential growth began, t = time in hours, N_t = cell number at time t , and N_0 = initial cell number (25).

Fluorescence-activated cell sorting analysis

FG and BxPC-3 cells were prepared for fluorescence-activated cell sorting (FACS) analysis of cell cycle distribution as described previously (26). Briefly, cells were seeded at 5×10^5 per dish in 60-mm cell culture dishes and incubated at 37°C for 12 h, after which Ad-KLF4 was added at 10 or 20 MOI for the FG cells and at 7.5 or 15 for the BxPC-3 cells, and Ad-EGFP was used to adjust the total MOI to be equal in each group. At 40 h after infection, the cells were harvested and fixed in 70% ethanol. Thirty minutes before FACS analysis, the cells were stained with propidium iodide and analyzed with a FACSCalibur equipped with the CellQuest software program (Becton Dickinson).

Northern blot analysis

Northern blotting was performed using total RNA extracted from cell cultures and using p27^{Kip1} and *KLF4* cDNA as probes. Equal loading of RNA samples was monitored by hybridizing the same membrane filter with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (27).

Western blot analysis

Standard Western blotting was done using whole-cell lysates and rabbit polyclonal antibodies against human KLF4, p27^{Kip1}, cyclin D1, p21^{CIP1} (Santa Cruz Biotechnology), and a peroxidase-linked, species-specific, anti-rabbit IgG F(ab')₂ fragment (Amersham). Equal protein sample loading was monitored by hybridizing the same membrane filter with an anti-GAPDH antibody. Proteins were detected with Amersham's enhanced chemiluminescence system. Kodak Digital Science Image station 440CF system was used for the acquisition of quantitative imaging data (27).

Small interfering RNA

RNA interference was done with synthetic small interfering RNA (siRNA) oligo to human KLF4 (Ambion). Briefly, BxPC-3 cells were seeded to 80% confluence in six-well plates in

triplicate and transiently transfected with KLF4 siRNA or a control scrambled siRNA (10 μ L/well, 20 μ mol/L) using Lipofectamine 2000. Cell or protein samples were harvested 48 h after transfection and processed for FACS or Western blot analysis. In some experiments, BxPC-3 cells were infected with Mission short hairpin RNA (shRNA) lentiviral particles (Sigma-Aldrich), which carry nontargeting or specific KLF4-targeting shRNA. After puromycin selection, the antibiotic-resistant cell clones were cultured in complete DMEM without puromycin for 3 to 4 d. The cells were then seeded at 5×10^5 per dish in 60-mm cell culture dishes and incubated in DMEM containing 1.0% FBS for 24 h at 37°C before being harvested and processed for protein extraction and FACS analysis, respectively. For *in vivo* tumorigenicity analysis, BxPC-3 cells and BxPC-3 cells that carry nontargeting or KLF4-specific targeting shRNA were seeded at 6×10^6 per dish in 150-mm cell culture dishes and incubated in complete DMEM for 24 h at 37°C, and then single-cell suspensions were prepared for *in vivo* assay using the similar procedure as described below.

Tumor growth and metastasis

Tumor cells in exponential growth phase were prepared for inoculation in mice. Single-cell suspensions that were >95% viable were used. Tumor cells (1×10^6 per mouse) were then injected into the subcutis or pancreas of nude mice (10 mice per group). The animals were killed 60 d after the tumor cell injection or when they seemed moribund. Primary pancreatic tumors were then harvested and weighed. Livers were also removed and fixed in Bouin's solution for 24 h to distinguish neoplastic lesions from organ parenchyma; metastases on the surface of the liver were counted under a dissecting microscope by an investigator blinded as to treatment condition.

Construction of *p27^{Kip1}* promoter reporter plasmids and mutagenesis

The *p27^{Kip1}* promoter constructs (both the full-length construct and a series of 5' deletion mutants) were originally obtained from Dr. Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan; ref. 28). To generate additional 5' deletion mutants, the following primers were used for PCR amplification: 5'-gataaggtacctgtttgttcggtttgtttttgag-3' (sense) and 5'-agatcgcatctcgagcccgg-3' (antisense). The PCR product was sub-cloned into the *Asp718/BgIII* sites of the pGVB2 vector. To generate site-directed mutants of KLF4 elements in the proximal region of the *p27^{Kip1}* promoter, the p27-N-MB435 vector was constructed with the following primers: 5'-gataaggtaccgaacttctctatgatcac-3' (sense) and 5'-agatcgcatctcgagcccgg-3' (antisense). The PCR product was then subcloned into the *Asp718/BgIII* sites of the pGVB2 vector, which was then used for site-directed mutation with the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. Primers (with the mutated sites shown in bold) used to generate mutation in the putative KLF4-binding site 1 (designated p27-N-MB435/KLF4-m1) were 5'-gaggggggtcgggccgctagg**TT**cgtttgtttgttcggtttg-3' (sense) and 5'-caaaaccgaacaaaacaagcg**AA**ctacgcccgaaccccctc-3' (antisense); for mutation in the KLF4-binding site 2 (designated p27-N-MB435/KLF4-m2), 5'-gactcggagggccttgcca**T**actctccgctgctggtcccc-3' (sense) and 5'-ggggaccaggcaagcg-gagagTAtggcaagcccgtccgagtc-3' (antisense); and for mutation in the KLF4-binding site 3 (designated p27-N-MB435/KLF4-m3), 5'-cgcgggaccg-cgggcttgca**TAA**gccagactcggaggggc-3' (sense) and 5'-gcccgtccgagtctgggc**TTA**tccaagcccgcggtcccgcg-3' (antisense). All constructs were verified by sequencing the inserts and flanking regions of the plasmids.

