

Targeted Inhibition of the KLF6 Splice Variant, KLF6 SV1, Suppresses Prostate Cancer Cell Growth and Spread

Goutham Narla,¹ Analisa DiFeo,² Shen Yao,¹ Asoka Banno,³ Eldad Hod,¹ Helen L. Reeves,¹ Rui F. Qiao,³ Olga Camacho-Vanegas,² Alice Levine,¹ Alexander Kirschenbaum,^{1,4} Andrew M. Chan,³ Scott L. Friedman,^{1,3} and John A. Martignetti^{1,2,3,5}

Departments of ¹Medicine, ²Human Genetics, ³Oncological Sciences, ⁴Urology, and ⁵Pediatrics, the Mount Sinai School of Medicine, New York, New York

Abstract

Prostate cancer is a leading cause of cancer death in men. Risk prognostication, treatment stratification, and the development of rational therapeutic strategies lag because the molecular mechanisms underlying the initiation and progression from primary to metastatic disease are unknown. Multiple lines of evidence now suggest that KLF6 is a key prostate cancer tumor suppressor gene including loss and/or mutation in prostate cancer tumors and cell lines and decreased KLF6 expression levels in recurrent prostate cancer samples. Most recently, we identified a common KLF6 germ line single nucleotide polymorphism that is associated with an increased relative risk of prostate cancer and the increased production of three alternatively spliced, dominant-negative KLF6 isoforms. Here we show that although wild-type KLF6 (wtKLF6) acts as a classic tumor suppressor, the single nucleotide polymorphism-increased splice isoform, KLF6 SV1, displays a markedly opposite effect on cell proliferation, colony formation, and invasion. In addition, whereas wtKLF6 knockdown increases tumor growth in nude mice >2-fold, short interfering RNA-mediated KLF6 SV1 inhibition reduces growth by ~50% and decreases the expression of a number of growth- and angiogenesis-related proteins. Together, these findings begin to highlight a dynamic and functional antagonism between wtKLF6 and its splice variant KLF6 SV1 in tumor growth and dissemination. (Cancer Res 2005; 65(13): 5761-8)

Introduction

Disseminated prostate cancer is a leading cause of cancer death in men. Although gene loci, candidate genes, and risk factors for subsets of familial forms of prostate cancer development are increasingly being identified, the molecular mechanisms underlying the initiation and transition from localized to metastatic phenotype are poorly understood (1). Identification of individual genes and biomarkers which correlate with clinical behavior and metastatic spread is an urgent priority in order to define both therapeutic targets and improve patient stratification for future treatment. KLF6/COPEB is one of a number of candidate genes consistently emerging as a potentially relevant molecular target (2–7).

KLF6 is a member of the Kruppel-like family of zinc finger transcription factors which are DNA-binding proteins regulating growth-related signal transduction pathways, cell proliferation,

apoptosis, and angiogenesis (8, 9). We previously showed that KLF6, a ubiquitously expressed zinc finger transcription factor, is a tumor suppressor gene inactivated by allelic loss and somatic mutation in sporadic prostate cancers that can mediate growth suppression both by a p53-independent, up-regulation of p21 (2), and by disrupting the interaction between cyclin D1 and CDK4 (10). Independent studies, albeit not all (11), have also identified inactivation in both sporadic prostate cancer and prostate cancer cell lines (3). In support of its more general role as a tumor suppressor in a number of human cancers, KLF6 mutation and loss have also been described in colorectal cancer, hepatocellular and nasopharyngeal carcinomas, and in malignant gliomas (2–7). Moreover, in separate high-throughput array studies aimed at identifying novel risk stratification markers and predictive gene cluster fingerprints, decreased KLF6 expression has also been shown to predict poor clinical outcomes in both prostate cancer (12) and pulmonary adenocarcinoma (13).

Beyond these findings in tumor samples, we recently identified a germline KLF6 single nucleotide polymorphism (SNP), IVS1 –27 G>A/IVSΔA, that is significantly associated with increased prostate cancer risk in men (14). This intronic SNP, which is the first reported high-prevalence, low-penetrance prostate cancer susceptibility allele, generates a novel functional SRp40 DNA binding site, ablating two other overlapping SR-protein binding sites, and increasing transcription of three alternatively spliced KLF6 isoforms, KLF6 SV1, SV2, and SV3. These alternatively spliced KLF6 proteins, present in both normal and cancerous tissues, antagonize the ability of wild-type KLF6 (wtKLF6) to up-regulate p21 expression and suppress cell proliferation (14). Although alternative splicing is present in both normal and cancerous cells, expression of the KLF6 splice variants seems to be up-regulated in prostate cancer (14). These results suggested that the IVSΔA SNP effectively disrupts a regulated pattern of KLF6 splicing and through overexpression of splice variants, could lead to an increased relative risk of prostate cancer. Given that KLF6 SV1 and SV2 antagonize the ability of wtKLF6 to up-regulate p21 expression and suppress cellular proliferation (14), these isoforms may provide the physiologic and biological link between this KLF6 germ line SNP and increased risk of prostate cancer.

Thus, the present studies explore the biological relevance and implications of one of the recently identified KLF6 splice variants, KLF6 SV1, on a number of cancer-related phenotypes. In addition, this study provides the first demonstration that suppressing wtKLF6 expression increases prostate cell growth in culture and tumor growth *in vivo*.

Materials and Methods

Cell culture and transient transfection. All cell lines were obtained from the American Tissue Culture Collection (Rockville, MD). Polyclonal

Note: S.L. Friedman and J.A. Martignetti share senior authorship.

Requests for reprints: John A. Martignetti, Department of Human Genetics, Mount Sinai School of Medicine, 1425 Madison Avenue, New York, NY 10029. Phone: 212-659-6744; E-mail: john.martignetti@mssm.edu.

©2005 American Association for Cancer Research.

pools of stable cell lines were generated by cotransfection of the pSUPER-si-luc, si-wtKLF6, si-SV1, or si-SV2 with a puromycin expressing plasmid. Transfected cells were selected with 2 µg/mL of puromycin. For each construct, three independent polyclonal pools of stable cell lines were generated and analyzed.

pSUPER plasmid construction and transfection. The pSUPER-siSV1 and pSUPER-siSV2 plasmids used to down-regulate KLF6 SV1 and KLF6 SV2 expression and the control pSUPER-luc construct were previously described (14). The pSUPER-si-wtKLF6 construct was generated as previously described using this pSUPER vector (ref. 15; generously provided by R. Agami). To insert the targeting sequence, DNA oligonucleotides were designed and cloned into the *BglII-HindIII* sites of the pSuper vector.

Si-wtKLF6-F: GATCCCCTGGCGATGCCTCCCCGACttcaagaga
GTCGGGGGAGGCATCGCCATTTTGGAAA

Si-wtKLF6-R: AGCTTTTCCAAAAATGGCGATGCCTCCCCGAC
tctcttgaaGTCGGGGGAGGCATCGCCAGGG

Immunohistochemistry. Immunohistochemical staining for factor VIII-related antigen and proliferating cell nuclear antigen (PCNA) were carried out as previously described (16, 17) using a factor VIII-related antigen (DAKO, Carpinteria, CA) antibody for the detection of tumor microvessel density and a PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for the detection of tumor cell proliferation, respectively. Measurements of PCNA staining and microvessel density were done as previously described (18). Briefly, microvessels that stained positive for factor VIII-related antigen were counted on four representative high-power fields (400×) for each tumor. Data was expressed as the average number of microvessels per 400× field for each experimental tumor group. PCNA staining was determined by counting the number of positive cells per 400× field and dividing that number by the total number of cells in that particular field. For each tumor, four high-powered fields were counted and the average for each experimental tumor group was determined.

