Roles of KLF6 and KLF6-SV1 in Ovarian Cancer Progression and Intraperitoneal Dissemination

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Abstract Purpose: We investigated the role of the KLF6 tumor suppressor gene and its alternatively spliced isoform KLF6-SV1 in epithelial ovarian cancer (EOC).

Experimental Design: We first analyzed tumors from 68 females with EOC for KLF6 gene inactivation using fluorescent loss of heterozygosity (LOH) analysis and direct DNA sequencing and then defined changes in KLF6 and KLF6-SV1 expression levels by quantitative real-time PCR. We then directly tested the effect of KLF6 and KLF6-SV1 inhibition in SKOV-3 stable cell lines on cellular invasion and proliferation in culture and tumor growth, i.p. dissemination, ascites production, and angiogenesis in vivo using BALB/c nu/nu mice. All statistical tests were two sided.

Results: LOH was present in 59% of samples in a cell type–specific manner, highest in serous (72%) and endometrioid (75%) subtypes, but absent in clear cell tumors. LOH was significantly correlated with tumor stage and grade. In addition, KLF6 expression was decreased in tumors when compared with ovarian surface epithelial cells. In contrast, KLF6-SV1 expression was increased ~5-fold and was associated with increased tumor grade regardless of LOH status. Consistent with these findings, KLF6 silencing increased cellular and tumor growth, angiogenesis, and vascular endothelial growth factor expression, i.p. dissemination, and ascites production. Conversely, KLF6-SV1 down-regulation decreased cell proliferation and invasion and completely suppressed in vivo tumor formation.

Conclusion: Our results show that KLF6 and KLF6-SV1 are associated with key clinical features of EOC and suggest that their therapeutic targeting may alter ovarian cancer growth, progression, and dissemination.

Epithelial ovarian cancer (EOC) is the most lethal of all gynecologic cancers and the fifth most frequent cause of cancer deaths in women (1). Although 5-year survival rates have increased over the past several decades to ~40%, overall mortality rates remain relatively constant (1, 2) largely because most women present late in disease course with widespread intra-abdominal metastasis. Thus, it will be critical to characterize the genetic defects that underlie key steps in the development and spread of ovarian cancer if advances are to be made in diagnosis, prevention, and treatment.

The tumor suppressor KLF6 is a member of the Kruppel-like zinc finger transcription factor family of proteins involved in regulating differentiation, development, cellular proliferation, growth-related signal transduction, and apoptosis (3, 4). KLF6 inactivation has been implicated in several human cancers, including prostate (5, 6), colorectal (7), non–small cell lung (8), gastric (9), astrocytic glioma (10), nasopharyngeal (11), and hepatocellular (12, 13) carcinomas. Furthermore, decreased KLF6 expression is associated with decreased patient survival in prostate (14, 15) and lung (16) cancers, whereas KLF6 overexpression decreases cell proliferation and reverts tumorigenicity in glioblastoma cell lines (17). Depending on cell type and context, the growth-suppressive properties of KLF6 have been associated with several highly relevant cancer pathways, including p53-independent up-regulation of p21 (5), disruption of cyclin D1 and cyclin-dependent kinase 4 interaction (18), induction of apoptosis (8), and c-jun inhibition (19).

Most recently, we have shown that a KLF6 single nucleotide polymorphism associated with increased prostate cancer risk increases alternative splicing of the gene into three...
biologically active, cytoplasmic isoforms, KLF6-SV1, KLF6-SV2, and KLF6-SV3, in both normal and cancerous tissues (20). Splice variant expression is up-regulated in prostate cancer tumors compared with normal prostate tissue (20) and the activity of one of the splice isoforms KLF6-SV1 directly opposes KLF6 effects on cell proliferation, colony formation, invasion, and in vivo tumor growth (21).

In view of these combined findings, we investigated the role of KLF6 and KLF6-SV1 in ovarian cancer. Most EOC tumor samples have KLF6 allelic loss, decreased wild-type expression, and increased expression of the dominant-negative KLF6-SV1 isoform. Targeted reduction of KLF6 in the ovarian cancer cell line SKOV-3 resulted in marked increases in cell proliferation, invasion, tumor growth, angiogenesis, and i.p. dissemination in vitro, modeling late stage EOC. In addition, these changes were associated with marked increases in vascular endothelial growth factor (VEGF) both in culture and in vivo. In contrast, KLF6-SV1 reduction resulted in decreased proliferation, invasion, angiogenesis, and tumorigenicity. Collectively, our results reveal an important association between KLF6 and KLF6-SV1 and the molecular basis of ovarian carcinogenesis.