Analysis of *p27^{Kip1}* promoter activity

The activity of the full-length and mutant *p27^{Kip1}* promoter constructs was analyzed as described previously (27). Briefly, plasmids containing firefly luciferase reporters were

cotransfected into tumor cells in triplicate, with pMiniTK-RL as an internal control, by using Lipofectamine 2000. The pMiniTK-RL contained a full-length *Renilla* luciferase gene under the control of a minimal thymidine kinase (TK) promoter (636–757 bp from pTK-RL; Promega). In some experiments, an *Sp1* promoter reporter (FOR2; ref. 29) and *p27^{Kip1}* reporters were cotransfected with pcDNA3.1-KLF4 or pcDNA3.1. The activity of both the firefly and *Renilla* luciferase reporters was determined 48 h later using a Dual Luciferase Assay kit (Promega). Specific promoter activity was expressed as the relative ratio of firefly luciferase activity to *Renilla* luciferase activity. The specific promoter activity was expressed as the fold changes of the experimental group versus the control group.

Chromatin immunoprecipitation assay

For these experiments, BxPC-3 and FG cells were subjected to chromatin immunoprecipitation (ChIP) with the ChIP Assay kit (Upstate Cell Signaling Solutions). Briefly, formaldehyde was used to cross-link proteins with DNA, and cells were lysed in SDS lysis buffer. The cell lysate was sonicated to shear the DNA to 400- to 600-bp lengths. Chromatin samples were then precleared with a salmon sperm DNA/protein A agarose-50% slurry for 30 min at 4°C and immunoprecipitated overnight with no antibody, an anti-KLF4 antibody (H-180), or an anti-FLAG antibody (M2). The region between –426 and –20 nucleotides of the *p27^{Kip1}* promoter was amplified with a pair of primers (designated as P1): 5'-aaggggctcgtcttttcg-3' (sense) and 5'-gagtgcgagagagcggtc-3' (antisense). The 3'-untranslated region (UTR) region of *p27^{Kip1}* gene was amplified as control for ChIP assay using the following primer set (designated as P2): 5'-aagcgttggatgtagcattatgc-3' (sense) and 5'-agatcaccagatctcccaaatgag-3' (antisense). The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were done with Promega's Gel Shift Assay System. Briefly, nuclear protein was extracted from BxPC-3 cells after 40 h of transduction with Ad-KLF4 at a MOI of 5 as described previously (27). Double-stranded oligonucleotides corresponding to KLF4-binding sites in the *p27^{Kip1}* promoter region were used as probes. For competition assays, a 50-fold molar excess of unlabeled oligonucleotides (cold probe) was added to the binding reaction. For the supershift assay, specific Sp1 (PEP2), Sp3 (D-29), KLF4 (H-180), and FLAG (M2; Sigma-Aldrich) antibodies were added to a final concentration of 0.1 µg/µL. Samples were then loaded onto a 4.5% polyacrylamide gel and subjected to electrophoresis. Gels were then dried and exposed to Kodak film (Eastman Kodak).

Statistical analysis

Each experiment was performed independently at least twice with similar results; findings from one representative experiment are presented. Significant differences between *in vitro* treatment conditions were assessed with Student's *t* test (two tailed), and differences between *in vivo* treatment groups were assessed with two-tailed Mann-Whitney *U* tests. To examine the relationship between two quantitative variables, the Pearson's linear regression analysis was performed using Statistical Package for the Social Sciences statistical software (version 11.05; SPSS, Inc.). $P \leq 0.05$ was deemed significant.

Results

KLF4 suppresses pancreatic cancer cell growth *in vitro*

First, we measured the expression of KLF4 in various pancreatic cancer cell lines at mRNA and protein levels (Fig. 1A). KLF4 was expressed at different levels by these cell lines. Then, we examined the correlation between endogenous KLF4 levels and *in vitro* growth behavior

of AsPC-1, BxPC-3, MiaPaCa-2, and FG cells and found that a higher expression level of KLF4 protein correlated with a longer cell doubling time (Supplementary Table S1 and Supplementary Fig. S1), indicating a possible negative effect of KLF4 on cell growth. Next, FG cells, which expressed relatively little endogenous KLF4, and BxPC-3, which expressed relatively high amount of endogenous KLF4, were transduced with adenoviral KLF4. Overexpression of KLF4 was consistent with growth suppression of both FG cells (Fig. 1B1) and BxPC-3 cells (Fig. 1B2) *in vitro*. These findings show that ectopic expression of KLF4 led to suppression of tumor cell growth.

KLF4 inhibits pancreatic cancer growth and metastasis *in vivo*

Next, FG or BxPC-3 cells were injected s.c. into nude mice. FG and BxPC-3 cells transduced with control Ad-EGFP showed progressive growth, whereas FG and BxPC-3 cells transduced with KLF4 grew more slowly (Fig. 1C1 and C2). In an orthotopic model, FG and BxPC-3 cells were injected into the pancreas of mice (10 per group). FG and BxPC-3 cells transduced with Ad-EGFP produced large tumors and hepatic metastases, whereas FG and BxPC-3 cells transduced with KLF4 produced only localized small tumors (data not shown). In addition, we also determined the effect of knocking down KLF4 expression on tumor growth. BxPC-3 cells transduced with specific KLF4 shRNA vector (T-16) had reduced KLF4 expression and grew larger tumors compared with that of BxPC-3 cells that transduced with nontargeting vector (Non-T; Fig. 1D). There was no significant difference in tumor growth when compared with the parental BxPC-3 with BxPC-3-Non-T cells (data not shown). In addition, we also examined the effect of physiologic up-regulation of KLF4 expression on tumor growth using retroviral gene transfer and found that stable KLF4-transduced FG cells grew much slower in nude mice than the parental FG cells or FG cells stably transduced with *Neo* gene (Supplementary Fig. S2). All these findings suggest that KLF4 in pancreatic cancer cells suppressed the growth and metastasis of those cells in these ectopic and orthotopic mouse models.