Colony formation assays. The ability of different transfectants to proliferate in an anchorage-independent manner was quantified by standard soft-agar assay. Approximately 10^5 cells were resuspended in 2 mL of 0.4% (w/v) Noble agar (Difco) and overlaid on top of 1% (w/v) agar as described previously (19). After 3 weeks of incubation at 37°C, continuously growing colonies were visualized by staining with 1 mg/mL of *p*-iodonitrotetrazolium violet. Colonies >2 mm in diameter were counted.

Tumorigenicity assay. Stable short interfering RNA (siRNA) PC3M cells (1×10^6) were injected into the left flank of 6- to 8-week-old female BALB/c *nu/nu* mice. Tumor volume was assessed every week and determined by the formula (length × width × width × 0.4). The mice were sacrificed 8 weeks after inoculation and tumors were excised for RNA, protein, and immunohistochemical analysis. All animal work and protocols were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee.

Migration and invasion assays. Standard invasion assays were done in Boyden chambers by using a reconstituted basement membrane (Matrigel, 0.5 mg/mL; Becton Dickinson, Mountain View, CA; ref. 20). Coated membranes were first blocked with 0.5% bovine serum albumin (BSA) in DMEM and equilibrated in 0.1% BSA/DMEM. Approximately 10^5 cells in serum-free DMEM were added to the upper chamber and conditioned medium derived from NIH 3T3 fibroblasts was used in the lower chamber as a chemoattractant. Following incubation for 19 hours at 37°C, cells in the upper chamber were thoroughly removed by gentle suctioning. Cells invaded through the barrier were fixed in 10% formalin and stained with 4',6-diamidino-2-phenylindole in PBS. Nuclei were visualized under a fluorescence microscope and images of five randomly selected nonoverlapping fields were counted.

Generation of KLF6 monoclonal antibodies. A 67 kDa glutathione *S*-transferase fusion peptide containing amino acids 28 to 199 of the human KLF6 activation domain (*pGEX-2-PM*) and the following peptide: EKSLT-DAHGKGVSGVLQEVMS were purified and used to generate the 2A2 and 9A2 monoclonal KLF6 antibodies, respectively.

Western blot analysis. Cell extracts for Western blotting were harvested in radioimmunoprecipitation assay buffer (standard protocols, Santa Cruz Biotechnology). Tumor tissue extracts were harvested and prepared in the T-PER reagent (Pierce, Rockford, IL). Equal amounts of protein (50 µg) as determined by the Bio-Rad (Richmond, CA) DC Protein quantification assay were loaded and separated by PAGE and transferred to nitrocellulose membranes. Western blotting was done using a goat polyclonal antibody to actin and VE-cadherin (SC-1616 and SC-6458, respectively), and monoclonal antibodies to p21 (Santa Cruz Biotechnology) and the KLF6 2A2 and 9A2 antibodies (Zymed).

Densitometric analysis. Enhanced chemiluminescent images of immunoblots were analyzed by scanning densitometry and quantified with a BIOQUANT NOVA imaging system. All values were normalized to actin and expressed as fold changes relative to control.

Analysis of proliferation. Proliferation was determined by estimating [³H]thymidine incorporation. PC3M stable cell lines expressing either si-luc, si-wtKLF6, si-SV1, or si-SV2 were plated at a density of 50,000 cells per well in 12-well dishes. Forty-eight hours after plating, 1 µCi/mL [³H]thymidine (Amersham) was added. After 2 hours, cells were washed four times with ice-cold PBS and fixed in methanol for 30 minutes at 4°C. After methanol removal and cell drying, cells were solubilized in 0.25% sodium hydroxide/0.25% SDS. After neutralization with hydrochloric acid (1 N), disintegrations per minute were estimated by liquid scintillation counting.

RNA and quantitative real-time PCR analysis. Cell line and tumor RNA was extracted using the RNeasy Mini and Midi kit (Qiagen, Chatsworth, CA). All RNA was treated with DNase (Qiagen). A total of 1 µg of RNA was reverse-transcribed per reaction using first-strand complementary DNA synthesis with random primers (Promega, Madison, WI). Quantitative real-time PCR was done using the following PCR primers on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA): Ki-67 forward, 5'-GAA GAG TTG TAA ATT TGC TTC T-3'; and Ki-67 reverse, 5'-ATG TTG TTT TGA CAC AAC AGG A-3'. Primer sequences for total and wtKLF6, p21, and glyceraldehyde-3-phosphate dehydrogenase (14) as well as for markers of angiogenesis including *Flt-1*, *VE-cadherin*, *Ang-2*, *Tie-1*, and *PECAM* have all been previously described (21). All experiments were done in triplicate and repeated three independent times. All values were normalized to glyceraldehyde-3-phosphate dehydrogenase levels. Levels of KLF6 alternative splicing was determined as previously described (14).

Results

KLF6 alternative splicing is differentially regulated in prostate cancer cell lines. Prior to their experimental manipulation, we established the baseline level of KLF6 alternative splicing in two well-characterized prostate cell lines, the benign prostatic hyperplasia cell line, BPH1, and the metastatic prostate cancer-derived cell line, PC3M. The levels of wtKLF6 and alternative splicing in both of these cell lines was determined and compared at both the mRNA and protein levels. We chose these two cell lines because of their highly contrasting genotypic and phenotypic features. The nontumorigenic human prostatic epithelial cell line, BPH1, was originally immortalized using the large T antigen oncogene and fails to form tumors in nude mice (22). PC3M was originally derived from liver metastases produced in nude mice subsequent to intrasplenic injection of PC3 cells (23).

Similar to previous findings in patient-derived tissues (14), wtKLF6 levels were lower, whereas alternatively spliced isoforms of KLF6 were higher in the metastatic, cancer-derived cell line PC3M than those in BPH1 cells (Fig. 1A and B). Densitometric analysis of the KLF6 protein isoforms in PC3M cells showed an average 2.8-fold increase in the ratio of alternatively spliced KLF6 to wtKLF6.

Interestingly, the endogenous splicing preferences between wild-type and splice forms in these two cell lines were maintained following transfection of a KLF6 minigene construct. Each cell line