Materials and Methods

Tumor samples, preparation, and DNA isolation. Tumor specimens were collected and analyzed under institutional review board approval. Histologic diagnosis was validated by pathology review at the University of Iowa Institutional Gynecological Oncology Tumor Board. Tumors were staged in accordance with the International Federation of Gynecology and Obstetrics surgical staging system. Tumor samples were snap frozen at the time of surgery in liquid nitrogen. DNA isolation and preparation techniques have been reported previously (22). Ten additional samples were collected and analyzed from the Regional Medical Center (San Jose, CA). Normal DNA was obtained from microdissected tissue in the region of the tumor or from margins with no evidence of cancer. In all cases, a 5-μm H&E section was used as a histologic reference for normal and tumor-derived tissues. Manual microdissection was done on serial 20-μm sections, and DNA was subsequently extracted using commercial reagents (Ambion, Austin, TX). Normal whole ovarian tissue cDNA was obtained from a commercial resource (Clontech, Mountain View, CA).

Loss of heterozygosity and DNA mutation analysis. Fluorescent loss of heterozygosity (LOH) analysis using genomic DNA from matched normal/tumor ovarian tissue and markers has been described previously (4). The exponential range of the PCR varied between 30 and 38 cycles and was specifically determined for each marker and sample. Data were analyzed using ABI Genescan and Genotyper software packages (Perkin-Elmer, Boston, MA) and allelic loss was scored by two independent observers. A relative allele ratio of ≤0.7, correlating with an allelic loss of ≤ 40%, was defined as LOH (5). Samples that had either loss of at least one of the KLF6-specific markers flanking the KLF6 gene, M1, M2, and M4, or a flanking marker when the contiguous KLF6-specific marker was noninformative were regarded as having LOH. All sample marker combinations were analyzed at least twice.

PCR products were directly sequenced, following purification (QiAquick PCR purification kit, Qiagen, Valencia, CA), and data were analyzed using the program Sequencher (Gene Codes Corp., Ann Arbor, MI). The following sets of intronic primers (sense and antisense, respectively) were used to amplify the coding region and intron/exon boundaries of KLF6 exon 2: forward 5’-CCGGCGAGCAT-GTATCTGTCCTTC-3’ and reverse 5’-CCCTCCAGGGCCTG TGCA-3’. PCR cycling conditions were 94°C (10 minutes) for 1 cycle, 94°C (30 seconds), 55°C (30 seconds), and 72°C (1 minute) for 45 cycles, and a final extension of 72°C (5 minutes).

Real-time PCR analysis. For quantifying target gene expression, RNA isolation from cultured cells and tumor xenografts was done using RNeasy Mini and Midi kits (Qiagen). All RNA was treated with DNase (Qiagen). RNA (1 μg) was reverse transcribed for each reaction using first-strand cDNA synthesis with random primers (Promega, Madison, WI). mRNA levels were quantified by real-time PCR (qRT-PCR) using the following PCR primers on an ABI PRISM 7900HT (Applied Biosystems): KLF6 forward 5’-CCGGCGAGCAGACACAG- GAGAAA-3’ and reverse 5’-CCCTCCAGGGCCTG TC-3’, total KLF6 forward 5’-CTGCCGTTCCTGAGAGCT-3’ and reverse 5’-CCACA-GATCTCTCGCTGTC-3’, glyceraldehyde-3-phosphate dehydrogenase forward 5’-CAAATGACCCCCTTCATGACC-3’ and reverse 5’-GATCT- CGGTCCTGAGAGG-3’, KLF6-SV1 forward 5’-CCCTGCCAGGAGAA- GAGAAA-3’ and reverse 5’-TCCACAGATCCCTCCGTGTC-3’, total KLF6-SV2 forward 5’-TCCGGAGACCCAGAGAA-3’ and reverse 5’-TCCACA-GATCTCTCGCTGTC-3’, KLF6-SV3 forward 5’-CCGGACCCAGACACA- GGTGT-3’ and reverse 5’-TCCACAGATCCCTCCGTGTC-3’, VEGF forward 5’-CCGGCGAGCTGTAATGCTCCT-3’ and reverse 5’-CGG- CTGTCACATCGCAAGTA-3’, and c-myc forward 5’-CAGGCTGTTA- GACGGTTAGT and reverse 5’-ACCCGGTCTGAGCAGAGGCTCAT. Primer sequences for FLI-1, KDR, platelet/endothelial cell adhesion molecule, Tie1, VE-cadherin, and ANG2 have been described previously (23).

