

# Kallikrein 4 Is a Proliferative Factor that Is Overexpressed in Prostate Cancer

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## Abstract

**Kallikrein 4 (KLK4) is a member of the human tissue KLK family. Whereas all other KLKs are secreted proteins with extracellular functions, KLK4 is primarily localized to the nucleus, indicating that it has a different function compared with other members of the KLK family. In addition, KLK4 expression is highly enriched in the prostate and is regulated by androgens. Here, we studied the possible functional role of KLK4 in prostate cancer cells and examined its expression at the protein level in prostate cancer specimens. Consistent with its mRNA expression, KLK4 protein is significantly overexpressed in malignant prostate compared with normal prostate. KLK4 expression is predominantly in the nucleus of basal cells in the prostate epithelium in keeping with its distribution in prostate cancer cells *in vitro*. Furthermore, adenovirus-mediated expression of KLK4 dramatically induces proliferation of prostate cancer cells, at least in part through significant alterations in cell cycle regulatory gene expression. Consistent with these data, small interfering RNA-mediated knockdown of endogenous KLK4 in LNCaP prostate cancer cells inhibits cell growth. These data identify KLK4 as the first member of the KLK family that is a proliferative factor with effects on gene expression and indicate that it may have an important role in prostate cancer development and progression.** [Cancer Res 2007;67(11):5221–30]

## Introduction

Prostate cancer is the most common non-skin cancer among men and is the second leading cause of cancer deaths in western men (1). Androgens play a critical role in the development and maintenance of both the normal prostate as well as in the development and progression of prostate cancer (2). Androgen ablation therapy is, therefore, the main course of treatment for the locally advanced and metastatic disease where most cancers respond by regression; however, they invariably progress to an androgen-independent phenotype for which there is currently no curative treatment available, although palliative options exist. The molecular mechanisms responsible for the effects of androgens on prostate biology and pathophysiology are largely unknown.

Recent studies have begun to identify androgen-regulated and prostate-enriched genes, which started to define the molecular details of androgen action in the prostate and in prostate carcinogenesis. The best known of these genes is *kallikrein 3* (*KLK3*), also known as prostate-specific antigen (PSA), that is a widely used clinical tumor marker for detection and monitoring of prostate cancer progression (3, 4). Another gene that is androgen regulated and highly enriched to prostate for expression is *KLK2*, which may also have utility as a prostate cancer marker in conjunction with PSA (5, 6). *KLK2* may be of particular interest in the discrimination of benign and malignant disease when the PSA levels are low as well as between locally advanced and organ-confined prostate cancer (7). However, the necessity to find additional markers still remains especially because the false-positive and false-negative rates using PSA and *KLK2* are high, and there is a need to differentiate aggressive from non-aggressive forms of prostate cancer.

KLKs, to which PSA and *KLK2* belong, are serine proteases with a high degree of similarity in gene structure and protein sequence. The human tissue *KLK* gene locus consists of 15 genes on chromosome 19q13–4 (8, 9). The proteins encoded by this locus (*KLK1*–*KLK15*) are expressed in a wide array of tissues, and many of them are current or emerging biomarkers for a variety of hormone-dependent cancers such as ovarian, breast, testis, and prostate cancer (for reviews, see refs. 10–12). *KLK4*, a more recently identified member of the KLK family, is a highly prostate-enriched gene that is also androgen regulated (13–17). The *KLK4* gene encodes several splice isoforms (16, 18–20), and it is now clear that it gives rise to at least eight different mRNA forms through alternative splicing and/or alternative transcription start sites (21). These transcripts are expected to encode at least seven different protein moieties (21). However, only two encoded proteins have been identified to date: one intracellular, predominantly nuclear form (17, 22), and one cytoplasmic, secreted form (22–24).

Previous studies from our laboratory (17) and others (25) have shown that *KLK4* mRNA is expressed both in normal and tumor prostate samples and is significantly overexpressed in prostate cancer. However, expression of *KLK4* protein in prostate cancer cell lines or tissue samples remains poorly documented. There have been two previous studies of secreted *KLK4* in limited number of tissues and biological fluids (23, 25), suggesting the existence of a secreted, full-length form of *KLK4* in prostate tissue and seminal fluid. *KLK4* expression in benign and malignant prostate tissue has also been examined in two studies, reaching different conclusions (23, 24). It is therefore still unclear whether there are differences in *KLK4* expression in normal prostate compared with prostate carcinoma.

In this study, we show that *KLK4* protein is expressed predominantly in the basal cells of the prostate epithelium and is significantly overexpressed in prostate carcinoma compared with

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benign prostate epithelium. Ectopic expression of KLK4 in prostate cancer cell lines dramatically increased proliferation and augmented cell motility. In addition, overexpression of KLK4 had significant effects on cell cycle-related gene expression. Furthermore, small interfering RNA (siRNA)-mediated knockdown of endogenous KLK4 in LNCaP cells clearly inhibited cellular proliferation. Taken together, these data strongly suggest that KLK4 has a proliferative effect on prostate cancer cells, possibly through cell cycle regulation. Given that KLK4 is overexpressed in prostate cancer, these data suggest that it may be a central player in the development and progression of prostate cancer.

## Materials and Methods

**Specimen preparation and immunohistochemistry.** The prostate tissue microarrays used to study KLK4 expression and the anti-KLK4 antiserum have been described previously (17).

The prostate tissue microarrays were first deparaffinized by xylene and serial ethanol dilutions. Antigen retrieval was done by autoclaving at 121 °C for 5 min in 0.01 mol/L citrate buffer (pH 6.4). The DAKO Envision System AP (DAKO) or the Supersensitive Detection kit (Biogenex) was used for antigen detection. Briefly, sections were equilibrated in TBS-Tween [0.05 mol/L Tris (pH 7.5), 0.3 mol/L NaCl, 0.1% Tween 20] for 10 min followed by blocking with 1% bovine serum albumin (BSA) in TBS. Rabbit anti-KLK4 antibody was applied at a concentration of 3 µg/mL in the blocking solution. After incubation for 30 min at room temperature, sections were washed by TBS-Tween thrice before applying the alkaline phosphatase-labeled polymer (DAKO) or horseradish peroxidase-labeled polymer (Biogenex) for 30 min at room temperature. Sections were washed again followed by application of the fast red substrate-chromogen (DAKO) or 3,3'-diaminobenzidine (DAB) chromogen (Biogenex). The counterstain was done with hematoxylin (DAKO), and the slides were mounted with DAKO Faramount aqueous mounting medium. Pre-immune serum from the same rabbit in which the antiserum was raised was applied as negative control, which resulted in no specific staining (data not shown).

**Immunohistochemistry scoring and statistical analysis.** SPSS 10.0 software was used for statistical analysis. To compare the expression level of KLK4 between benign and malignant tissue, Mann-Whitney test was applied. The Kruskal-Wallis test was used for analysis of correlation between KLK4 expression and tumor grade.