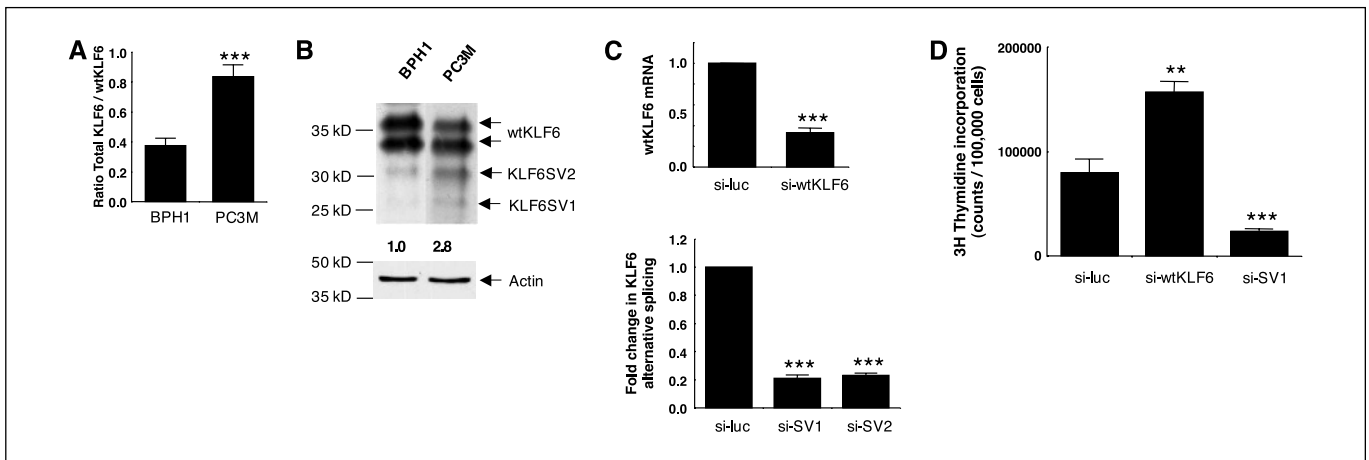


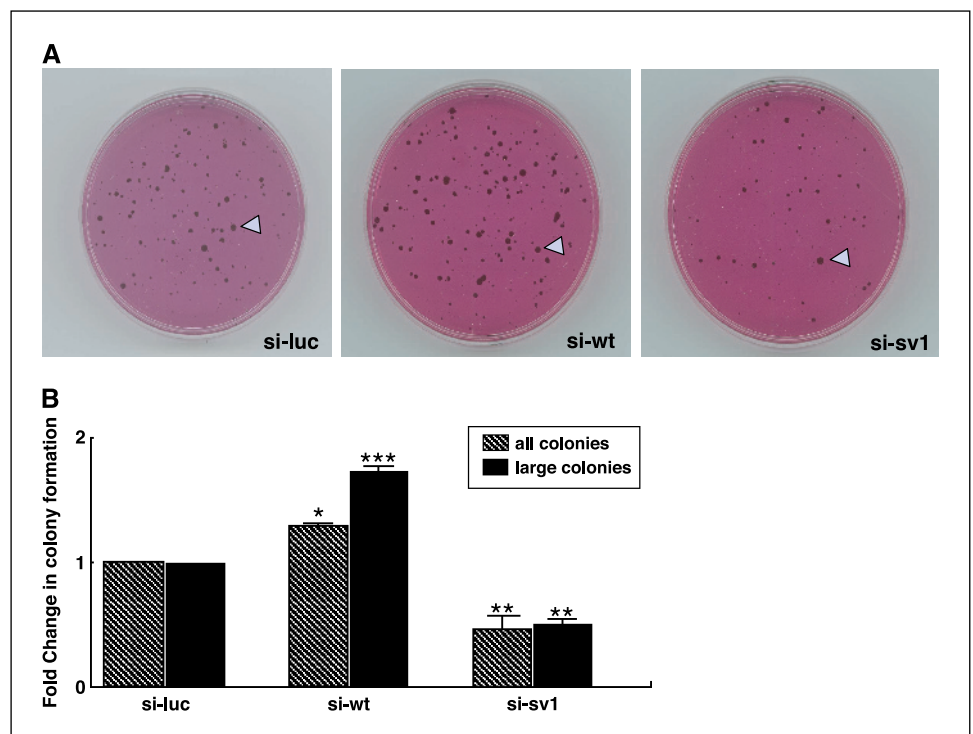
Figure 1. The *KLF6* gene is differentially spliced in prostate cell lines. *A*, quantitative real-time PCR was done on the benign prostatic hyperplasia cell line (BPH1) and the metastatic prostate cancer cell line (PC3M) to determine the baseline level of endogenous *KLF6* alternative splicing in these prostate-derived cell lines. The PC3M cell line had a significantly higher endogenous splicing ratio ($x = 0.83$) than did the BPH1 cell line ($x = 0.41$; ***, $P < 0.0001$). *B*, Western blot analysis for *KLF6* expression in BPH1 and PC3M cells using the 2A2 *KLF6* monoclonal antibody. Consistent with the RNA findings, the PC3M cell line expresses increased levels of both *KLF6* variant proteins. The ratio of splice variants to wild-type protein was determined by densitometry to be ~ 2.8 -fold. *C*, transient expression of the pSUPER-si-wtKLF6, si-luc, si-SV1, and si-SV2 in PC3M cells. Three independent transfection experiments were done and analyzed for each pSUPER-siRNA construct. Transient si-wtKLF6 expression results in a 70% decrease in wtKLF6 mRNA (***, $P < 0.0001$), whereas targeted reduction of si-SV1 and SV2 expression results in an $\sim 80\%$ decrease in *KLF6* alternative splicing as measured by quantitative real-time PCR (***, $P < 0.0001$). *D*, cell proliferation analysis of transiently transfected PC3M cells. The pSUPER-si-wtKLF6 expressing cells proliferate 80% more than pSUPER-luc expressing cells (**, $P < 0.001$), whereas the pSUPER-si-SV1 expressing cells proliferate 65% less than control cells (***, $P < 0.0001$).

was transfected with an expression vector containing the entire *KLF6* gene including the 5' and 3' untranslated regions (14). Again, wtKLF6 levels were decreased, whereas alternatively spliced forms were increased in the PC3M cells when compared with the BPH1 cell line (Fig. 1A).

Targeted silencing of wtKLF6 and *KLF6* SV1 divergently affects cellular proliferation. Having shown differential regulation in *KLF6* alternative splicing levels in these two cell lines, we next examined the effect of targeted down-regulation of each

isoform specifically by RNAi-mediated gene silencing on growth- and metastasis-related features of the PC3M cell line. We have previously shown that both *KLF6* SV1 and *KLF6* SV2 proteins mislocalize to the cytoplasm and fail to either up-regulate endogenous p21 or suppress cell proliferation (14). Silencing of either the endogenous *KLF6* SV1 or SV2 transcripts increases p21 mRNA and protein levels (14). However, the isoforms are not biologically equivalent because silencing SV1, but not SV2, results in decreased cell proliferation in culture (14).

Figure 2. Stable abrogation of *KLF6* SV1 by siRNA decreases anchorage-independent growth of PC3M. Stable cell lines expressing siRNA to either luciferase (luc), wtKLF6 (si-wt), or SV1 (si-SV1) and were assessed for their ability to grow in soft agar (arrowheads), as described in Materials and Methods. *A*, targeted reduction of full-length *KLF6* (si-wtKLF6) results in increased colony formation in soft agar as compared with the control cell line, pSUPER-si-luc. Targeted reduction of *KLF6* SV1 results in a significant decrease in colony formation. *B*, the total number of all colonies and large colonies was counted by two independent observers in three independent experiments, each done in triplicate. Targeted reduction of wtKLF6 resulted in a 30% increase in the number of total colonies (*, $P < 0.01$) and a 90% increase in large colony formation compared with the si-luc control cell line (***, $P < 0.0001$). Furthermore, targeted reduction of *KLF6* SV1 leads to a 50% reduction in both the number of all colonies and large colonies formed compared with controls (**, $P < 0.001$).



First, we generated stable cell lines expressing siRNAs specific to either wtKLF6 (si-wtKLF6), KLF6 SV1 (si-SV1), or KLF6 SV2 (si-SV2). Each pSUPER-derived siRNA specifically targets the respective KLF6 mRNA, with no effect on the other isoforms (14). Multiple polyclonal cell line pools for each construct were generated and analyzed by quantitative real-time PCR and Western blotting. As shown in Fig. 1C, wtKLF6 levels were reduced ~50%, whereas each targeted KLF6 alternative splice form was reduced ~60% in the respective stable cell lines expressing pSUPER-si-SV1 and si-SV2 as compared with pSUPER-Luc expressing cell lines. Proliferation rates were drastically and divergently affected in two of the generated lines. Cell proliferation increased by almost 90% in the si-wtKLF6 cells but reduced by almost 60% in the si-SV1 cell lines when compared with controls (Fig. 1D). No changes in proliferation rates were noted in the pSUPER-si-SV2 stable cell lines (data not shown).