All values were calculated by normalizing the levels of each target for each cDNA to glyceraldehyde-3-phosphate dehydrogenase and then using this normalized value to calculate fold change compared with control. KLF6 splicing fold change was calculated by dividing the fold changes in total KLF6 (KLF6 + alternatively spliced KLF6 transcripts) by that in KLF6 alone. All experiments were done in triplicate and repeated three independent times. The expression level of each amplicon was calculated by normalizing each cDNA to glyceraldehyde-3-phosphate dehydrogenase and then using this normalized value to calculate fold change. All experiments were done at least three and each in triplicate. Statistical significance was determined by one-way ANOVA using a Bonferroni correction.

Ovarian surface epithelium gene expression analysis. A set of 10 normal ovarian surface epithelium cytobrushing specimens was obtained from the normal ovaries of donors during surgery for other gynecologic diseases at Brigham and Women’s Hospital according to an institutional review board–approved protocol as described previously (24). Total RNA was subsequently isolated using a RNeasy Micro kit (Qiagen). All purified total RNA specimens were quantified and checked for quality with a Bioanalyzer 2100 System (Agilent, Palo Alto, CA) before further manipulation.

As described previously, two rounds of amplification were used to generate sufficient product from 25 ng ovarian surface epithelial total RNA for Affymetrix (Santa Clara, CA) GeneChip analysis (25). Briefly, total RNA was reverse transcribed using the Two-Cycle cDNA Synthesis kit (Affymetrix) and oligo-dT24-T7 (5'-GCCGCGAGCTAGAGAATAG- TAATAGCAGCTCATACTAGGAGGCGG-3') primer according to the manufacturer’s instructions followed by amplification with the MEGAScript T7 kit (Ambion). After cleanup of the cRNA, second round double-stranded cDNA was generated and amplified using the IVT labeling kit (Affymetrix). cRNA was hybridized to a U133 Plus 2.0 oligonucleotide array (Affymetrix) and stained with phycoerythrin-conjugated streptavidin ( Molecular Probes, Eugene, OR) in a Fluidics Station 450 (Affymetrix). The arrays were then scanned using a confocal laser GeneChip Scanner 3000 with GeneChip Operating Software (Affymetrix) and normalized to a target value of 500 using MAS 5.0. Normalized data were uploaded into the National Cancer Institute Microarray Analysis Database for quality-control screening and collation of KLF6 and housekeeping gene (Gusb, Acta2, Actb, Pph1, and Ppia) signal intensities. The expression values for KLF6 and the housekeeping genes were called present for all of the arrays according to the MAS 5.0 algorithm.

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Cell culture and transfection. SKOV-3 cells were purchased from the American Type Culture Collection (Manassas, VA). Stable cell lines were generated by cotransfection of the pSUPER-siRNA-luciferase (si-Luc), pSUPER-si-KLF6, and pSUPER-si-SV1 with a puromycin expression plasmid and selected with 2 µg/mL puromycin as described previously (21). Polyclonal pools of each small interfering RNA (siRNA)–infected cell lines were collected and KLF6 and KLF6-SV1 knockdown was determined by qRT-PCR and Western blot.

Western blot analysis and densitometric analysis. Cell extracts were harvested in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Santa Cruz, CA; standard protocol). Equal amounts of protein (50 µg) determined by the Bio-Rad DC protein quantification assay, Hercules, CA) were loaded and separated by PAGE and transferred to nitrocellulose membranes. Actin (SC-1616), KLF6 (SC-7158), and VEGF (SC-507) antibodies were obtained from Santa Cruz Biotechnology, c-myc (OP10) antibody was from Oncogene (Carpinteria, CA) and proliferating cell nuclear antigen (M-0879) antibody from DAKO (Carpinteria, CA).