**Adenovirus expression system.** An adenovirus vector expressing a His-tagged KLK4 under a tetracycline-inducible promoter was created. KLK4 cDNA starting from an inserted ATG start site in the second exon of *KLK4* mRNA was cloned into a pcDNA4-HisMax vector. The cloned cDNA encodes KLK4 protein with methionine in the place of the starting glutamine of the peptide used for immunization (protein NH<sub>2</sub>-terminal sequence: MIINGEDCSPHSQPW). The KLK4 cDNA was made by PCR amplification with Taq polymerase from pCRII-K4 (full-length KLK4 cDNA) with the following primers: forward 5'-ATGATCATAAACGGCGAGAGACTG-3' and reverse 5'-TTAACTGGCCTGGACGGTTTTCTC-3'. The PCR product was cloned into pcDNA4-HisMax by TOPO cloning (Invitrogen). The plasmid was cut by *Hind*III and *Eco*RV, and the 976-bp fragment was cloned into the same sites in the pTRE-tight vector (BD Biosciences). A 1,575-bp fragment containing KLK4 cDNA with His-tag, Xpress-tag, and polyadenylated tail was cut from pTRE-tight-KLK4 by *Xho*I and cloned into the *Xho*I site of the AdEasy shuttle vector. The resulting AdEasy-shuttle-KLK4 vector was transformed into competent bacteria already containing the Tet-inducible viral vector AdEasy-Tet-Rev. The AdEasy-Tet-Rev-containing bacteria and the AdEasy shuttle vector were kindly provided by Göran Akusjärvi (University of Uppsala). The KLK4 fragment of AdEasy-KLK4 was inserted into the AdEasy-Tet-Rev vector by bacterial recombination. The resulting viral vector expressing His-Xpress-KLK4 under a Tet-inducible promoter was then linearized with *Pac*I and transfected into 293T cells using Lipofectamine according to the manufacturer's recommendation (Invitrogen). Five to 7 days after transfection, viral plaques were collected. Recombinant virus was verified by PCR and restriction enzyme cleavage by

Hirt-extracted viral DNA (26). One plaque was selected for production of a high-titer stock in 293T cells. The virus producing cells were collected and lysed, and the virus lysate (Ad-KLK4) was stored at -80 °C until further use. The titer of Ad-KLK4 was determined by DAB staining using Fast DAB tablets according to the manufacturer's recommendation (Sigma).

**Protein extraction and Western analysis.** At the desired time points, cells were harvested, and whole-cell extract was prepared and subjected to Western analysis as described previously (17). The primary antibodies used were mouse monoclonal anti-His (Invitrogen; 1:5,000), mouse monoclonal anti-tubulin (Sigma; 1:1,000), rabbit polyclonal KLK4 (1:600), and mouse monoclonal anti-proliferating cell nuclear antigen (PCNA; Cell Signaling; 1:2,000).

**Proliferation assays.** The human prostate cell lines LNCaP, PC-3, and DU145 were all obtained from the American Type Culture Collection and were routinely maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 5 mg/mL penicillin/streptomycin, and 200 mmol/L L-glutamine (Life Technologies, Inc.).

The cells were seeded in 10-cm tissue culture plates, and the medium was changed to RPMI 1640 supplemented with 2% FBS just before infection; 6 µL (~1 × 10<sup>6</sup> ifu) of the 293T viral cell lysate Ad-KLK4 (see above) were added to the cells and incubated at 37 °C for 3 to 4 h before the medium was replaced by fresh medium supplemented with 10% FBS. Doxycycline was then added to the cells at a concentration of 1 µg/mL to induce expression of KLK4, or cells were left untreated. The day after infection, cells were split into six-well plates at a density of 2,500 per well for the colony formation assay. Cells were cultured for about 3 weeks with replenishing of the medium and doxycycline every 2 days until colonies were formed, which were then stained with crystal violet. The area covered by the colonies was quantified using GeneTools software from SynGene. For proliferation rate assays, the cells were split at a density of 50,000 per well after infection, and total cell number was counted every 2 days.

**Cell motility assay.** PC-3 cells were infected with Ad-KLK4 as described above. One day after infection, the cells were resuspended in growth medium with 0.1% BSA and seeded into Transwell membrane inserts (Corning) in 24-well plates. Growth medium with 20% serum was added to the wells under the inserts. The cells were grown in the absence or presence of 1 µg/mL doxycycline as for the proliferation assays. Four days after infection, the cells were fixed and stained according to the manufacturer's recommendations. The stained cells on the membrane inserts were imaged and quantified by using GeneTools software from SynGene.

**Cell cycle oligonucleotide array.** A cell cycle-specific oligonucleotide array (Oligo GEArray, SuperArray) representing 96 genes involved in cell cycle regulation and progression was used according to the manufacturer's recommendation. The final signals were acquired by Typhoon 9000 scanner (Perkin-Elmer) and by exposure to X-ray film (Kodak). ImageQuant software (Perkin-Elmer) and GEArray Expression Analysis Suite (SuperArray) were used for quantification. The signals were normalized to β-actin levels on the same array.

**Quantitative reverse transcription-PCR.** Cells were harvested at different time points after infection with Ad-KLK4 or transfection with siRNA and subjected to RNA extraction using an Absolute RNA Microprep kit (Stratagene) with DNase treatment. The RNA concentration was measured by spectrophotometric analysis (Lambda 25 UV/VIS spectrometer, Perkin-Elmer), and RNA quality was verified by gel electrophoresis. The RNA was used for cDNA synthesis with Superscript reverse transcriptase (Invitrogen). The cDNA was used as template in quantitative PCR using SYBR green dye and LightCycler machine (Roche). The sequences of the primers used are available upon request. The housekeeping gene *TBP* was used for normalization of the data.

**siRNA.** Synthetic siRNAs specific for KLK4 (target sequence 5'-AATCCGTGCCGAGTCTGAC-3') and luciferase (target sequence: 5'-AACTTACGCTGAGTACTCGA-3') were obtained from Qiagen. Two days before transfection, LNCaP cells were seeded in six-well plates in full medium. Just before transfection, the medium was changed with RPMI 1640 without antibiotics and FBS, and the cells were transfected with 100 nmol/L siRNA per well using the Oligofectamine reagent (Invitrogen) according to the manufacturer's recommendation. The day after transfection, RPMI 1640

with 20% FBS was added to the cells. Cells were harvested at different time points after transfection for RNA or protein extraction, or assayed for proliferation as described above.