Differential effects of wtKLF6 and SV1 gene silencing on tumor cell colony formation, migration, and invasion. We next explored the effect of targeted reduction on the ability of different KLF6 siRNA stable cell lines to proliferate in an anchorage-independent manner as quantified by soft-agar assay. Consistent with its function as a tumor suppressor gene, targeted reduction of wtKLF6 (si-wtKLF6) led to a >50% increase in colony formation (Fig. 2A and B; $P < 0.01$). Reduction of KLF6 SV1, on the other hand, had exactly the opposite effect, resulting in a >50% decrease in colony formation (Fig. 2A and B; $P < 0.001$).

Given the role of cell migration and invasion in the progression of localized cancer to disseminated disease, we next analyzed these

phenotypes. Interestingly, these were only affected by one of the two KLF6 isoforms. Targeted reduction of the KLF6 SV1 protein resulted in a 60% decrease in both cell migration (Fig. 3A; $P < 0.01$) and invasion (Fig. 3B; $P < 0.001$) of this highly metastatic cell line. In contrast to its markedly opposite effects to SV1 on cellular proliferation and colony formation, reduction of the wtKLF6 protein had no effect on either cell migration or invasion (data not shown).

Inhibition of wtKLF6 and KLF6 SV1 differentially affect tumorigenicity *in vivo*. Based on the marked functional differences between the various KLF6 isoform siRNA cell lines, we next explored whether wtKLF6 or the splice variants affected tumorigenicity *in vivo*. Beyond the previous patient-derived tumor findings—that decreased wtKLF6 expression correlated with worse prognosis (12) and KLF6 SV1 expression is increased in prostate cancer tissue (14)—the tumorigenicity data provides the first experimental evidence that these isoforms differentially regulate tumor growth *in vivo*.

PC3M stable cell lines expressing specific siRNAs to either luciferase, wtKLF6, SV1, or SV2 were injected s.c. into nude mice and after 8 weeks, the mice were sacrificed and tumor mass was determined. Tumor take rates were consistent between the various groups, with less than two of the injected mice in each group failing to produce a measurable tumor after 8 weeks of growth. Consistent with its role as a tumor suppressor gene, reduction of wtKLF6 mRNA led to >2-fold increase in tumorigenicity (Fig. 4A and B; $P < 0.01$). In marked contrast, silencing of the KLF6 SV1 transcript resulted in a 40% reduction in tumorigenicity *in vivo* (Fig. 4A and B; $P < 0.001$). Targeted reduction of the KLF6 SV2 transcript had no effect on tumor growth (data not shown).

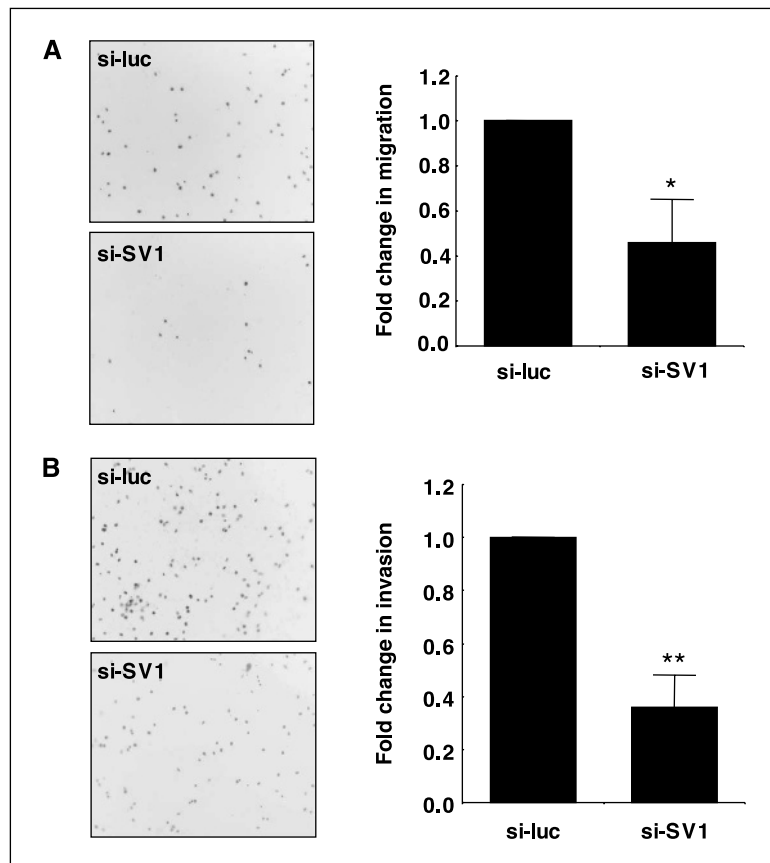


Figure 3. Reduced migration and invasion following abrogation of KLF6 SV1 by siRNA in PC3M cells. Cell lines stably expressing either siRNA to luciferase or KLF6 SV1 were assessed for their ability to either migrate and to invade through a Matrigel-coated insert, as described in Materials and Methods. Left, the underside of the insert stained with 4',6-diamidino-2-phenylindole; right, fold-change. A, targeted reduction of the KLF6 SV1 protein resulted in a 60% decrease in cell migration compared with the control luc cell line (*, $P < 0.01$). The number of cells that migrated were counted from four fields from three independent experiments done on two separate and independent polyclonal pools of si-luc, si-wtKLF6, si-SV1, and si-SV2 stable cell lines. B, si-SV1 stable cell lines were 50% less invasive through a Matrigel basement membrane (**, $P < 0.001$). The number of invasive cells were counted from four fields from four independent experiments on two separate and independent polyclonal pools of cells as described above.