Enhanced chemiluminescent immunoblot images were analyzed by scanning densitometry and quantified with a BIOQUANT NOVA imaging system. Values were expressed as fold change relative to control and normalized to actin as a loading control.

Analysis of proliferation. Proliferation was determined by measuring [3H]thymidine incorporation. The siRNA SKOV-3 stable cell lines containing si-Luc, si-KLF6, or si-SV1 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, Piscataway, NJ) 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% NaOH/0.25% SDS, samples were then neutralized with hydrochloric acid (1 N), and disintegrations per minute were measured by liquid scintillation counting (5).

Invasion assays. Standard invasion assays were done in Boyden chambers using a reconstituted basement membrane (Matrigel, 0.5 mg/mL). Coated membranes were first blocked with 0.5% bovine serum albumin/DMEM and equilibrated in 0.1% bovine serum albumin/DMEM. 10^5 cells in serum-free DMEM were added to the upper chamber and conditioned medium was derived from NIH 3T3 fibroblasts in the lower chamber as a chemoattractant. Following incubation for 19 hours at 37°C, cells in the upper chamber were thoroughly removed by suctioning. Cells that had invaded through the barrier were fixed in 10% formalin and stained with 0.1% Crystal violet.

Histologic grade

Table 1. Clinical profile of patient tumor samples, n (%)

<table>
<thead>
<tr>
<th>Histologic type</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>Epithelial</td>
<td></td>
</tr>
<tr>
<td>Serous carcinoma</td>
<td>46 (68)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>8 (12)</td>
</tr>
<tr>
<td>Mucinous carcinoma</td>
<td>9 (13)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>5 (7)</td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7 (10)</td>
</tr>
<tr>
<td>II</td>
<td>4 (6)</td>
</tr>
<tr>
<td>III</td>
<td>39 (57)</td>
</tr>
<tr>
<td>IV</td>
<td>18 (26)</td>
</tr>
<tr>
<td>Primary residual tumor</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>15 (22)</td>
</tr>
<tr>
<td>≤2 cm</td>
<td>20 (29)</td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>19 (28)</td>
</tr>
<tr>
<td>No data</td>
<td>14 (21)</td>
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and nasopharyngeal (11) carcinomas, no somatic mutations were detected in any of the EOC samples analyzed.

We next explored whether allelic loss was associated with decreased KLF6 mRNA expression. Fifteen of the 26 tumor samples showing LOH that had RNA available for analysis had, on average, an 80% reduction in expression when compared with tumors without LOH ($P < 0.001$) or when compared with normal ovarian tissue ($P < 0.01$; Fig. 1B; average levels of wild-type KLF6 are 0.2 ± 0.11 in LOH samples, 1.2 ± 0.2 in no LOH samples, and 1.5 ± 0.23 in normal ovarian tissues). KLF6 expression levels in tumors without LOH trended lower but were not significantly different when compared with a panel of cDNAs isolated from normal whole ovaries (Fig. 1B). These findings showing

### Table A

<table>
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<tr>
<td>D10S249</td>
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</tr>
<tr>
<td>D10S894</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td></td>
</tr>
<tr>
<td>D10S91</td>
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### Papillary serous adenocarcinoma

<table>
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<tr>
<td>D10S94</td>
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<tr>
<td>M1</td>
<td></td>
</tr>
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<td>M2</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td></td>
</tr>
<tr>
<td>D10S91</td>
<td></td>
</tr>
</tbody>
</table>