**Results**

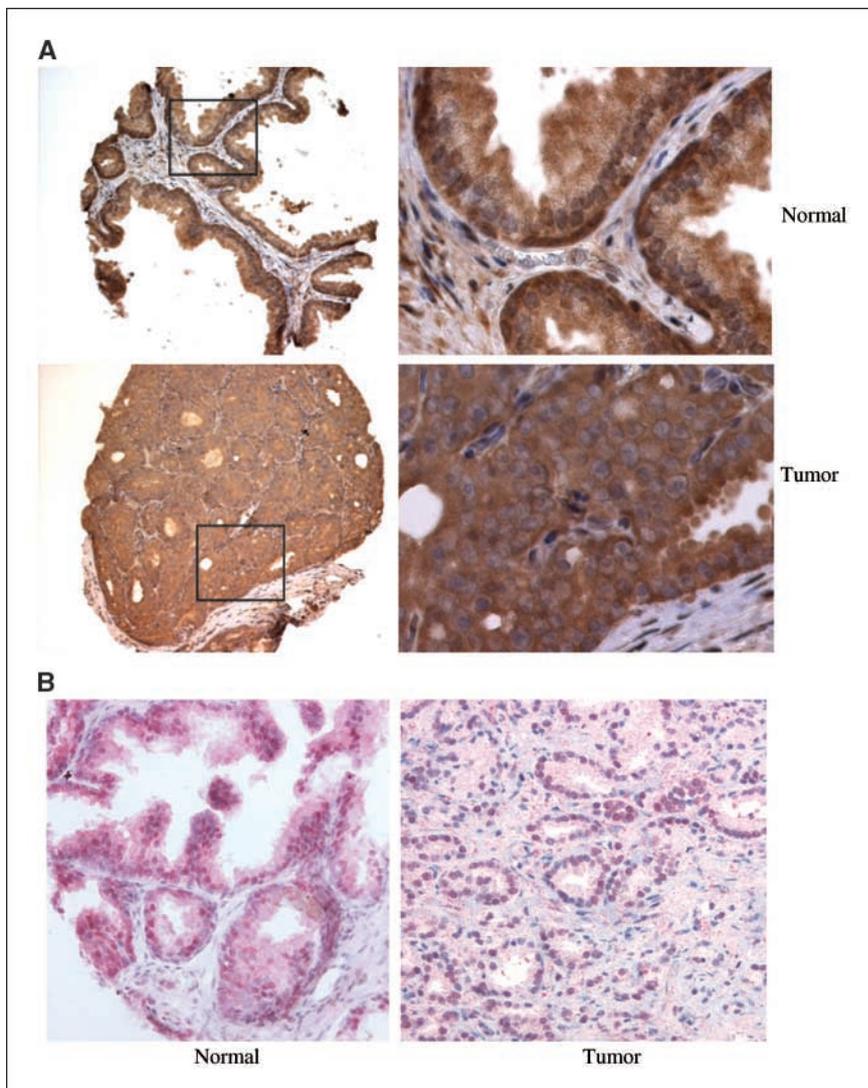
**KLK4 is overexpressed in malignant prostate carcinoma.** To date, the studies that examined KLK4 expression in normal compared with cancerous prostate used limited number of tissue samples and resulted in conflicting findings (23, 24). We therefore examined KLK4 expression on a tissue microarray that contained benign ( $n = 42$ ) and malignant prostate tissue ( $n = 207$ ) by immunohistochemical analysis. The tissue microarray included samples representing various stages of prostate cancer progression and normal prostate glands, and a well-characterized anti-KLK4 antibody was used for the analysis (17).

As shown in Fig. 1, in normal prostate tissue, KLK4 protein was expressed solely in epithelial cells. KLK4 staining was predominantly nuclear, with some cytosolic staining in normal prostate epithelial cells, which was clearly seen among the slides mounted without hematoxylin counterstaining (Fig. 1B). The nuclear staining of KLK4 in prostate tissue is consistent with our previous findings obtained in prostate cancer cell lines, either endogenous

KLK4 in LNCaP cells, or ectopically expressed KLK4 in DU145 cells (17). Basal cells, in general, showed higher KLK4 levels compared with the luminal cells. This is in agreement with the mRNA *in situ* hybridization results, which showed predominantly basal cell localization of *KLK4* mRNA in prostate tissue (17). Together, these data establish KLK4 as a predominantly nuclear protein expressed primarily in the basal cells of the prostate epithelium, suggesting a dramatically different function compared with other members of the KLK family.

To better compare KLK4 expression between normal and neoplastic prostatic epithelium, we quantified KLK4 levels based on the methodology described previously (27). The mean staining intensity in neoplastic prostatic epithelium was significantly higher compared with normal prostatic epithelial cells (Table 1;  $P < 0.001$ , Mann-Whitney test). Intensity scoring was also carried out to investigate whether there is an association between KLK4 expression and tumor grade (Gleason score). Consistent with our previous mRNA *in situ* hybridization results, done on a different cohort of patients (17), KLK4 expression was not significantly different among different tumor grades (data not shown;  $P = 0.178$ , Kruskal-Wallis test). These data establish that KLK4 is overexpressed in prostate cancer,

**Figure 1.** KLK4 expression in normal and malignant prostate. Tissue microarrays with normal and tumor glands from human prostate were stained with KLK4 antiserum and detected by Supersensitive Detection kit (A) or DAKO Envision System AP (B). KLK4 expression was predominantly observed in the basal cells of the prostate epithelium, with stronger staining in tumor glands. The expression was predominantly nuclear (B). Two different magnifications in (A).



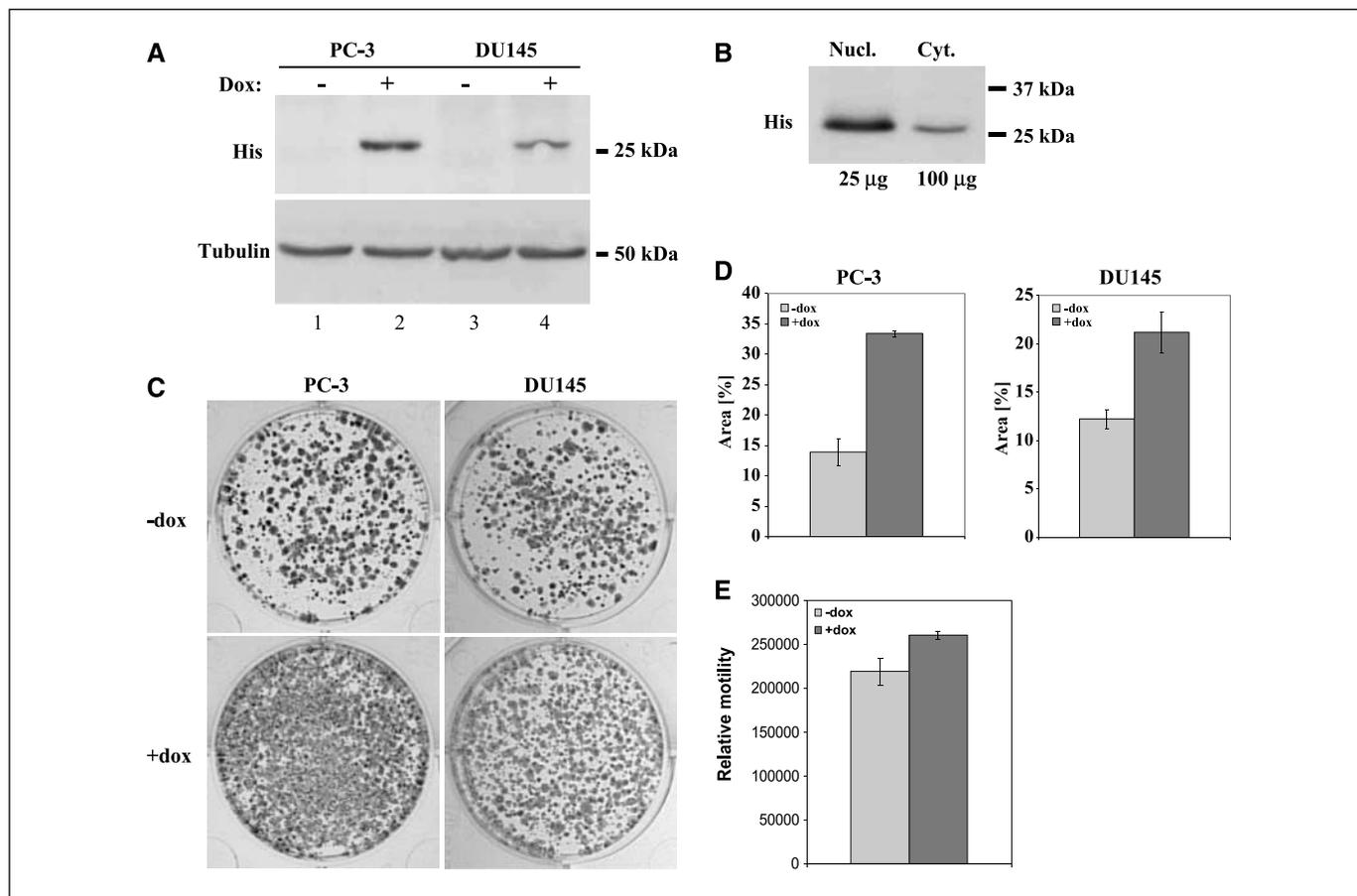
**Table 1.** KLK4 expression is higher in malignant prostate glands compared with benign glands ( $P < 0.001$ )