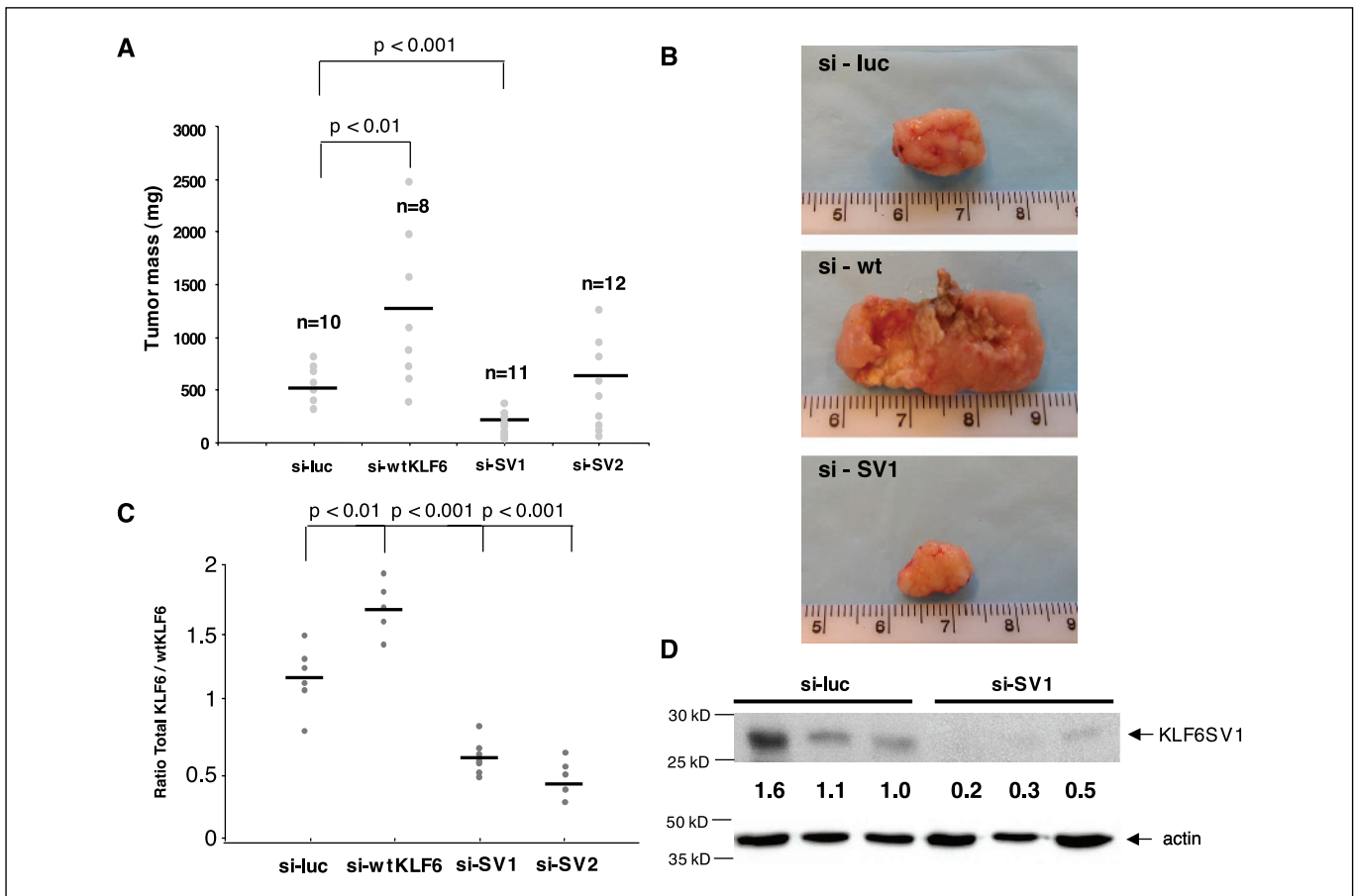


Figure 4. Divergent effects of stably expressed siRNAs to KLF6 wild-type and SV1 on PC3M xenograft growth *in vivo*. Stable cell lines expressing siRNAs to either luciferase wtKLF6, or splice forms SV1 or SV2 were injected into nude mice and their volume assessed as described in Materials and Methods. **A**, reduction of the full-length wtKLF6 mRNA resulted in >90% increase in tumorigenicity *in vivo* ($P < 0.01$); whereas PC3M cells expressing siRNA to KLF6 SV1 were 50% less tumorigenic *in vivo* ($P < 0.001$). **B**, a representative tumor from each group. **C**, KLF6 alternative splicing was quantified by quantitative real-time PCR as previously described (14) from RNA isolated from these tumors. The si-wtKLF6 derived tumors had 60% more alternative splicing relative to wtKLF6 mRNA than did control luc tumors ($P < 0.01$), whereas tumors derived from the si-SV1 and si-SV2 cell lines had a 50% and 70% decrease in KLF6 alternative splicing, respectively, compared with the control tumors ($P < 0.001$). **D**, Western blot analysis of tumor proteins extracted from independent animals injected with PC3M cells expressing pSUPER-luc ($n = 3$) and si-SV1 ($n = 3$) using the KLF6 9A2 monoclonal antibody specific to the KLF6 SV1 protein. Si-SV1 tumors expressed significantly less KLF6 SV1 protein than control si-luc tumors, as measured by densitometry relative to an actin-loading control.

To determine if the siRNA-derived tumors maintained their down-regulation of KLF6 isoforms *in vivo*, the ratio of alternatively spliced KLF6 transcripts to wtKLF6 levels was quantified by quantitative real-time PCR from tumor-isolated RNA at the end of the experimental time course. As predicted, tumors derived from the si-wtKLF6 cell line had decreased wtKLF6 levels compared with si-luc tumors (Fig. 4C). This resulted in a significant increase in the ratio of KLF6 splice variants to wtKLF6 mRNA (Fig. 4C; $P < 0.01$). On the other hand, tumors derived from the si-SV1 and si-SV2 cell lines had a reduction in KLF6 alternative splicing which effectively increased the wtKLF6 to total ratio of KLF6 (Fig. 4C; $P < 0.001$). Western blot analysis of the si-SV1 tumors with the KLF6 SV1 specific monoclonal (9A2) revealed a significant reduction in SV1 protein level *in vivo* (Fig. 4D). Interestingly, although si-SV2 tumors paralleled the significantly decreased ratio in KLF6 splice forms to wtKLF6 seen in the si-SV1 tumors, SV2 reduction had no effect on tumorigenicity (Fig. 4A-C).

Decreased si-SV1 tumor growth is associated with an antiproliferative and antiangiogenic gene expression profile. To explore potential mechanisms underlying the si-SV1-mediated reduction in tumorigenicity, we examined the expression patterns of a number of key genes regulating cell proliferation, angiogenesis,

and apoptosis in the stable cell-derived tumors. Consistent with our previous findings in patient-derived tumors (14), p21 mRNA and protein levels were increased over 2-fold in si-SV1-derived tumors (Fig. 5A and B; $P < 0.01$). This up-regulation of p21 was associated with decreased expression of markers of cellular proliferation as assessed by decreased PCNA staining and a ~30% reduction in Ki-67 mRNA levels (Fig. 5B-D). On the other hand, si-wtKLF6-derived tumors were significantly larger than control tumors and displayed higher PCNA staining, as well as a 50% increase in Ki-67 mRNA levels (Fig. 5C and D). Targeted reduction of the SV2 transcript had no effect on tumorigenicity or p21 levels (data not shown).

Tumor growth *in vivo* is known to be dependent on the ability of cancer cells to stimulate blood vessel growth through up-regulation of angiogenic mediators. Platelet/endothelial cell adhesion molecule-1 (PECAM-1)/CD31 is highly expressed by endothelial cells and is a reproducible marker of angiogenesis in transplanted tumor models. Given the relatively pallid appearance of the si-SV1-derived tumors (Fig. 4B), we analyzed the effects of SV1 silencing on a number of angiogenic markers, including CD31. Si-SV1-derived tumors had a >50% reduction in

CD31 expression compared with control tumors (Fig. 6A; $P < 0.0001$) consistent with an overall inhibition of *in vivo* angiogenesis. To broaden this finding, we then included an analysis of the expression levels of a panel of genes, *VEGF*, *Ang-1*, *Ang-2*, *Flt-1*, *KDR*, *Tie-1*, *VE-cadherin*, and *PECAM-1*, that together have been shown to more accurately reflect angiogenesis than any single marker gene alone (21). Consistent with the PECAM-1/CD31 protein immunohistochemical data, expression of five angiogenic genes, *Ang-2*, *Flt-1*, *Tie-1*, *VE-cadherin*, and *PECAM-1* was significantly reduced in si-SV1 tumors (Fig. 6B). *VEGF*, *Ang-1*, and *KDR* levels were not significantly changed. This potentially reflects the differences in the regulation of angiogenesis in different tumor types and tissues (21). Nonetheless, levels of VE-cadherin, a key molecule in endothelial cell-cell interaction and in the formation of mature, functional blood vessels, were decreased on average by >70% as assessed by Western blotting (Fig. 6C).