### Endometrioid

### Mucinous

### Diagrams

**Fig. 1.** A, LOH status of KLF6. LOH of the KLF6 locus was analyzed using microsatellite markers (Y-axis) from the 10p15 region and KLF6-specific markers KLF6M1, KLF6M2, and KLF6M4, which have been described previously (5). Cases (X-axis) are ranked according to degree of loss with respect to KLF6 and separated by histologic type. Black squares, LOH; gray squares, noninformative; white squares, no evidence of loss; - , a PCR that failed three or more times. Tumors were defined as having LOH if one or all of the markers flanking the KLF6 locus were lost. B, correlation of LOH and RNA levels. KLF6 mRNA levels in 26 samples were analyzed by qRT-PCR and correlated to their respective LOH status. All samples with LOH ($n = 15$) showed ~80% down-regulation in KLF6 mRNA levels when compared with tumors ($n = 9$) without LOH ($P < 0.001$) or when compared with normal whole ovary tissue ($n = 5$; $P < 0.01$). C, KLF6 expression in ovarian surface epithelium (OSE) brushings compared with select housekeeping genes. KLF6 microarray signal intensity values were averaged for 10 ovarian surface epithelium brushings and compared with average expression levels of 5 housekeeping genes: GUSB, ACTA2, ACTB, PPIH, and PPIA. KLF6 expression was comparable with housekeeping gene levels, indicating that KLF6 is reliably expressed in ovarian epithelial cells. D, KLF6 expression was decreased in 62 (82%) tumor samples when compared with normal ovarian surface epithelial cells. The overall decrease was 1.66-fold. Statistical analysis was done using a Student's $t$ test with two-tailed distribution and two-sample equal variance. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 

with LOH relative to normal ovarian tissue and was increased, on average, 5-fold (samples analyzed. Most importantly, KLF6-SV1 expression splice forms. KLF6-SV3 was not detected in any of the cancers. Specifically, KLF6 expression in a set of 10 ovarian surface epithelial cells was compared with its expression across 76 stage III EOC samples by Affymetrix GeneChip analysis (24, 25). As shown in Fig. 1C and D, KLF6 expression was decreased in 62 (82%) tumor samples when compared with ovarian surface epithelial cells and the overall decrease was 1.66-fold (P < 0.05). Taken together, these findings showing KLF6 LOH and/or decreased expression in most samples are consistent with its role as a tumor suppressor in EOC.

**KLF6 gene splicing is up-regulated in ovarian tumors.** Based on our previous identification of increased alternative splicing of the KLF6 gene into biologically active KLF6 alternative splice forms in prostate tumors (20), we next explored whether these variants were also expressed in ovarian tissue and whether KLF6 alternative splicing was dysregulated in EOC. RNA from 33 tumors and a panel of 5 normal ovarian samples were analyzed by qRTPCR using highly specific and sensitive sets of PCR primers for each KLF6 isoform (21). All samples were analyzed by qRT-PCR using highly specific and sensitive sets of PCR primers for each KLF6 isoform (21). All but one of the cancer samples, 32 of 33 (97%), expressed KLF6-SV1 and 25 of 33 (75%) expressed KLF6-SV2 alternative splice forms. KLF6-SV3 was not detected in any of the samples analyzed. Most importantly, KLF6-SV1 expression was increased, on average, 5-fold (P < 0.001) in EOC samples with LOH relative to normal ovarian tissue and ~3-fold in those tumors without LOH (SV1, 0.08 ± 0.04 in normal tissue, 0.22 ± 0.03 in no LOH samples, and 0.58 ± 0.04 in LOH samples). Increased KLF6-SV1 expression was independent of LOH status (P < 0.01; Fig. 2A) and was particularly notable in view of the overall decrease in KLF6 expression in EOC (Fig. 1B).

**KLF6-SV1 overexpression correlates with advanced tumor grade.** We next explored possible correlations between increased KLF6 alternative splicing and EOC clinicopathologic factors, including tumor grade, histology, and International Federation of Gynecology and Obstetrics stage. Our findings suggest that increased KLF6 isoform expression is associated with more aggressive tumors and may be specific to the histologic tumor type. To quantitatively assess the relative amount of splice variants in each tumor sample, we first determined the ‘KLF6 splicing index’: the ratio of all KLF6 transcripts compared with only wtKLF6. We used qRT-PCR primers specific to either conserved or unique regions within the KLF6 transcript such that only KLF6 or both wild-type and variant transcripts (total) were amplified (20). We detected a 2-fold increase in KLF6 splicing index in poorly differentiated grade III tumors compared with well to moderately differentiated grade I or II tumors (P < 0.05; Fig. 2B; average KLF6 splicing index is 1.2 ± 0.06 in grade I/II tumors versus 1.8 ± 0.11 in grade III tumors). Additionally, a 30% increase in KLF6 alternative splicing was detected in stage III/IV tumors when compared with stage I/II, but due to the low number of stage I/II tumors statistical significance was not achieved. Differences in splicing were also evident when comparing serous with nonserous tumors. Serous tumors had a 50% increase in the splicing index (P < 0.05; data not shown).