KLF4 suppresses cell cycle progression of pancreatic cancer cells

To investigate the mechanism by which KLF4 inhibited pancreatic cancer cell growth, we tested the effects of KLF4 expression on cell cycle distribution. Overexpression of KLF4 induced G₁ arrest in both FG cells (Fig. 2A, left) and BxPC-3 cells (Fig. 2B) and reduced the proportion of FG cells in S phase (Fig. 2A, right) and the percentage of BxPC-3 cells in G₂-M (Fig. 2B). Additionally, lentivirus-mediated shRNA transient transfection specifically knocked down KLF4 expression in BxPC-3 cells (data not shown). After serum deprivation, knocking down of KLF4 expression in those cells significantly reduced the percentage of cells in G₁ and G₂ phases but increased the percentage of cells in S phase when compared with those in parental BxPC-3 cells or cells that carry nontargeting shRNA (Non-T; Fig. 2C). Alternatively, a direct transduction of KLF4 siRNA into BxPC-3 cells resulted in a similar effect on cell cycle progression in a dose-dependent manner (Supplementary Fig. S3). These results are consistent with those from our *in vitro* proliferation experiments, in which KLF4 expression suppressed tumor cell proliferation.

KLF4 up-regulates p27^{Kip1} expression in pancreatic cancer cells

Next, to investigate the mechanism by which KLF4 induced cell cycle arrest, we assessed the expression of various cell cycle proteins. Following the expression of exogenous KLF4, the expression of p27^{Kip1} was significantly increased (Fig. 3A). Over-expression of KLF4 by FG cells reduced the expression of cyclin D1 and S-phase kinase-associated protein 2 (Skp2) but increased that of p21^{CIP1}. Then, we undertook dose-response experiments and found that overexpression of KLF4 increased the expression of p27^{Kip1} in both cell types (Fig. 3B), with the extent of the increase paralleling the MOI of Ad-KLF4. To determine whether endogenous KLF4 specifically regulates p27^{Kip1} expression, we used siRNA-mediated gene knockdown

in the relatively high KLF4-expressing BxPC-3 cells and found corresponding dose-dependent reductions in both KLF4 and $p27^{Kip1}$ expression (Fig. 3C). Because the ability of $p27^{Kip1}$ to regulate cell cycling requires that it be in the nucleus, we next considered whether KLF4 could induce the nuclear accumulation of $p27^{Kip1}$ protein. We found that the EGFP-KLF4 fusion protein was located mainly in the nuclei of FG cells, where it colocalized with $p27^{Kip1}$ (Fig. 3D). Collectively, these findings indicate that KLF4 positively regulated $p27^{Kip1}$ expression in pancreatic cancer cells.

KLF4 transcriptionally activates $p27^{Kip1}$ promoter

Because $p27^{Kip1}$ protein levels can be regulated by several mechanisms (28,30,31), we next sought to determine if KLF4 transcriptionally regulates $p27^{Kip1}$ expression. Indeed, transduction with Ad-KLF4 induced the expression of $p27^{Kip1}$ RNA as determined by Northern blotting (Fig. 4A) and induced $p27^{Kip1}$ promoter activity in luciferase reporter analyses (Fig. 4B). These findings indicate that KLF4 can transcriptionally regulate $p27^{Kip1}$ expression. Next, we identified the region in the $p27^{Kip1}$ promoter responsible for transcriptional activation by KLF4 by transfecting HEK 293 cells with a series of 5' deletion mutants based on the 3,568-bp $p27^{Kip1}$ promoter, along with a KLF4 expression vector or a control vector, and testing the effect on promoter activity. Loss of the region spanning -3,358 to -462 nucleotides in $p27^{Kip1}$ promoter construct did not substantially affect the induction of $p27^{Kip1}$ promoter activity by KLF4. However, further deletion of the 5' promoter region up to -435 nucleotides significantly increased the induction of $p27^{Kip1}$ promoter activity, and further deletion of the nucleotides to -60 completely abolished the induction of $p27^{Kip1}$ promoter activity, suggesting that the -435/-60 region contains the regulatory elements that are required for KLF4 to induce $p27^{Kip1}$ promoter activity.

KLF4 binds to the $p27^{Kip1}$ promoter *in vivo*

Next, we sought to determine if KLF4 binds to the promoter region of the $p27^{Kip1}$ gene *in vivo* using ChIP assays. The PCR forward and reverse primers flanking the essential regulatory region are shown in Fig. 5A. First, to determine whether endogenous KLF4 binds to the $p27^{Kip1}$ promoter, we immunoprecipitated chromatin fragments from BxPC-3 cells with control IgG or a specific anti-KLF4 antibody and found that the DNA fragment of the $p27^{Kip1}$ promoter was immunoprecipitated by the specific anti-KLF4 antibody (Fig. 5B1, lane 6), but the DNA fragment of $p27^{Kip1}$ 3'-UTR was not immunoprecipitated by the specific anti-KLF4 antibody (Fig. 5B1, lane 7). Next, to determine whether exogenous KLF4 (i.e., that produced via gene transfer) binds to the $p27^{Kip1}$ promoter, we transduced FG with Ad-KLF4 or Ad-EGFP and immunoprecipitated chromatin fragments with specific anti-FLAG antibody. Compared with EGFP-transduced cells or control FG cells, exogenous KLF4 clearly bound to the $p27^{Kip1}$ promoter (Fig. 5B2, lane 12) but not to 3'-UTR of $p27^{Kip1}$ gene (Fig. 5B2, lane 15). Thus, both exogenous and endogenous KLF4 bound to the $p27^{Kip1}$ promoter in pancreatic cancer cells *in vivo*.