	Intensity score				Total
	0	1	2	3	
Benign	14	20	8	0	42
Malignant	22	57	88	40	207

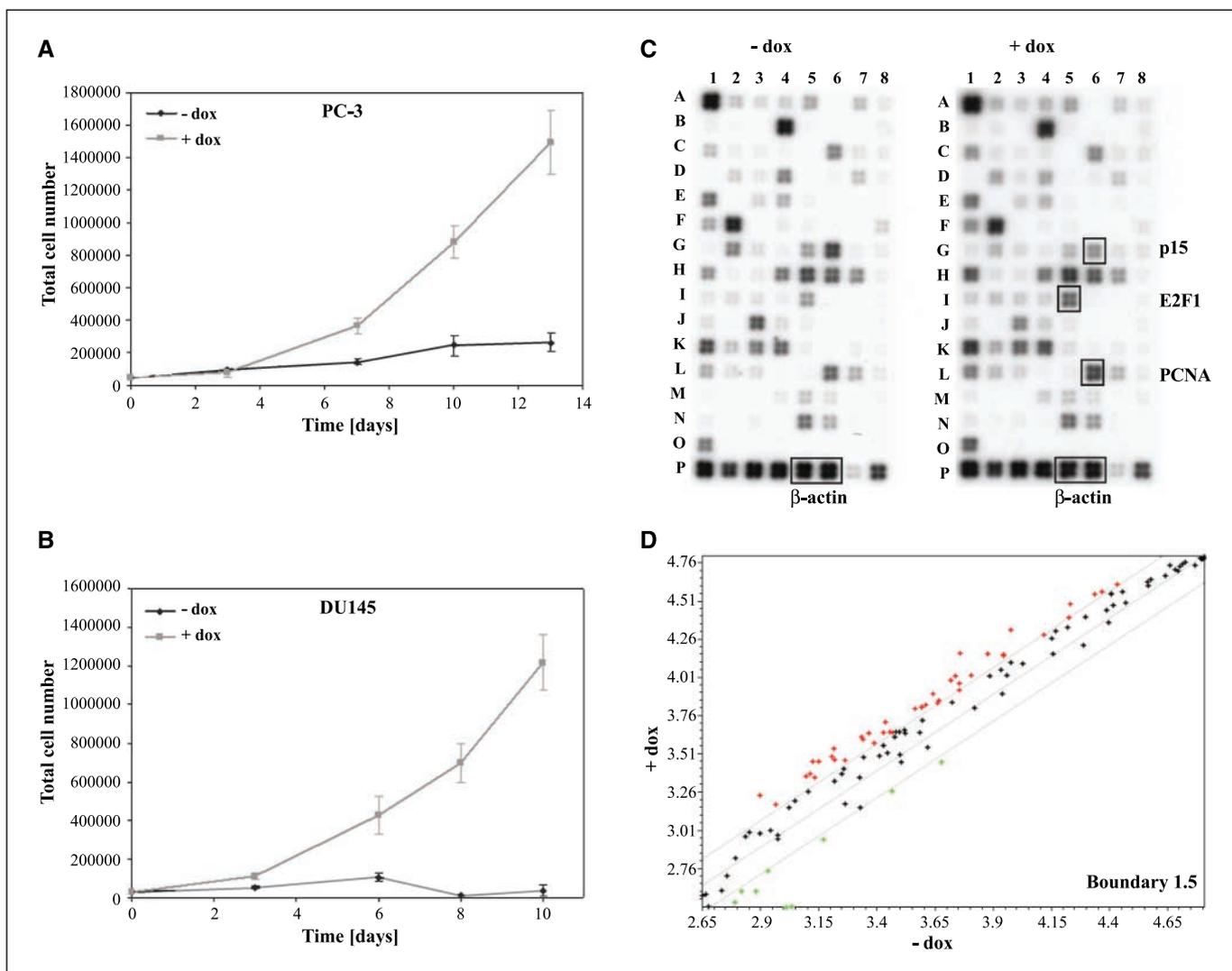
NOTE: Tissue microarrays with benign ( $n = 42$ ) and malignant ( $n = 207$ ) glands from human prostate were evaluated for KLK4 staining by immunohistochemical analysis with anti-KLK4 antiserum. A pathologist scored the intensity of staining in the tissue microarrays, and the data were analyzed using the Mann-Whitney test. The number of glands for each intensity score (0–3) for benign and malignant glands are shown.

with no significant differences in expression levels among well- or poorly differentiated tumors.