**Targeted down-regulation of KLF6 and KLF6-SV1 alters cellular proliferation and invasion.** Having identified correlations between KLF6 family members and clinicopathologic variables in patient samples, we next directly explored the biological role of KLF6 and KLF6-SV1 using siRNA-mediated gene silencing to specifically down-regulate their expression. We used the human serous cystadenocarcinoma-derived SKOV-3 cell line, which is capable of inducing i.p. carcinomatosis in xenograft models and generated stable cell lines expressing siRNAs to luciferase (si-Luc), wild-type KLF6 (si-KLF6), and KLF6-SV1 (si-SV1). In their respective cell lines, KLF6 protein decreased expression levels with allelic loss are in accord with previous studies in non–small cell lung cancer wherein 14 of 14 samples with LOH had lower KLF6 expression levels (8).

Finally, given that the vast majority of ovarian cancer is believed to arise from ovarian surface epithelial cells, we also compared the level of KLF6 expression in these cells to EOC. KLF6 expression was decreased in most high-grade ovarian cancers. Specifically, KLF6 expression in a set of 10 ovarian surface epithelial cells was compared with its expression across 76 stage III EOC samples by Affymetrix GeneChip analysis (24, 25). As shown in Fig. 1C and D, KLF6 expression was decreased in 62 (82%) tumor samples when compared with ovarian surface epithelial cells and the overall decrease was 1.66-fold (P < 0.05). Taken together, these findings showing KLF6 LOH and/or decreased expression in most samples are consistent with its role as a tumor suppressor in EOC.

<table>
<thead>
<tr>
<th>Histologic type</th>
<th>Alloic loss of 10p15 (%)</th>
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<tbody>
<tr>
<td>Serous</td>
<td>33/46 (72)*</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>6/9 (75)*</td>
</tr>
<tr>
<td>Mucinous</td>
<td>1/9 (11)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>40/68 (69)</td>
</tr>
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</table>

*Allelic loss of KLF6 was significantly more frequently found in serous and endometrioid carcinoma (P < 0.05).
levels were reduced ~50%, whereas KLF6-SV1 was decreased 75% compared with si-Luc control (Fig. 3A and B).

Our findings show that KLF6 and KLF6-SV1 affect cellular proliferation and invasion of the SKOV-3 cell line. We first examined cellular proliferation differences between these siRNA stable cell lines. In the si-KLF6-expressing cell line, proliferation was increased ~2-fold ($P < 0.001$). Conversely, targeted reduction of KLF6-SV1 resulted in a 25% reduction in cellular invasion compared with control ($P < 0.01$), whereas cells with decreased KLF6 levels showed a ~2.5-fold increase in cellular invasion ($P < 0.001$). Cell number was determined from analysis of four representative visual fields from three independent experiments. Statistical analysis was done using a Student’s $t$ test with two-tailed distribution and two-sample equal variance.

Next we tested the effects of KLF6 and KLF6-SV1 silencing on invasive capacity. Targeted KLF6 reduction resulted in a 2.5-fold increase in invasive capacity ($P < 0.001$) in cellular invasion, whereas KLF6-SV1 showed a 60% decrease in cell invasion ($P < 0.001$). Conversely, targeted reduction of KLF6 levels showed a 2.5-fold increase in cell invasion ($P < 0.001$), whereas cells with decreased KLF6 levels showed a 40% decrease in proliferation ($P < 0.01$), whereas si-KLF6-SV1 cells showed an ~40% decrease in proliferation ($P < 0.01$).