KLF4 binds to the $p27^{Kip1}$ promoter *in vitro*

To identify KLF4-specific binding sites in the proximal promoter region of $p27^{Kip1}$, we analyzed previously reported KLF4-binding motifs (32,33) and found four putative KLF4-binding sites in the essential region of $p27^{Kip1}$ promoter. We then used EMSA to determine if KLF4 binds to these sites *in vitro* using synthesized oligonucleotides containing the core sequences shown in Fig. 5A. First, the putative KLF4-binding site #3 was radiolabeled and used as a hot probe in EMSA, resulting in four major shifted DNA-protein complexes (Fig. 5C). The shifted complexes were then competed with cold wild-type site #3 oligonucleotides (lane 4) but not with mutant oligonucleotides (lane 5). In supershift assays, the putative KLF4 band was further shifted by anti-KLF4 antibody (lane 3). Similarly, we showed that KLF4 also

bound to putative KLF4-binding site #1 and #2 of *p27^{Kip1}* promoter *in vitro* (Supplementary Fig. S4). Thus, we were able to identify three putative KLF4-binding sites in the proximal region of the *p27^{Kip1}* promoter.

Finally, to provide direct evidence that these putative KLF4-binding sites within the proximal *p27^{Kip1}* promoter contribute to the activity of the *p27^{Kip1}* promoter, we performed site-directed mutagenesis of the three putative KLF4-binding sites (or the combination of all three of them). Disruption of the putative KLF4-binding sites #1 and #2 led to slight decreases in the induction of *p27^{Kip1}* promoter activity by KLF4 (Fig. 5D); disruption of site #3 led to a substantial reduction. Mutation of all three sites totally eliminated the induction of *p27^{Kip1}* promoter activity by KLF4 (Fig. 5D). These findings suggest that all three putative KLF4-binding sites must be intact for the full induction of *p27^{Kip1}* promoter activity by KLF4. Finally, because KLF4 can positively or negatively regulate the expression of several genes, we selected proximal promoters of Sp1 and *p27^{Kip1}* and cotransfected them with control or KLF4 expression vectors into HEK 293 cells. In these experiments, KLF4 positively regulated *p27^{Kip1}* promoter activity but negatively regulated Sp1 promoter activity (Supplementary Fig. S5). This result is consistent with our present and previous report (34) and indicates that the effect of KLF4 on gene expression (positive or negative) is specific to the gene promoters.

Discussion

In the present study, we found that KLF4 was expressed at various levels in human pancreatic cancer cell lines and that ectopic overexpression of KLF4 significantly inhibited pancreatic cancer cell growth *in vitro* and tumorigenicity in animal models, whereas knocking down of KLF4 expression promoted tumor growth *in vivo*. Our mechanistic investigations suggested that this antitumor activity resulted from cell cycle arrest, that ectopic overexpression of KLF4 by pancreatic cancer cells led to significant induction of *p27^{Kip1}* mRNA and protein, that KLF4 bound to the promoter region of *p27^{Kip1}*, and that the intact of three KLF4-binding sites in the *p27^{Kip1}* promoter region is required for the full induction of *p27^{Kip1}* promoter activity by KLF4. Collectively, these findings are the first report of the mechanism of action of KLF4 in pancreatic cancer, supporting the notion that KLF4 acts as a tumor suppressor (23).

KLF4 has been implicated in regulation of the differentiation of pancreatic ductal cells (35, 36). A recent microarray analysis indicated that KLF4 was up-regulated in pancreatic intraepithelial neoplasia (PanIN) lesions (37), but whether the alteration of KLF4 expression plays a constraining or promoting role in pancreatic cancer remains unknown. Given that the effects of oncogenic KRAS in isolated pancreatic ductal cells derived from keratin 19 promoter-driven K-ras^{G12V} transgenic mice were constrained by up-regulation of certain cell cycle regulators, such as *p16^{INK4A}/p19^{Arf}* and *p27^{Kip1}*, resulting in controlled cellular proliferation (38); *p16^{INK4A}* has been found to inhibit the progression of pancreatic adenocarcinoma in mice (2,39); and homozygous deletion of the *CDKN2A* locus where *p16^{INK4A}* resides resulted in significant down-regulation of KLF4 in melanoma cell lines (40), it is reasonable to speculate that up-regulation of KLF4 in the early premalignant lesions of PanIN may represent a defense mechanism against oncogene activation. In the present study, an ectopic overexpression of KLF4 significantly inhibited tumor cell growth *in vitro* and suppressed tumor growth and metastasis *in vivo*, whereas lentiviral-mediated knocking down of KLF4 expression promoted tumor growth in animal model. These results suggest that KLF4 has a tumor-suppressive effect. However, many questions remain about whether KLF4 is a bona fide tumor suppressor in pancreatic cancer, including whether the expression of KLF4 is different at various disease stages or whether genetic alterations of KLF4 are common in pancreatic cancer.

Several lines of evidence suggest that KLF4 is a negative regulator of cellular proliferation and an inducer of epithelial terminal differentiation (23). Among the cell cycle genes regulated by KLF4, many of those that are up-regulated are inhibitors of proliferation, whereas genes that promote proliferation are repressed (41). In the current study, transduction of KLF4 caused significant cell growth inhibition owing to blockage of G₁-S and G₂-M cell cycle progression. Importantly, we found that KLF4 up-regulated *p27^{Kip1}* and, to a lesser extent, *p21^{CIP1}* but down-regulated cyclin D1 expression. Previous studies have established the importance of KLF4 in regulation of *p21^{CIP1}* expression (7,11,42,43). Mechanistically, KLF4 activates *p21^{CIP1}* through a specific Sp1-like *cis*-element in the *p21^{CIP1}* proximal promoter; interestingly, the same element is also required for *p53* to activate the *p21^{CIP1}* promoter, although *p53* does not bind to it (44). Similarly, KLF4 negatively regulates cyclin D1 expression through binding to the Sp1 motif in the proximal region of the cyclin D1 promoter (45).

p27^{Kip1} is a universal cyclin-dependent kinase inhibitor that directly inhibits the enzymatic activity of cyclin-CDK complexes, resulting in cell cycle arrest at G₁. In the present study, we established that KLF4 positively regulated *p27^{Kip1}* expression. Our results are consistent with those from a previous study of redox-controlled vascular smooth muscle cell proliferation, in which KLF4 was found to be involved in the expression of *p21^{CIP1}*, *p53*, and *p27^{Kip1}* induced by H-Fe (46). Moreover, our study provides a molecular mechanism by which KLF4 regulates *p27^{Kip1}* expression.