**Ectopic expression of KLK4 increases colony formation in prostate cancer cells.** The overexpression of KLK4 in prostate cancer compared with normal prostate suggests that it has a role in the development and progression of prostate cancer. One possible role of KLK4 in this regard is to act as a proliferative factor because increased rate of proliferation is a general characteristic of cancer cells. We addressed this possibility by using adenovirus-mediated ectopic expression of KLK4 in the prostate cancer cell lines PC-3 and DU145, which are KLK4 negative (17). An inducible adenovirus vector for KLK4 expression was generated and used to make adenovirus-expressing KLK4 (Ad-KLK4). This was then used to infect cells that were either left untreated or treated with doxycycline to induce KLK4 expression. Cells were harvested, and KLK4 expression was assessed by Western analysis. As shown in Fig. 2A, there was no KLK4 expression in the absence of doxycycline in PC-3 (*lane 1*) or DU145 cells (*lane 3*). Upon addition



**Figure 2.** KLK4 expression increases colony formation and motility of prostate cancer cells. *A*, an adenovirus system (see Materials and Methods) was used to ectopically express His-tagged KLK4 in PC-3 (*lanes 1* and *2*) and DU145 (*lanes 3* and *4*) cells. KLK4 expression was induced by the addition of doxycycline (+*dox*), and whole-cell protein extracts were subjected to Western analysis with an anti-His antiserum. Tubulin was used as loading control. Molecular weight markers (*right*). *B*, nuclear localization of KLK4 expressed in PC-3 cells. Nuclear (*Nucl.*) and cytoplasmic (*Cyt.*) extracts from KLK4-expressing PC3 cells were subjected to Western analysis with an anti-His antiserum. Molecular weight markers (*right*). *C*, KLK4 expression increases colony formation in PC-3 and DU145 cells. PC-3 and DU145 cells expressing (+*dox*) or not expressing (–*dox*) KLK4 were seeded in six-well plates and assayed for colony formation ability by crystal violet staining after 3 wks of growth. The experiment was done in triplicate and repeated twice with similar results. *D*, quantification of colony formation. The area covered by the colonies in (*C*) was quantified by GeneTools (SynGene). The experiment was done in triplicate and repeated at least twice with similar results. *Columns*, area; *bars*, SD.  $P < 0.05$ , compared with non-expressing cells (–*dox*), assessed by Student's *t* test. *E*, increased motility of KLK4-expressing PC-3 cells. PC-3 cells infected with KLK4-expressing adenovirus were seeded onto Transwell membrane insert in the absence or presence of doxycycline. Four days after infection, the membranes were stained with hematoxylin and quantified by GeneTools (SynGene). The experiment was done in triplicate and repeated at least twice with similar results. *Columns*, relative motility; *bars*, SD.  $P < 0.05$ , compared with non-expressing cells (–*dox*), assessed by Student's *t* test.



**Figure 3.** KLK4 increases proliferation of PC-3 and DU145 cells and affects expression of cell cycle regulatory genes. An adenovirus system (see Materials and Methods) was used to ectopically express His-tagged KLK4 in PC-3 (A) and DU145 (B) cells. At different time points after infection, the cells were counted, and the proliferation curves of KLK4-expressing cells (+dox) was compared with the curves of non-expressing cells (-dox). The experiment was done in triplicate and repeated at least twice with similar results. Points, total cell number; bars, SD. C, PC-3 cells were treated as in (A), and 10 d after infection, the cells were harvested, and total RNA was extracted. cDNA was made by reverse transcription, used for biotin-labeled cRNA synthesis that was used to probe Cell Cycle Oligonucleotide Arrays (see Materials and Methods). The expression profile of KLK4-expressing cells (+dox) was compared with the expression profile of non-expressing cells (-dox). Spots containing oligonucleotides for  $\beta$ -actin were used as loading control, and some of the genes showing different expression in the two arrays are indicated. The experiment was repeated once with similar results. D, GEArray Expression Analysis Suite software (SuperArray) was used to analyze the data in (C). A boundary of 1.5 was set in a scatterplot analysis. Genes with a >1.5-fold up-regulation (red spots) and genes with a >1.5-fold down-regulation (green spots).

of doxycycline, significant levels of KLK protein accumulated in both cell lines (Fig. 2A, lanes 2 and 4). Furthermore, adenovirus-expressed KLK4 was predominantly localized to the nucleus as shown by cellular fractionation (Fig. 2B), consistent with the intracellular localization of endogenous KLK4 (17).

We then determined whether ectopic KLK4 expression affects colony formation ability of PC-3 and DU145 cells. As shown in Fig. 2C and D, KLK4 expression significantly increased colony formation of PC-3 and DU145 cells (2.5- and 1.7-fold, respectively) compared with non-expressing cells ( $P < 0.05$ ).

To assess whether KLK4 affects cell motility, another variable often changed in cancer cells (28, 29), Ad-KLK4-infected PC-3 cells were seeded onto Transwell inserts, allowed to grow for 4 days in the presence or absence of doxycycline, and thereafter fixed and stained, and the amount of cells in the membrane was

quantified. Expected expression of KLK4 in response to doxycycline was confirmed by Western analysis (data not shown). As shown in Fig. 2E, there was a significant increase (18%) in the motility of the cells expressing KLK4 compared with non-expressing cells ( $P < 0.05$ ).

**KLK4 expression increases proliferation rate of PC-3 and DU145 cells.** To more directly assess the possible effect of KLK4 on cellular proliferation, PC-3 and DU145 cells were assayed for growth rates in the presence or absence of KLK4 expression (Fig. 3). As shown in Fig. 3A, KLK4 expression dramatically increased the rate of proliferation of PC-3 cells compared with non-expressing cells starting at about day 6 and reaching 10-fold higher cell numbers at day 14. Similar results were obtained in DU145 cells where the differences in growth began as early as 3 days and reached more than a 20-fold difference in cell numbers at day 10

(Fig. 3B). These data indicate that KLK4 has a direct effect on prostate cancer cell growth.

**KLK4 affects cell cycle-related gene expression.** The colony formation and cellular proliferation assays presented above showed that KLK4 increases cellular proliferation in prostate cancer cell lines. However, it is not clear how KLK4 may exert this effect. KLK4 expression is regulated by androgens (16, 17), and it is known that the androgen receptor (AR) stimulates cell cycle progression in prostate cancer cells (30, 31). However, how AR exerts this effect remains unclear. It has previously been shown that androgens, through AR, can increase the expression of G1 cyclins and cyclin-dependent kinases (CDK) and decrease the expression of CDK inhibitors (32–36). Other cell cycle-related genes, such as the CDK inhibitor *p21*, has actually been shown to be a direct target for AR (37). It is also possible that the proliferative effect of AR is not direct, but indirect, possibly through androgen-regulated genes such as *KLK4*.

To examine the latter possibility, we used a cell cycle-specific oligonucleotide array to screen for genes that were up-regulated or down-regulated in response to KLK4 expression. Biotin-labeled cRNA from PC-3 cells expressing KLK4, harvested 10 days after infection, was used to probe arrays that contained oligonucleotide probes for 96 different genes involved in cell cycle regulation and compared with arrays probed with cRNA made from non-induced cells (Fig. 3C). As before, the expression of KLK4 in adenovirus-infected PC-3 cells was confirmed by Western analysis, and the positive effect on proliferation was confirmed by proliferation assay (data not shown). The intensity of the different spots were quantified and normalized to  $\beta$ -actin levels at the same array (indicated at the array in Fig. 3C). The genes that were up-regulated or down-regulated >1.5-fold were identified as shown in the scatterplot analysis in Fig. 3D. Some of the highly regulated genes are summarized in Table 2. Of interest, several proliferative factors, such as cyclin B1 and E2F1, were clearly up-regulated as well as the proliferation markers PCNA and Ki67. CDK inhibitors p21 (Cdk2B), p15 (Cdk1A), and p16 (Cdk1B) that are involved in cell cycle inhibition were markedly down-regulated in response to KLK4 expression. Similar results were obtained when cRNA from cells collected at 5, 7, or 13 days after infection was used (data not shown). These results suggest that the proliferative effect of KLK4 expression on prostate cancer cells is, at least in part, through the regulation of cell cycle.