Discussion

A growing number of studies have highlighted the mechanisms by which the KLF6 tumor suppressor gene may be functionally inactivated in human cancer. Somatic KLF6 mutations and allelic

loss have been identified in several primary human neoplasms, including prostate (2, 3), colorectal (4), hepatocellular carcinoma (5), malignant glioma (6), and nasopharyngeal carcinoma (7). Additionally, KLF6 is transcriptionally silenced in esophageal cancer cells through promoter hypermethylation (24) and is significantly down-regulated in primary lung cancer samples and lung cancer cell lines (13, 25), as well as in some prostate cancer cell lines (3), whereas the mechanism(s) of inactivation in these cases have yet to be defined. Furthermore, KLF6 protein expression is absent in a number of glioblastoma cell lines and primary glioma patient samples and reconstitution of wtKLF6 protein expression reverts tumor growth both *in vitro* and *in vivo* (26).

Our experimental findings suggest an important role for the KLF6 tumor suppressor gene in regulating prostate cancer development and progression through two distinct yet complementary pathways. First, decreased expression of wtKLF6 leads to increased proliferation, colony formation, and tumorigenicity *in vivo*. This is the first report demonstrating the biological function of the KLF6 tumor suppressor gene by siRNA-mediated targeted gene silencing in culture and *in vivo*. These findings provide a biologically relevant link to independent studies

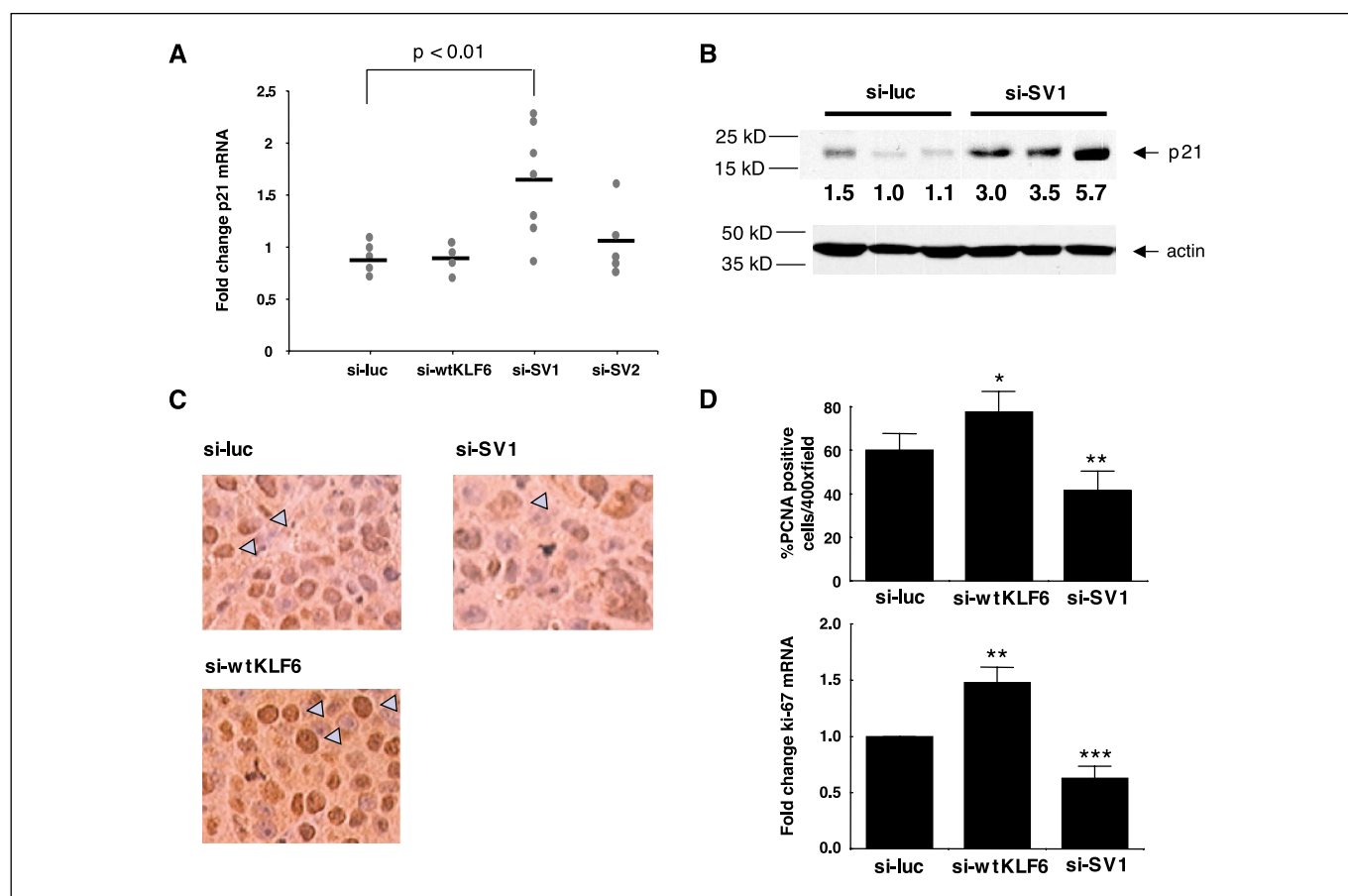


Figure 5. Correlation of cell growth effects with p21 and proliferation markers in tumors stably expressing siRNAs for wtKLF6 or KLF6 SV1. Tumors were analyzed for their expression of p21 mRNA or protein, PCNA, and Ki-67 in response to abrogation of wtKLF6 or KLF6 SV1. **A**, targeted reduction of the KLF6 SV1 protein results in a significant up-regulation of both p21 mRNA and protein as measured by quantitative real-time PCR and Western blotting, respectively. **B**, immunohistochemistry for PCNA in siRNA tumors. Targeted reduction of wtKLF6 results in increased PCNA staining *in vivo* ($P < 0.01$), whereas tumors derived from si-SV1 cells express significantly less PCNA ($P < 0.001$). For each group of tumors, si-luc ($n = 6$), si-wtKLF6 ($n = 5$), and si-SV1 ($n = 7$) six independent high-powered fields for each tumor were counted, assessing both the total number of cells and the number of PCNA-positive cells. The graph represents the average percentage of PCNA-positive cells for each group. **C**, quantitative real-time PCR analysis for Ki-67 mRNA levels in siRNA-derived tumors. Si-SV1 tumors ($n = 7$) expressed significantly less Ki-67 mRNA ($P < 0.0001$) than control luc ($n = 6$) controls, whereas targeted reduction of wtKLF6 ($n = 5$) *in vivo* resulted in an almost 50% increase in Ki-67 mRNA ($P < 0.001$).