p27^{Kip1} protein levels are regulated by several mechanisms. Although transcriptional regulation is the primary mechanism, cellular abundance of *p27^{Kip1}* was previously thought to be regulated predominantly by ubiquitin/proteasome-mediated proteolysis (47) in a process that requires *p27^{Kip1}* to associate with cyclin/cyclin-dependent kinase complexes and be phosphorylated at Thr¹⁸⁷, which is then recognized by SCF^{Skp2}, a member of the F-box family of the specific substrate recognition subunit of Skp1/Cull/F-box ubiquitin-protein ligase complexes (30). However, more recently, loss of *p27^{Kip1}* was shown to promote tumorigenesis in the lung independently of SCF^{Skp2}-mediated *p27^{Kip1}* degradation, with loss of *p27^{Kip1}* protein expression resulting from reduced expression of *p27^{Kip1}* mRNA in the tumors (31). These findings were consistent with the observation that down-regulation of *p27^{Kip1}* protein was caused by decreased abundance of *p27^{Kip1}* mRNA in more than half of human breast cancers tested (31). Therefore, in the present study, we focused on how KLF4 directly regulated transcription of *p27^{Kip1}*, although it is possible that KLF4 may indirectly affect *p27^{Kip1}* stability and hence *p27^{Kip1}* protein levels through regulating Skp2 expression. Our data strongly suggested that KLF4 transcriptionally regulates *p27^{Kip1}* expression by directly binding to the proximal region of *p27^{Kip1}* promoter. Although this region has been reported to have promoter activity (48), the physiologic role of this region in the regulation of *p27^{Kip1}* expression remained to be defined. Therefore, our study provides novel insight into the functional role of the proximal region of *p27^{Kip1}* promoter in regulation of *p27^{Kip1}* gene expression. Interestingly, it is possible that KLF4 has an inhibitory effect on the other binding sites by recruiting other molecules to the promoter region of *p27*, which has elegantly been shown by a recent report (49). Thus, transcriptional regulation of *p27^{Kip1}* by KLF4 is complex and warrants further investigation.

In summary, KLF4 was expressed at various levels in pancreatic cancer cells and that ectopic overexpression of KLF4 suppressed pancreatic cancer cell growth *in vitro* and *in vivo* via induction of *p27^{Kip1}* expression and cell cycle arrest, suggesting that KLF4 has tumor suppressor functions in pancreatic cancer. Our findings also provide new insights into the transcriptional regulation of *p27^{Kip1}* expression by KLF4 and may help to clarify the role of KLF4 in pancreatic cancer progression.

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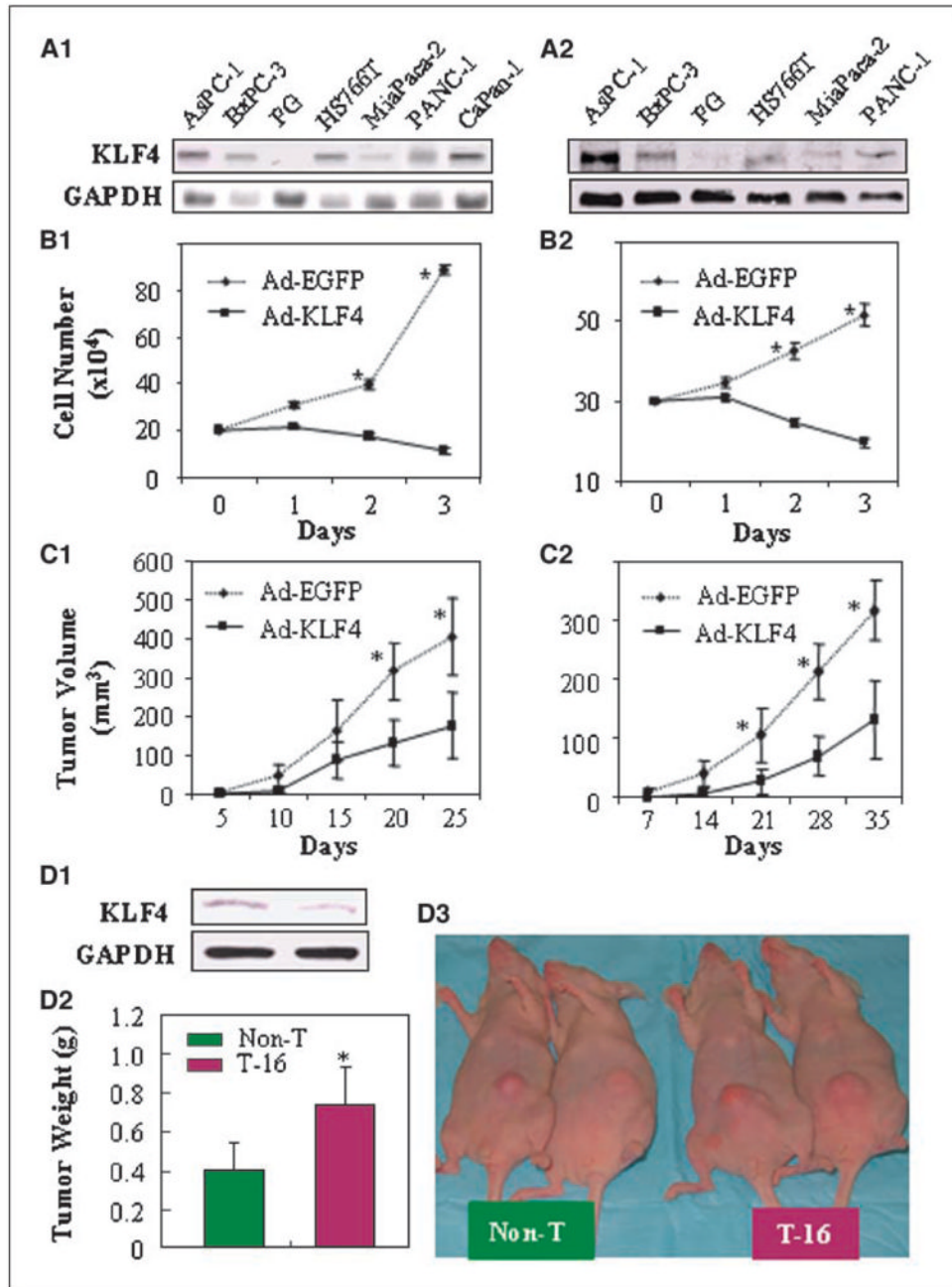


Figure 1.