To confirm the expression data obtained in the array analysis, the change in expression of some of the genes listed in Table 2 were tested by quantitative reverse transcription-PCR (RT-PCR). As shown in Fig. 4A, quantitative RT-PCR results largely confirmed the regulation pattern observed on the arrays. Although the exact fold induction varied somewhat between the array and the quantitative RT-PCR, the fold inductions were in the same range. We also determined the regulation pattern of the same genes in samples harvested at 5, 7, and 13 days after infection. Although the down-regulated genes (*cyclin H*, *p15*, *p16*, *p21*, and *GADD45*) all behaved relatively similar at all four time points (data not shown), some of the up-regulated genes were regulated more significantly at earlier time points (Fig. 4B). In particular, *cyclin B1*, *E2F1*, and *Ki67* were strongly up-regulated at 7 days, with 10-, 7-, and 10-fold up-regulation, respectively. The up-regulation of the proliferation marker PCNA was also observed at the protein level (Fig. 4C). Protein extract from cells harvested 11 and 14 days after infection were subjected to Western analysis with a PCNA-specific antibody, and the level of PCNA was increased in the KLK4-expressing cells

**Table 2.** Cell cycle-related genes regulated by ectopic expression of KLK4 in PC-3 cells

Array position	Gene	Protein	Fold regulation
Up-regulated genes			
C1	<i>CCNB1</i>	Cyclin B1	2.9
C2	<i>CCNB2</i>	Cyclin B2	2.6
D2	<i>CCNG1</i>	Cyclin G1	2.7
H1	<i>CDKN3</i>	Cdk3	2.4
H2	<i>CHEK1</i>	CHK1 checkpoint homologue	2.3
I1	<i>CUL4A</i>	Cullin 4A	2.3
I4	<i>DNM2</i>	Dynamin 2	2.4
I5	<i>E2F2</i>	E2F1	1.8
L2	<i>MKI67</i>	Ki67 antigen	2.3
L6	<i>PCNA</i>	Proliferating cell nuclear antigen	1.7
M4	<i>RAD9A</i>	RAD9 homologue 1	2.0
Down-regulated genes			
D4	<i>CCNH</i>	Cyclin H	0.42
E4	<i>CDC34</i>	Cell division cycle 34	0.74
F8	<i>CDK7</i>	CDK7	0.71
G2	<i>CDKN1A</i>	Cdk1A (p21)	0.58
G5	<i>CDKN2A</i>	Cdk1A (p16)	0.65
G6	<i>CDKN2B</i>	Cdk1B (p15)	0.46
J3	<i>GADD45A</i>	Growth arrest and DNA damage-inducible- $\alpha$	0.58

NOTE: Each of the signals on the Cell Cycle Oligonucleotide Arrays in Fig. 3C were quantified [ImageQuant software (Perkin-Elmer) and GEArray Expression Analysis Suite (SuperArray)] and normalized to  $\beta$ -actin levels at the same array. The fold regulation for each gene was calculated by dividing the results from the KLK4-expressing cells (+*dax*) by the results for the non-expressing cells (–*dax*). The fold regulation for a selection of genes is given, together with their position on the array (see Fig. 3C) and their protein name.

both at 11 days (Fig. 4C, compare lanes 1 and 2) and at 14 days (compare lanes 3 and 4).

**siRNA-mediated knockdown of endogenous KLK4 inhibits proliferation of LNCaP cells.** To investigate if the effect of KLK4 expression on cellular proliferation also applied to endogenous KLK4, siRNA was used to knock down the expression of KLK4 in LNCaP cells, an androgen-responsive cell line in which KLK4 is expressed (17, 22). LNCaP cells were either transfected with luciferase siRNA as control, or with siRNA directed against KLK4. As shown in Fig. 5, KLK4-specific siRNA treatment effectively eliminated KLK4 expression at both the mRNA (Fig. 5A) and protein (Fig. 5B) levels. Of interest, it can be noted that there was no significant knockdown of KLK4 protein at 4 days after transfection, but only after 6 days (Fig. 5B, compare lanes 3 and 6). This is in contrast to the knockdown at the mRNA level, which is significant already 3 days after transfection (Fig. 5A). This suggests a high stability of the KLK4 protein, which was indeed observed in a run-and-chase experiment with metabolically labeled KLK4 that indicated a protein half-life of ~2.5 days (data not shown).

Proliferation analysis under conditions where KLK4 level is decreased by siRNA showed that KLK4 is required for cellular proliferation of LNCaP cells (Fig. 5C). At 6 days after transfection,

the number of cells transfected with KLK4 siRNA was 60% less than the number of control cells. Interestingly, the knockdown of KLK4 corresponds with a reduction in the level of PCNA (Fig. 5B, middle), which correlated well with the reduced cell proliferation. Coupled to the increase in proliferation when KLK4 is ectopically expressed, these data strongly suggest that KLK4 is a proliferative factor in prostate cancer cells.