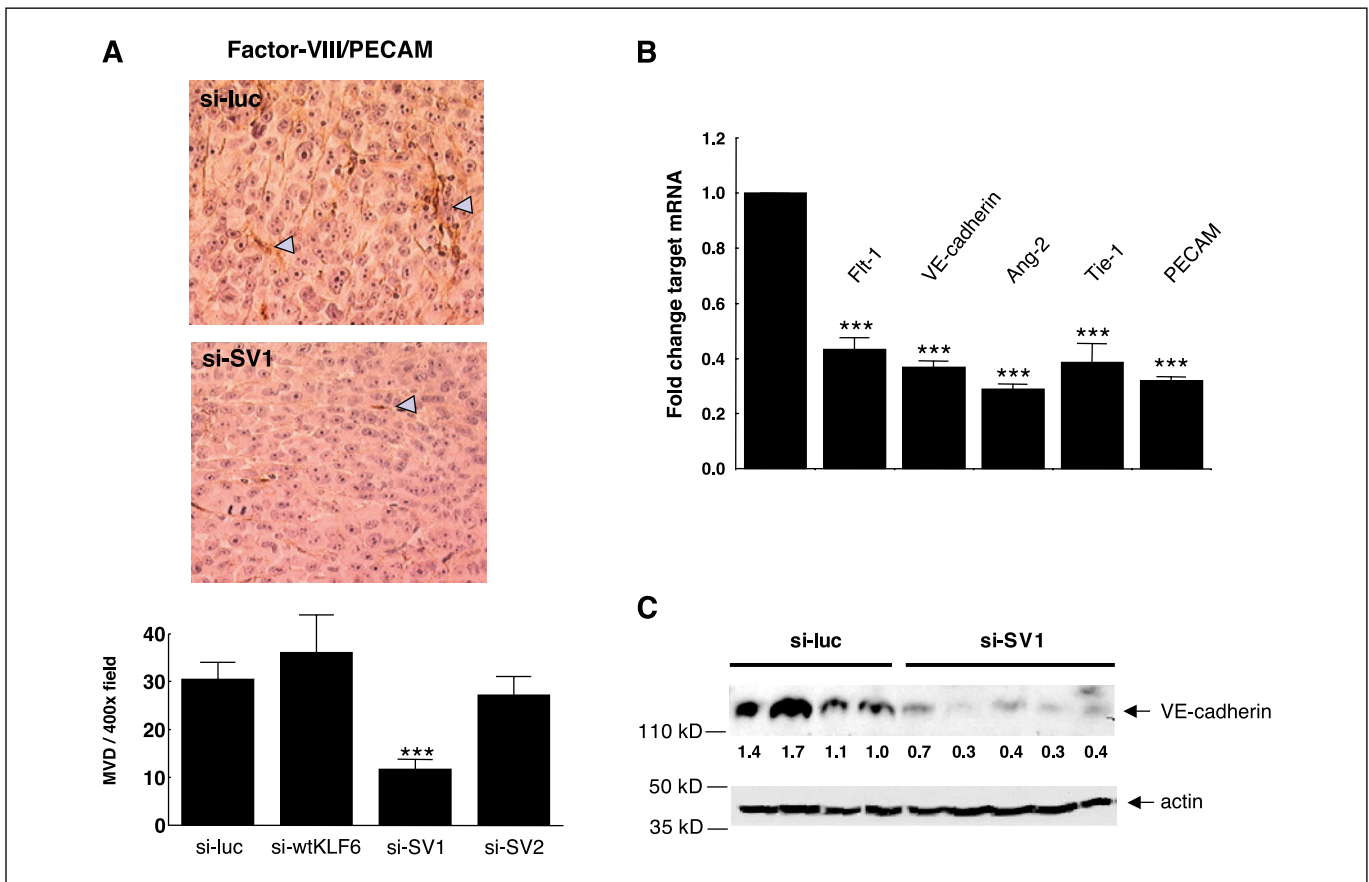


Figure 6. Reduced KLF6 SV1 expression leads to decreased angiogenesis by PC3M-derived tumors *in vivo*. Tumors generated as described in Fig. 5 were analyzed for expression of angiogenesis-related markers *in vivo*. **A**, CD31/PECAM staining of stable siRNA cell line-derived tumors. Targeted reduction of the KLF6 SV1 protein results in a 60% decrease in microvessel density ($P < 0.0001$) as measured by the number of CD31/PECAM-positive endothelial cells per 400 \times field. For each siRNA-derived tumor, four independent 400 \times fields were counted for CD31-positive endothelial cells. **B**, quantitative real-time PCR analysis of a panel of markers of angiogenesis. Si-SV1-derived tumors expressed significantly lower levels of Flt-1, VE-cadherin, Ang-2, Tie-1, and PECAM compared with luc control tumors ($P < 0.0001$). **C**, Western blot analysis of pSUPER-luc and si-SV1-derived tumor protein using a VE-cadherin antibody. Si-SV1 tumors expressed significantly less VE-cadherin protein than control si-luc tumors as measured by densitometry relative to an actin loading control. Three independent si-luc tumors compared with three independent si-SV1 tumors are shown.

demonstrating that decreased KLF6 expression correlates with poor outcomes in lung and prostate cancer (12, 13).

Second, enhanced alternative splicing in a metastatic-derived prostate cancer cell line leads to increased production of the dominant-negative splice variant KLF6 SV1. This isoform antagonizes the ability of wtKLF6 to suppress cell proliferation and tumorigenicity *in vivo*. siRNA-mediated gene silencing experiments suggest that the KLF6 SV1 variant significantly reduces colony formation, migration, invasion, and tumorigenicity. Complementing these studies and suggesting the global importance of these findings to prostate cancer, we have recently shown that the germ line *KLF6* gene IVSΔA polymorphism results in increased prostate cancer risk and increased production of KLF6 SV1 and SV2. Thus, the biological activity of KLF6 SV1 in these studies identifies a possible mechanistic basis for the association of the KLF6 SNP with increased lifetime prostate cancer risk. Indeed, enhanced generation of KLF6 alternative splice forms may contribute to a tumorigenic phenotype independent of either allelic loss, inactivating somatic mutation, or promoter methylation, possibly through a dominant-negative activity on wtKLF6 function. The molecular basis for this effect has yet to be determined.

Similarly, the mechanisms by which the KLF6 family regulates cancer development and progression are still being elucidated. Emerging cell cycle-related mechanisms include up-regulation of the cyclin-dependent kinase inhibitor p21 in a p53-independent manner (2) and disruption of the CDK4/cyclin D1 interaction (10). KLF6 may induce apoptosis and suppress colony formation independent of the p53 tumor suppressor gene (25). Of potential interest, KLF6 and p53 have also recently been shown to physically interact and to cooperate in the transcriptional up-regulation of the insulin-like growth factor-IR gene (27); however, the importance of this interaction in human cancer remains unknown. Combined, these studies highlight not only the general role of KLF6 in cancer pathogenesis but also the mechanisms of its action and regulation on key pathways regulating cell proliferation and angiogenesis in culture and *in vivo*.

The role of dysregulated alternative splicing in disease progression is now being shown in a range of human diseases (28) and cancer in particular (29). Similarly, genomic analysis suggests the existence of cancer-induced splice forms for a number of genes (29). Examples of other known tumor suppressor genes that are alternatively spliced include WT-1 (30), mdm-2

(31), WWOX (32), NF1 (33), Men1 (34), and PTEN (35). In general, dysregulated splicing of many genes and the subsequent generation of alternatively spliced transcripts may reflect a by-product of underlying defects in the genes regulating splice site selection and control and may therefore play little, if any role, in the development of cancer. No reports have suggested biological differences secondary to changes in these genes. In contrast, our findings suggest that cancer-induced/overexpressed splice forms can promote tumorigenesis rather than simply exist as by-products of cancer development.

Although KLF6 alternative splicing is present in both normal and cancerous tissue (14), we suggest that the KLF6 family tumor suppressor gene function is regulated by a critical balance between wild-type and alternatively spliced forms. We show that targeted and specific alterations of this ratio, combined with

specific regulation of KLF6 SV1 levels, have profound effects on many key processes regulating cancer cell growth and metastasis including colony formation, migration, invasion, proliferation, and angiogenesis.