KLF4 suppresses human pancreatic cancer cell growth *in vitro* and *in vivo*. *A*, KLF4 expression in human pancreatic cancer cell lines at the mRNA level (by Northern blot analysis; *A1*) and at the protein level (by Western blot analysis; *A2*). *B*, FG and BxPC-3 cells were transduced with Ad-KLF4 or Ad-EGFP at MOIs of 20 (FG cells; *B1*) or 15 (BxPC-3 cells; *B2*) and assessed for proliferation by cell counting at 1 to 3 d after transduction. *C*, growth kinetics of KLF4-transduced FG cells (*C1*) and BxPC-3 cells (*C2*) injected into the subcutis of nude mice. *D*, BxPC-3 cells with reduced KLF4 protein expression (by Western blot analysis; *D1*) grew larger tumors in nude mice (*D2*, representative tumor sizes; *D3*, averages of tumor weight). Bars, SE. *, $P < 0.05$ versus Ad-EGFP or nontarget control (*Non-T*).

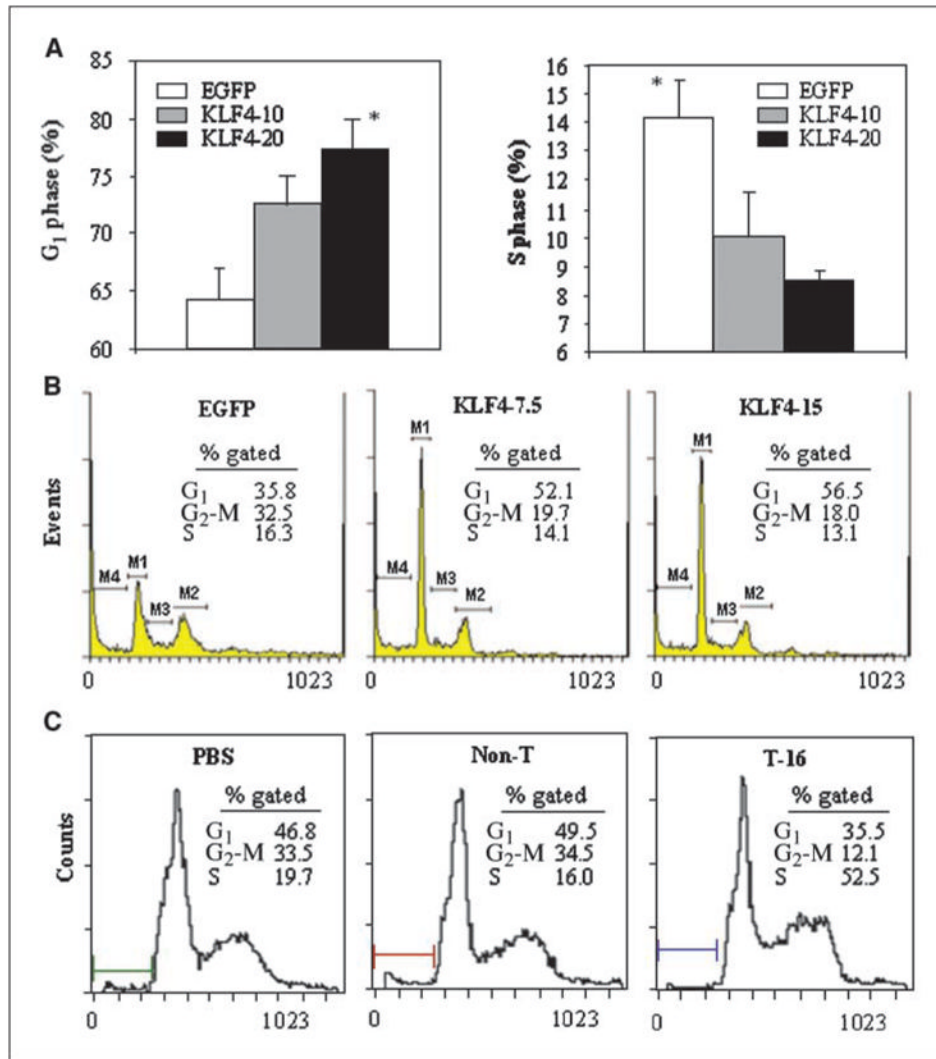
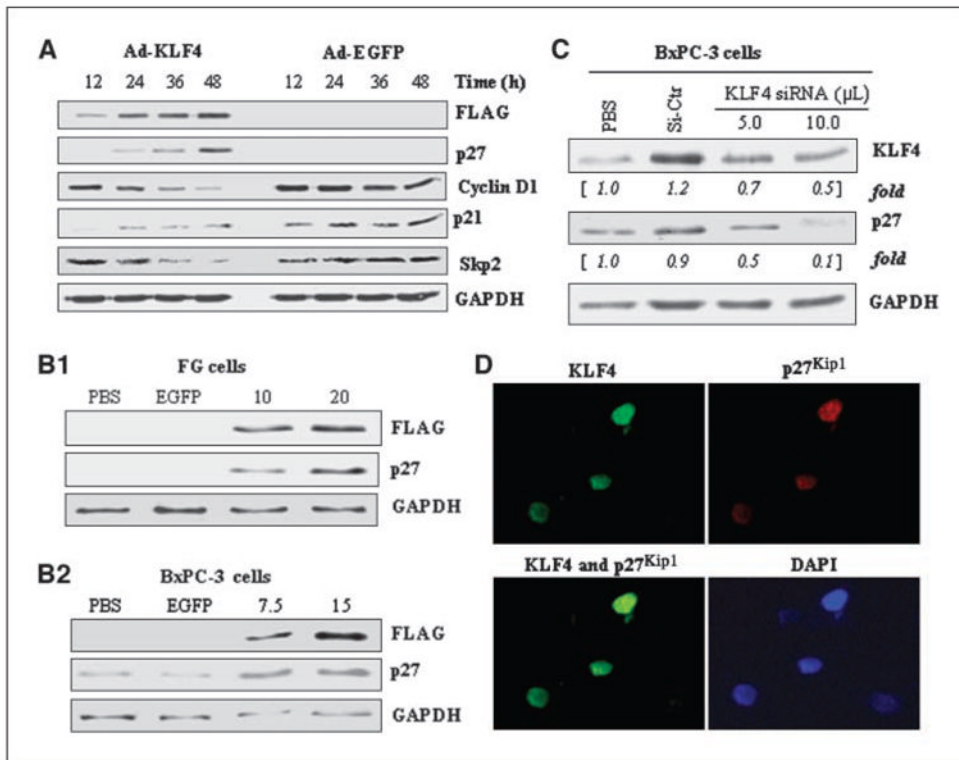