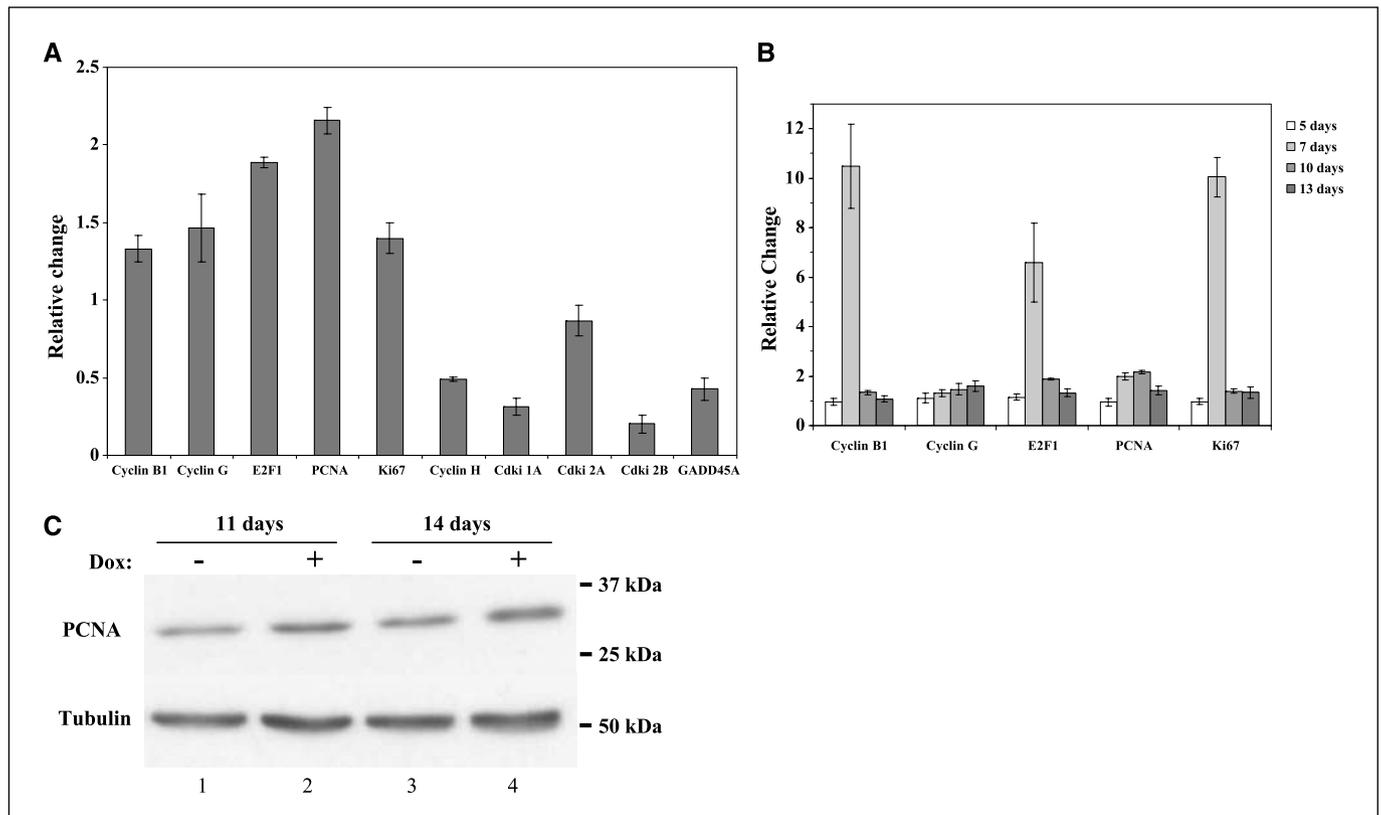
**Discussion**

**KLK4 is overexpressed in prostate cancer.** The immunohistochemical data that we provide here show that KLK4 expression is increased in prostate cancer compared with normal prostate; this is in agreement with our previous findings at the mRNA level (17). Despite a clear increase in cancer tissue compared with normal prostate, there was no significant difference in KLK4 expression among different grade tumors.

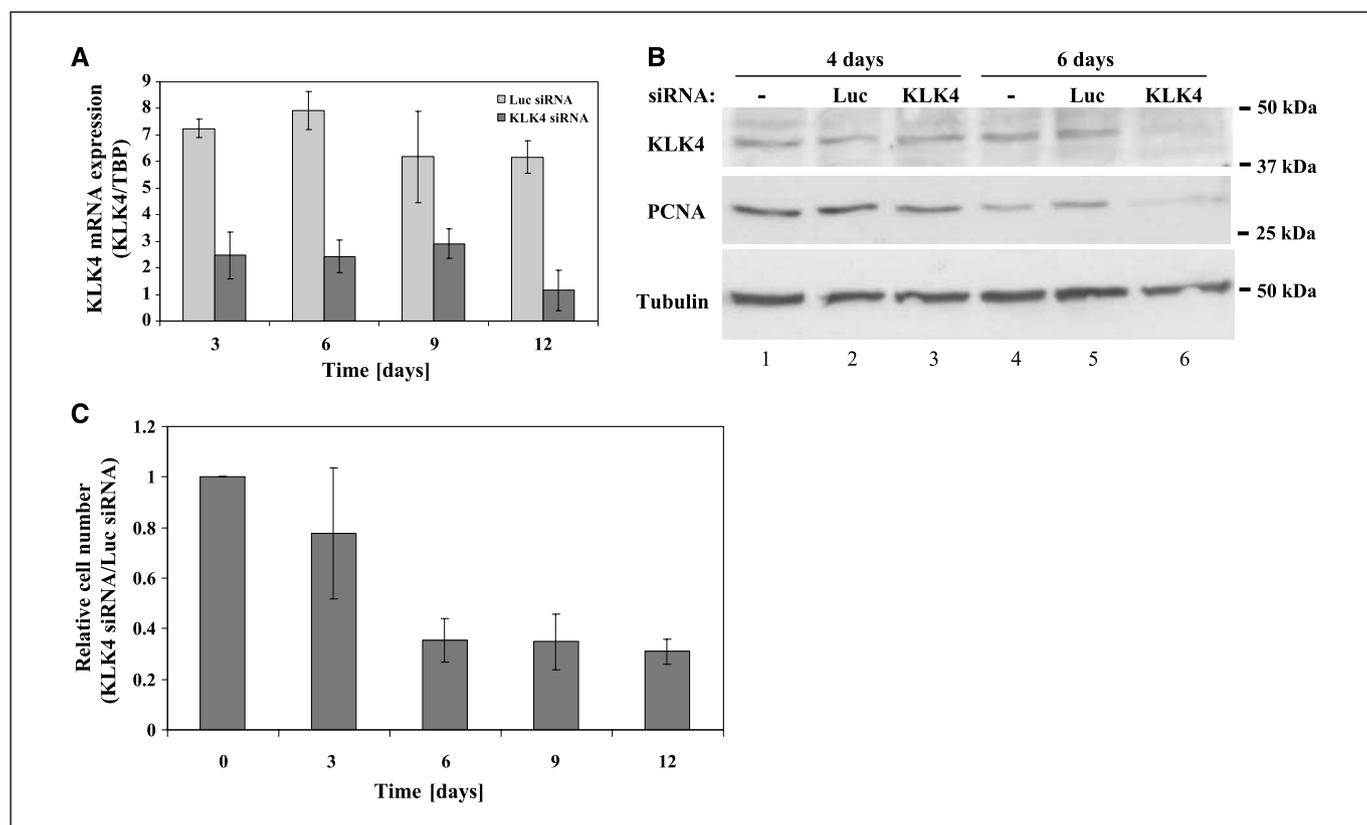
The expression of KLK4 protein in prostate cancer tissue has not been thoroughly examined previously. In one study, no significant differences were found in KLK4 expression between benign and malignant prostate tissues (23), using an immunoassay-based system. In contrast, another study (24) found increased expression of KLK4 in prostate cancer compared with normal tissue, consistent with our findings. Both these reports were based on relatively small sample sets ( $n = 16/18$  and  $n = 6$ , respectively),

which may explain the inconsistency between the results. Furthermore, both these reports examined the expression of the cytoplasmic and secreted forms of KLK4, which is different from the predominant nuclear form that we have studied here. The differences between the reports might, therefore, also indicate different expression patterns of the two isoforms of KLK4 with distinct cellular localization and function.