Acknowledgments

Received 1/21/2005; revised 3/11/2005; accepted 4/13/2005.

Grant support: Prostate Cancer Foundation grants (to J.A. Martignetti and S.L. Friedman), DAMD17-02-1-0720 (to J.A. Martignetti and S.L. Friedman), DAMD17-03-1-0129 (to J.A. Martignetti), DAMD17-03-1-0100 (to S.L. Friedman), NIH grant DK37340 (to S.L. Friedman); by grants-in-aid from the T.J. Martell Foundation for Leukemia, Cancer, and Aids Research; and the Manfred Lehmann Cancer Research Foundation (A. Levine).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The authors are grateful to Linda Tringo (Mount Sinai School of Medicine) for technical assistance.

References

- Dhanasekaran SM, Barrette TR, Ghosh D, et al. Delineation of prognostic biomarkers in prostate cancer. *Nature* 2001;412:822-6.
- Narla G, Heath KE, Reeves HL, et al. KLF6, a candidate tumor suppressor gene mutated in prostate cancer. *Science* 2001;294:2563-6.
- Chen C, Hyytinen ER, Sun X, et al. Deletion, mutation, and loss of expression of KLF6 in human prostate cancer. *Am J Pathol* 2003;162:1349-54.
- Reeves HL, Narla G, Ogunbiyi O, et al. Kruppel-like factor 6 (KLF6) is a tumor-suppressor gene frequently inactivated in colorectal cancer. *Gastroenterology* 2004;126:1090-103.
- Kremer-Tal S, Reeves HL, Narla G, et al. Frequent inactivation of the tumor suppressor Kruppel-like factor 6 (KLF6) in hepatocellular carcinoma. *Hepatology* 2004 Nov;40:1047-52.
- Jeng YM, Hsu HC. KLF6, a putative tumor suppressor gene, is mutated in astrocytic gliomas. *Int J Cancer* 2003;105:625-9.
- Chen HK, Liu XQ, Lin J, Chen TY, Feng QS, Zeng YX. Mutation analysis of KLF6 gene in human nasopharyngeal carcinomas. *Ai Zheng* 2002;21:1047-50.
- Bieker JJ. Kruppel-like factors: three fingers in many pies. *J Biol Chem* 2001;276:34355-8.
- Black AR, Black JD, Azizkhan-Clifford J. Sp1 and Kruppel-like factor family of transcription factors in cell growth regulation and cancer. *J Cell Physiol* 2001;188:143-60.
- Benzeno S, Narla G, Allina J, et al. Cyclin dependent kinase inhibition by the KLF6 tumor suppressor protein through interaction with cyclin D1. *Cancer Res* 2004;64:3885-91.
- Muhlbauer KR, Grone HJ, Ernst T, et al. Analysis of human prostate cancers and cell lines for mutations in the TP53 and KLF6 tumour suppressor genes. *Br J Cancer* 2003;89:687-90.
- Glinsky GV, Glinskii AB, Stephenson AJ, Hoffman RM, Gerald WL. Gene expression profiling predicts clinical outcome of prostate cancer. *J Clin Invest* 2004;113:913-23.
- Kettunen E, Anttila S, Seppanen JK, et al. Differentially expressed genes in nonsmall cell lung cancer: expression profiling of cancer-related genes in squamous cell lung cancer. *Cancer Genet Cytogenet* 2004;149:98-106.
- Narla G, Difeo A, Reeves HL, et al. A germline DNA polymorphism associated with increased prostate cancer risk enhances alternative splicing of the KLF6 tumor suppressor gene. *Cancer Res* 2005;65:1213-22.
- Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002;296:550-3.
- Weidner N, Carroll PR, Flax J, et al. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993;401:143-6.
- O'Reilly MS, Boehm T, Shing Y, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997;88:277-85.
- Igawa T, Lin FF, Rao P, et al. Suppression of LNCaP prostate cancer xenograft tumors by a prostate-specific protein tyrosine phosphatase, prostatic acid phosphatase. *Prostate* 2003;55:247-58.
- Chan AM, Miki T, Meyers KA, et al. A new human oncogene of the ras superfamily unmasked by expression cDNA cloning. *Proc Natl Acad Sci U S A* 1994;91:7558-62.
- Albini A, Iwamoto Y, Kleinman HK, et al. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987;47:3239-45.
- Shih SC, Robinson GS, Perruzzi CA, et al. Molecular profiling of angiogenesis markers. *Am J Pathol* 2002;161:35-41.
- Hayward SW, Dahiya R, Cunha GR, et al. Establishment and characterization of an immortalized but non-transformed human prostate epithelial cell line: BPH-1. *In Vitro Cell Dev Biol Anim* 1995;31:14-24.
- Kozlowski JM, Fidler IJ, Campbell D, et al. Metastatic behavior of human tumor cell lines grown in the nude mouse. *Cancer Res* 1984;44:3522-9.
- Yamashita K, Upadhyay S, Osada M, et al. Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. *Cancer Cell* 2002;2:485-95.
- Ito G, Uchiyama M, Kondo M, et al. Kruppel-like factor 6 (KLF6) is frequently downregulated and induces apoptosis in non-small cell lung cancer cells. *Cancer Res* 2004;11:3838-43.
- Kimmelman AC, Qiao RF, Narla G, et al. Suppression of glioblastoma tumorigenicity by the Kruppel-like transcription factor KLF6. *Oncogene* 2004;29:5077-83.
- Rubinstein M, Idelman G, Plymate SR, et al. Transcriptional activation of the insulin-like growth factor I receptor gene by the Kruppel-like factor 6 (KLF6) tumor suppressor protein: potential interactions between KLF6 and p53. *Endocrinology* 2004;145:3769-77.
- Faustino NA, Cooper TA. Pre-mRNA splicing and human disease. *Genes Dev* 2003;15:419-37.
- Venables JP. Aberrant and alternative splicing in cancer. *Cancer Res* 2004;64:7647-54.
- Hastie ND. Life, sex, and WT1 isoforms—three amino acids can make all the difference. *Cell* 2001;106:391-4.
- Sigalas I, Calvert AH, Anderson JJ, Neal DE, Lunec J. Alternatively spliced mdm2 transcripts with loss of p53 binding domain sequences: transforming ability and frequent detection in human cancer. *Nat Med* 1996;8:912-7.
- Driouch K, Prydz H, Monese R, Johansen H, Lidereau R, Frengen E. Alternative transcripts of the candidate tumor suppressor gene, WWOX, are expressed at high levels in human breast tumors. *Oncogene* 2002;21:1832-40.
- Vandenbroucke I, Callens T, De Paep A, Messiaen L. Complex splicing pattern generates great diversity in human NF1 transcripts. *BMC Genomics* 2002;3:13.
- Forsberg L, Zablewska B, Piel F, et al. Differential expression of multiple alternative spliceforms of the Men1 tumor suppressor gene in mouse. *Int J Mol Med* 2001;8:681-9.
- Sharrard RM, Maitland NJ. Alternative splicing of the human PTEN/MMAC1/TEP1 gene. *Biochim Biophys Acta* 2000;1494:282-5.

Targeted Inhibition of the KLF6 Splice Variant, KLF6 SV1, Suppresses Prostate Cancer Cell Growth and Spread

Goutham Narla, Analisa DiFeo, Shen Yao, et al.

Cancer Res 2005;65:5761-5768.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/65/13/5761>

Cited articles This article cites 32 articles, 9 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/65/13/5761.full#ref-list-1>

Citing articles This article has been cited by 15 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/65/13/5761.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.