Figure 2.

Altered KLF4 expressions affect cell cycle progression of pancreatic cancer cells. FG cells (A) or BxPC-3 cells (B) were transduced with Ad-KLF4 at the indicated MOI; Ad-EGFP was used as a control or to adjust the total MOI to be equal in each treatment condition. Cells were harvested at 40 h after transduction, fixed in 70% ethanol, and then stained with propidium iodide for FACS analysis. C, parental BxPC-3 cells and BxPC-3 cells stably carrying nontarget (*Non-T*) or specific target (*T-16*) shRNAs were cultured in DMEM containing 1% colony-stimulating factor for 24 h; cells were harvested and processed for FACS analysis. Bars, SE. *, $P < 0.05$ versus Ad-EGFP control.

**Figure 3.**

KLF4 up-regulates p27^{Kip1} expression in human pancreatic cancer cells. *A*, FG cells were transduced with adenoviral KLF4 or EGFP (control) at a MOI of 20 and expression of KLF4 and cell cycle-related proteins was assessed at the indicated times by Western blotting. *B*, p27^{Kip1} expression was determined in FG and BxPC-3 cells at 48 h after transduction with Ad-KLF4 at indicated MOI as noted for Fig. 2. *C*, BxPC-3 cells were treated with control (scrambled) siRNA or the indicated volumes of KLF4 siRNA (with control siRNA used to adjust the total volume to 10.0 μL/well); p27^{Kip1} expression was determined 48 h later by Western blotting. *D*, transduction of FG cells with an EGFP-KLF4 fusion protein followed by immunofluorescence showed colocalization of KLF4 and p27^{Kip1} in the nuclei. DAPI, 4',6-diamidino-2-phenylindole.