D. invasion capacity in siRNA stable cells. Top, representative photomicrographs of the underside of a Matrigel insert stained with 4',6'-diamidino-2-phenylindole; bottom, number of cells per x400 magnified visual field. Reduction of KLF6-SV1 resulted in a 60% decrease in cell invasion compared with control ($P < 0.01$), whereas cells with decreased KLF6 levels showed a 2.5-fold increase in cellular invasion ($P < 0.001$). Cell number was determined from analysis of four representative visual fields from three independent experiments. Statistical analysis was done using a Student’s $t$ test with two-tailed distribution and two-sample equal variance.

Targeted KLF6 and KLF6-SV1 reduction alters VEGF expression and secretion. Given the striking effects of targeted KLF6 and KLF6-SV1 reduction on in vitro tumor growth, we examined their effect of reducing KLF6 and KLF6-SV1 on VEGF expression and secretion, because VEGF is a critical regulator of angiogenesis and EOC tumor development (26). Differences in all four transcripts encoding monomeric VEGF, excluding isoform 121, were all found to be significant ($P < 0.001$; Fig. 5C). In further support of these findings, a panel of angiogenesis-related genes, consisting of ANG1, ANG2, FLT-1, KDR, TIE1, VE-cadherin, and platelet/endothelial cell adhesion molecule-1, shown previously to more accurately reflect angiogenesis than single marker genes alone (23), were all significantly increased in si-KLF6-derived tumors (Fig. 5D).

KLF6 and KLF6-SV1 directly affect tumor mass and growth rate in vivo. SKOV-3 stable cell lines expressing si-Luc, si-KLF6, or si-SV1 were s.c. injected into nude mice, monitored weekly for tumor growth, and then sacrificed at the end of 6 weeks. Most conspicuously, all mice transplanted with si-SV1 cells failed to form persistent tumors. The small tumors that initially developed regressed after 3 weeks (Fig. 4A). In contrast, reduction of KLF6 doubled tumor growth rate and mass, consistent with its tumor-suppressive functions ($P < 0.01$; Fig. 4A and B). In these tumors, two known markers of cellular proliferation, c-myc and proliferating cell nuclear antigen, were consistently up-regulated ($P < 0.01$; Fig. 4C).

No further experiments could be done on si-SV1-expressing tumors because they completely regressed.
Decreased KLF6 increases i.p. dissemination in vivo. Finally, the role of KLF6 on EOC progression was examined in an in vivo ovarian cancer mouse model that recapitulates advanced-stage disease with i.p. carcinomatosis and ascites production (27, 28). Combined observations on patient samples and experimental studies all suggested that KLF6 loss might contribute to metastasis because it was associated with increasing EOC stage and grade. The SKOV-3 si-Luc or si-KLF6 cell lines were injected into the peritoneal cavity of BALB/c nu/nu mice and tumor growth was followed by weekly measurement of abdominal circumferences. All si-KLF6 mice developed pronounced abdominal swelling from ascites accumulation and i.p. carcinomatosis at 6 weeks post-tumor cell inoculation, at which point animals from both groups were euthanized and tumor burdens were assessed. In the si-KLF6 mice, tumors were present on the peritoneal, intestinal, and diaphragmatic surfaces (Fig. 6A). Average ascites volume was 600 μL (Table 3), with an average VEGF concentration of 870 pg/mL (data not shown). These VEGF levels were comparable with those reported in EOC ascites fluid from patients (29). In marked contrast, no overt metastatic tumors or ascitic fluid collections were observed in the si-Luc control mice at this time point.

Discussion

Our findings in patient samples and experimental models define a high frequency of KLF6 allelic loss, decreased KLF6 expression, and increased expression of the dominant-negative isoform KLF6-SV1 and suggest that dysregulation of KLF6 and KLF6-SV1 may be an important event in the development and spread of EOC. Mounting evidence in other tumors has highlighted a variety of KLF6-inactivating mechanisms relevant to tumor growth and spread. These mechanisms include (a) LOH and somatic mutation in prostate (5, 6), colorectal (7), malignant glioma (10), nasopharyngeal carcinomas (11), HCC (12, 13), and gastric cancer (9); (b) transcriptional...
silencing through promoter hypermethylation in esophageal cancer cell lines (30); and (c) dysregulated alternative splicing in prostate cancer (20). This study is the first to identify loss and dysregulation of KLF6 and KLF6-SV1 in ovarian cancer development. Of particular interest, therefore, is a recent microarray study that associated decreased KLF6 expression with cisplatin resistance in ovarian cancer cell lines (31). Taken together, these results suggest that not only is KLF6 loss a frequent event but also this loss may also be predictive of staging and long-term outcome. Indeed, reports in prostate and lung cancers have identified decreased KLF6 expression as a predictor of survival (14–16). Future studies are therefore warranted to examine this question.