**KLK4 is involved in the proliferation of prostate cancer cells.** Overexpression of KLK4 in prostate cancer cells compared with normal prostate suggested that it may have a role in prostate cancer progression. The data we have presented here on cellular proliferation and colony formation clearly indicate that KLK4 is involved in growth of prostate cancer cells. Our results on proliferation are in contrast to what was reported in another study (24) where KLK4 expression in PC-3 cells resulted in a decreased growth rate. As with the immunohistochemical results, the inconsistency may be due to the fact that this study examined a secreted form of KLK4 and not the predominant intracellular form as in the present study. Our previous data (17) and the immunohistochemical data presented here show that the predominant form of KLK4 in prostate cancer cells is nuclear; therefore, its biological activity is expected to be more significant compared with other minor forms. In this context, it is important to assess the various forms of KLK4 and the possible effects of KLK4



**Figure 4.** Validation of cell cycle regulatory gene expression changes in response to KLK4 expression. *A*, an adenovirus system (see Materials and Methods) was used to ectopically express His-tagged KLK4 in PC-3 cells. Ten days after infection, the cells were harvested, and total RNA was extracted and used for cDNA synthesis. The cDNA was used as template in quantitative RT-PCR using SYBR Green Dye with primers specific for the genes indicated. The expression level of each gene was normalized to the housekeeping gene *TBP* in each sample and is presented as the expression level in KLK4-expressing cells relative to the expression in non-induced cells. *Columns*, average of three experiments; *bars*, SD. *B*, samples harvested 5, 7, 10, and 13 d after infection were treated as in (*A*), and quantitative RT-PCR was done with primers specific for the genes indicated. *Columns*, average of three experiments; *bars*, SD. *C*, increased PCNA protein levels upon KLK4 expression. Whole-cell extracts from PC-3 cells expressing KLK4 (+dox) or not (-dox) harvested 11 and 13 d after infection were subjected to Western analysis with PCNA antiserum. Tubulin was used as loading control. Molecular weight markers (*right*).



**Figure 5.** siRNA-mediated knockdown of endogenous KLK4 in LNCaP cells. **A**, LNCaP cells were transfected with siRNA specific for KLK4 or the luciferase gene (Luc siRNA), and the cells were harvested after 3, 6, 9, and 12 d. Total RNA was extracted and used for cDNA synthesis. The cDNA was then used as template in quantitative PCR using SYBR Green Dye with primers specific for KLK4 and the housekeeping gene *TBP*. The expression level of KLK4 was normalized to *TBP* in each sample. The experiment was done in triplicate. *Columns*, KLK4 mRNA expression; *bars*, SD. **B**, LNCaP cells were either left untreated (–) or transfected with KLK4 siRNA or Luc siRNA. Four and 6 d after transfection, the cells were harvested, and the whole-cell protein extract was subjected to Western analysis with either KLK4 or PCNA antiserum. Tubulin was used as loading control. Molecular weight markers (*right*). **C**, LNCaP cells transfected with KLK4 siRNA or Luc siRNA were counted at 3, 6, 9, and 12 d after transfection. The number of KLK4 siRNA-transfected cells are presented relative to the number of Luc siRNA-transfected cells. The experiment was done in triplicate and repeated twice with similar results. *Columns*, relative cell number; *bars*, SD.

overexpression, or its knockdown, on “normal” prostate cells such as RWPE-1.

Interestingly, the size of KLK4 expressed in the adenovirus system was around 25 kDa, close to its calculated molecular weight. This is smaller than the endogenous KLK4 expressed in LNCaP cells (45 kDa; refs. 17, 22), possibly due to differential posttranslational modifications in the different cell lines. A ~25-kDa size was also observed for KLK4 upon transient transfection of PC-3 cells (22), suggesting that KLK4 is differentially modified in PC-3 and LNCaP cells. This difference in modifications might potentially affect the functional properties of the protein. However, the fact that knockdown of endogenous KLK4 in LNCaP cells has opposite consequences compared with its ectopic expression in PC-3 and DU145 cells suggests that the 25-kDa KLK4 has largely maintained its functional properties. Further work is required to identify the exact nature of the KLK4 isoforms, their posttranslational modifications, and the effect of these on KLK4 function, which will definitively test this possibility.

A role for prostatic KLK in growth regulation has previously been predicted based on *in vitro* biochemical analysis. PSA and KLK2 have both been shown to cleave and inactivate members of the insulin-like growth factor (IGF)-binding proteins (IGFBP; ref. 38). IGFs are mitogenic regulators of normal and neoplastic prostate cell growth (39), and high levels of circulating IGF correlate with

the development of prostate carcinoma (40). Recombinant KLK4 of the minor secreted form can cleave and degrade IGFBPs *in vitro* with rapid proteolysis of IGFBP-5 and IGFBP-4 (41). The overexpression of the secreted KLK4 may, therefore, induce growth of prostate cells by increasing the bioavailability of the IGFs and thereby their mitogenic action in the prostate microenvironment. However, because our experiments involved the predominant intracellular KLK4, this mechanism cannot account for its effect on cell proliferation that we have observed.

**Cell cycle regulators as targets for KLK4.** How does KLK4 affect proliferation of prostate cancer cells? Cell cycle-specific oligonucleotide array analysis indicated that whereas several positive regulators of the cell cycle were up-regulated, cell cycle inhibitors, such as several CDK inhibitors, were down-regulated in KLK4-expressing cells. Both PCNA and Ki67, which are common markers of cell proliferation, were significantly up-regulated, confirming the proliferative effect of KLK4. Interestingly, the CDK inhibitors p15, p16, and p21 were all down-regulated. These act as tumor suppressor genes, and their down-regulation or loss is commonly seen in cancers (for reviews, see refs. 42, 43). For instance, p16 of the INK4 family is an important CDK inhibitor, specifically inhibiting CDK4 and CDK6. The inactivation of *p16* by genetic mutations, allelic loss, or hypermethylation of the promoter is a common event in prostate cancer as well as in other cancers

(e.g., see ref. 44). In fact, homozygous loss of *p16* gene was found in 40% of prostate cancers (45). The adenovirus-mediated overexpression of p16 in PC-3 cells resulted in a 70% reduction in cell number compared with controls (46), indicating that the regulation of this gene alone may account for the observed changes in cellular proliferation. p21 of the CIP/KIP family is another CDK inhibitor with a wider substrate specificity than the INK4 family (47). Loss of heterozygosity of the *p21* chromosomal location (6p) and *p21* somatic mutations have also been described in prostate cancer (48), and similarly to p16, its adenoviral overexpression has a growth-inhibitory effect on prostate cancer cells (49). Because KLK4 is not a transcription factor, how it affects the expression of these genes is not clear which needs to be investigated further. Screening for KLK4 interaction partners may in part help answering this question. For example, it is possible that KLK4 interacts with and controls the activity of a transcription factor or a coactivator/corepressor. Alternatively, KLK4 may affect chromatin structure of specific genes and thereby modify their expression. The crystal structure of KLK4 was recently solved (50), which may also

give useful information about potential enzymatic activities and substrate specificity.

In summary, this is the first time that a significant and unique function for a member of the KLK family has been shown. These data suggest that KLK4 may play important roles in prostate cancer development and progression. Further studies are needed to assess whether KLK4 may be a potential target for prostate cancer therapy, or have utility as a diagnostic or prognostic marker for prostate cancer.

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