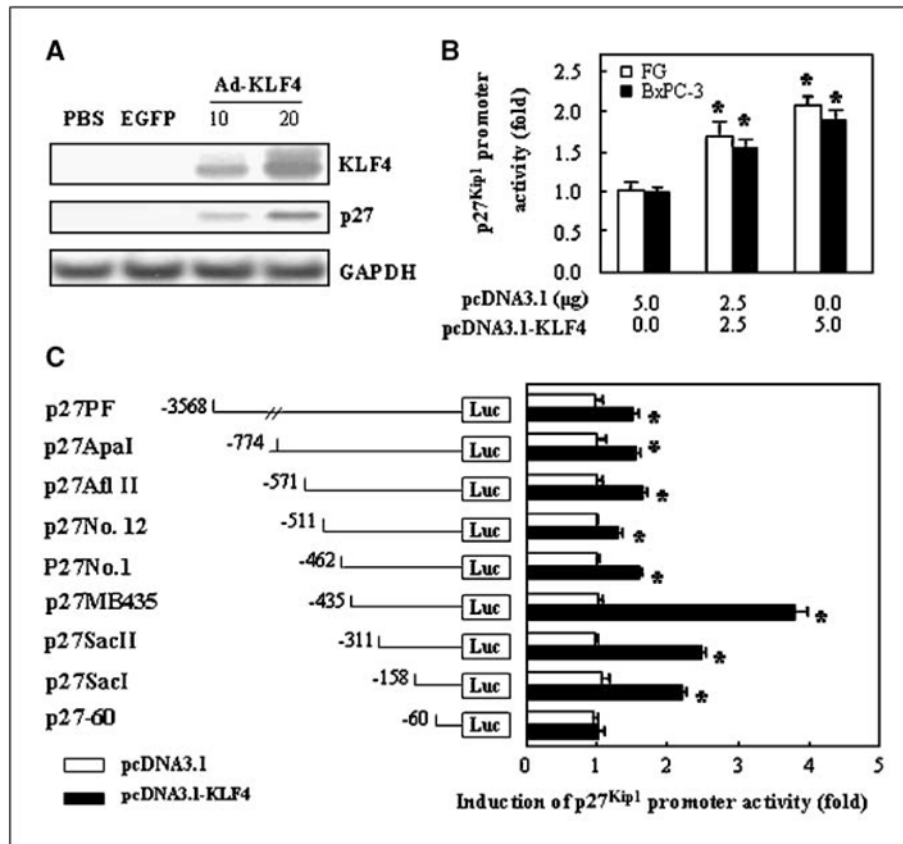


Figure 4. KLF4 transcriptionally activates *p27^{Kip1}* promoter. **A**, FG cells were transduced with adenoviral KLF4 or EGFP as described for Fig. 2, and KLF4 and *p27^{Kip1}* expression was measured 36 h later by Northern blotting. **B**, a full-length *p27^{Kip1}* promoter reporter (p27PF) was transfected into FG and BxPC-3 cells with pcDNA3.1-KLF4 or pcDNA3.1 (control) as shown and *p27^{Kip1}* promoter activity was determined 48 h later by dual luciferase assay. Results are expressed as the “fold” changes of the RR of pcDNA3.1-KLF4 vector versus pcDNA3.1 control vector. **C**, a series of 5' deletion mutants based on the 3,568-bp *p27^{Kip1}* promoter were transfected into HEK 293 cells by using a KLF4 expression vector or control vector, and promoter activities were measured with a dual luciferase assay. Results are expressed as fold changes of the RR of KLF4 expression vector versus pcDNA3.1 control vector. Bars, SE. *, $P < 0.05$ versus the value of pcDNA3.1 control vector.

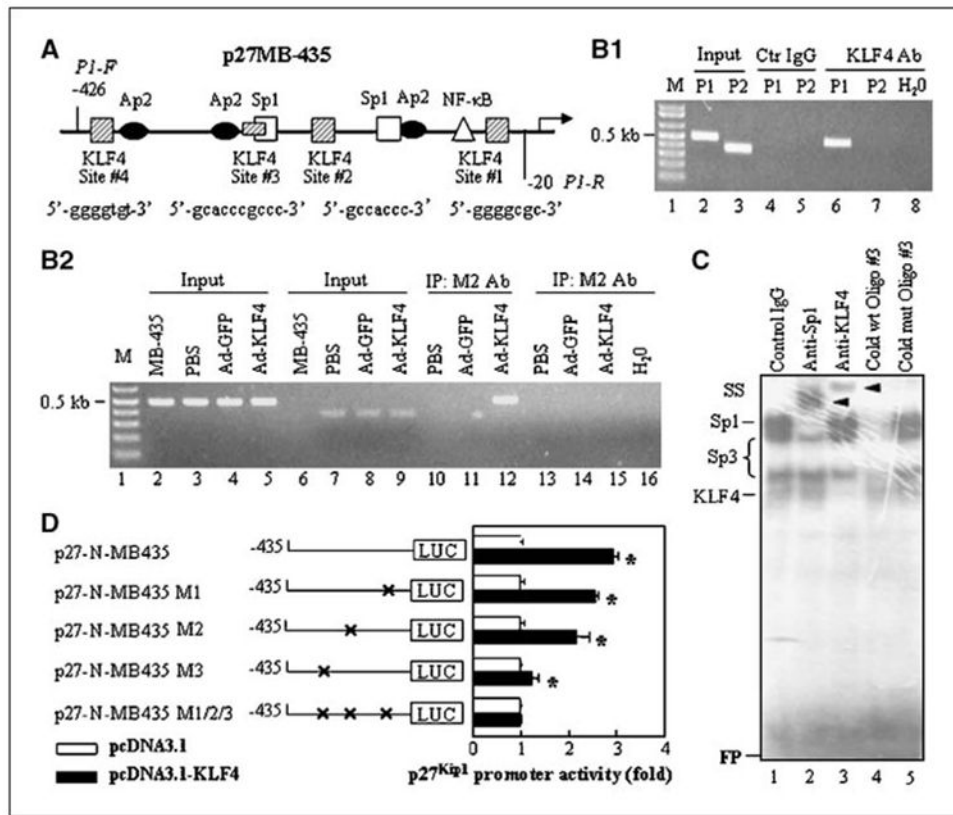


Figure 5. Binding of KLF4 to the p27^{Kip1} promoter *in vivo* and *in vitro*. **A**, schematic structure of p27^{Kip1} promoter. The locations and sequences of putative KLF4-binding sites and forward (P1-F) and reverse (P1-R) PCR primers flanking the p27^{Kip1} promoter were shown. **B1**, binding of endogenous KLF4 to p27^{Kip1} promoter. Chromatin fragments were prepared from BxPC-3 cells and ChIP assay was performed using a control IgG or anti-KLF4 antibody. Chromatin fragments without IgG or antibody were used as input controls. The PCR was performed using two sets of primers (P1, for p27^{Kip1} promoter; P2, for p27^{Kip1} 3'-UTR as a control) as described in Materials and Methods. **B2**, binding of exogenous KLF4 to p27^{Kip1} promoter. Chromatin fragments were prepared from FG cells (PBS), FG cells transduced with Ad-EGFP (Ad-EGFP), or Ad-KLF4 harboring FLAG-tagged KLF4 gene (Ad-KLF4) and ChIP assay was performed using a specific anti-FLAG antibody. The PCR was performed using two sets of primers (P1, for lanes 2–5 and 10–12; P2, for lanes 6–9 and 13–16). **C**, EMSA. Nuclear protein was extracted from BxPC-3 cells transduced with Ad-KLF4. Radioisotope-labeled oligo #3 corresponding to #3 KLF4-binding site on p27 promoter was used as a probe. The probe was incubated with nuclear protein in the presence of control IgG (lane 1), anti-Sp1 (lane 2), anti-KLF4 (lane 3), cold wild-type oligo #3 (1:50 ratio; lane 4), or mutant oligo #3 (1:50 ratio; lane 5). Shifted bands were marked as “Sp1,” “Sp3,” or “KLF4”; supershifted bands “SS” were indicated by arrows; and free probes were marked as “FP.” **D**, p27 promoter activity. The p27 promoter reporter constructs with site-directed mutagenesis of three putative KLF4-binding sites were transfected into BxPC-3 cells in triplicate with KLF4 expression vector or control pcDNA3 vector and incubated for 48 h. The relative p27 promoter activities were determined and the activities in KLF4 groups were expressed as fold changes of that in their respective controls. This was a representative experiment of three with similar results. *, statistical significance ($P < 0.01$) compared with respective control groups.