In our study, two of the most common EOC histologic subtypes, serous papillary adenocarcinoma and endometrioid carcinoma, accounting for >70% of all EOC tumors, had the highest LOH frequency, 72% and 75%, respectively. Interestingly, KLF6 was not lost in clear cell samples. This genetic variance between subtypes is consistent with other reports showing differences in p53 mutation/LOH status (32) and gene expression profiles (32–34) between clear cell and the other histologic subtypes.

Our findings also show the importance and prevalence of KLF6-SV1 up-regulation in EOC tumor proliferation. We
showed previously that, unlike KLF6, KLF6-SV1 does not directly up-regulate p21 or suppress cellular proliferation but may antagonize KLF6 function in a dominant-negative manner (20, 21). In ovarian tumors, KLF6-SV1 isoform expression was increased in poorly differentiated, higher-staged serous tumors and this increase correlated with increased KLF6 cytoplasmic staining. Most notably, targeted siRNA-mediated KLF6-SV1 knockdown in highly tumorigenic SKOV-3 cells blocked tumor growth. Therefore, potential treatment of ovarian cancer through targeted inhibition of KLF6-SV1 deserves further attention.

In addition to marked in vitro biological changes, in vivo effects of KLF6 manipulation were also shown and correlated with their effect on angiogenesis. Tumor angiogenesis is an essential determinant for primary and metastatic tumor growth (35, 36) and VEGF is known to act as a potent angiogenic factor with additional important pathophysiologic consequences both in vitro and in vivo (37, 38). Over-expression of VEGF in ovarian cancer correlates with poor survival and metastatic spread (39). Therefore, it is particularly significant that in vivo s.c. tumors expressing si-KLF6 were 2-fold larger, had extensive ascites, expressed 5-fold more VEGF, and, on average, had an 8-fold increase in VEGF concentration. Conversely, in this same nude mouse model system, si-SV1 stable SKOV-3 cells were not tumorigenic and regressed after 3 weeks. One possibility, which would not exclude additional potential effects on proliferation and apoptosis, is that early-forming si-SV1 tumors were unable to sustain continued or increased growth secondary to deficient angiogenic potential. Consistent with this hypothesis, si-SV1 stable cells grown in culture expressed significantly less VEGF (Fig. 5A). Suppression of VEGF expression by KLF6 is potentially important because several clinical studies have shown the effectiveness of inhibiting VEGF-mediated angiogenesis in treating ovarian cancer (3–6, 40).

In contrast to effects of decreased KLF6-SV1, targeted decreases in KLF6 expression result in a more aggressive cancer phenotype. Using a well-characterized genetically engineered model of dissemination, si-KLF6 SKOV-3 tumors were marked by increased i.p. dissemination and ascites formation, mimicking the late-stage features of ovarian cancer. At the 6-week time point, only mice in the si-KLF6 group developed multiple, visible peritoneal tumors with marked VEGF-containing ascites.

In summary, these findings show that the tumor suppressor gene KLF6 and its splice variant KLF6-SV1 play an important role in ovarian cancer development and progression. Ultimately, we believe these findings have clinical relevance in identifying novel pathogenic associations for the KLF6 tumor suppressor family, defining interactions with clinically relevant pathways, and highlighting down-regulation of KLF6-SV1 as a potential therapeutic target.

Table 3. Summary of in vivo tumor growth and ascites accumulation in ovarian cancer mouse models

<table>
<thead>
<tr>
<th>Group</th>
<th>No. mice that form subcutaneous peritoneal dissemination</th>
<th>No. mice with ascites formation mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>si-Luc</td>
<td>9/9</td>
<td>0/5</td>
</tr>
<tr>
<td>si-KLF6</td>
<td>12/12</td>
<td>9/9</td>
</tr>
</tbody>
</table>

References

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