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Prognostic value and function of KLF4 in prostate cancer: RNAa and vector-mediated overexpression identify KLF4 as an inhibitor of tumor cell growth and migration

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Abstract

KLF4/GLKF4 is a transcription factor that can have divergent functions in different malignancies. The role of KLF4 in prostate cancer etiology remains unclear. We have recently reported that small double-stranded RNA (dsRNA) can induce gene expression by targeting promoter sequence in a phenomenon referred to as RNA activation (RNAa). In the present study, we examine KLF4 levels in prostate cancer tissue and utilize RNAa as a tool for gene overexpression to investigate its function. Expression analysis indicated that KLF4 is significantly downregulated in prostate cancer cell lines compared to non-tumorigenic prostate cells. Meta-analysis of existing cDNA microarray data also revealed that KLF4 is frequently depleted in prostate cancer tissue with more pronounced reduction in metastases. In support, tissue microarray analysis of tumors and patient-matched controls indicated downregulation of KLF4 in metastatic tumor samples. Logistic regression analysis found that tumors with a KLF4 staining score <5 had a 15-fold higher risk for developing metastatic prostate cancer ($P = 0.001$, 95% CI: 3.0–79.0). *In vitro* analysis indicated that RNAa-mediated overexpression of KLF4 inhibited prostate cancer cell proliferation and survival, as well as altered the expression of several downstream cell cycle-related genes. Ectopic expression of KLF4 *via* viral transduction recapitulated the RNAa results validating its inhibitory effects on cancer growth. Reactivation of KLF4 also suppressed migration and invasion of prostate cancer cells. These results suggest that KLF4 functions as an inhibitor of tumor cell growth and migration in prostate cancer and decreased expression has prognostic value for predicting prostate cancer metastasis.

Keywords

KLF4; RNAa; tumor suppressor gene; prostate cancer; metastasis

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Introduction

KLF4/GKLF (gut-enriched Krüppel-like factor) is a member of the Krüppel-like factor subfamily of zinc finger proteins. Inactivation or silencing of KLF4 has been observed in a number of human cancers including gastrointestinal, pancreas, bladder, and lung cancer (1–4). Deletion of KLF4 in mouse models leads to abnormal differentiation, increased proliferation, and formation of intestinal adenomas in the colon and gastric epithelia (5–7). Ectopic expression of KLF4 has been shown to inhibit cell proliferation (8,9), induce apoptosis (9), and promote cell cycle arrest (3,9,10). These observations provide evidence that KLF4 has putative tumor suppressor function in a variety of malignancies and its inactivation may play a pivotal role in cancer progression. However, in the case of squamous cell carcinoma and breast cancer, KLF4 has been reported to promote growth and/or cellular de-differentiation (11,12). The ability for KLF4 to behave as a tumor suppressor or oncogene is largely dependent on genetic context of the tissue and/or cancer. For instance, the oncogenic function of KLF4 has been attributed to its ability to directly suppress p53 transcription (11).

Recently, we and others have reported that double-stranded RNA (dsRNA) can activate gene expression by targeting promoter sequence in a process termed RNA activation (RNAa, refs. 13–16). This technique alters chromatin structure leading to robust and prolonged expression of the endogenous target gene (13,14). As such, RNAa has potential to be a useful tool for interrogating gene function by serving as an alternative to traditional vector-based systems and an attractive strategy to activate tumor suppressor genes for the treatment of cancer.

The role of KLF4 in prostate cancer etiology has never been examined. In the present study, we show that KLF4 is downregulated in prostate cancer with metastases and utilize RNAa and vector-mediated overexpression to investigate its function. Our results indicate that KLF4 functions as an inhibitor of tumor progression in prostate cancer and decreased expression has prognostic value for predicting prostate cancer metastasis. We also demonstrate that RNAa can be utilized as a research tool to evaluate gene function in a manner similar to vector-mediated overexpression.

Materials and Methods

dsRNA Design

One kilobase (kb) of the human KLF4 promoter was scanned for dsRNA target sites based on rational design rules as previously reported (13,17). All dsRNA sequences are listed in Table S1.

Cell Culture and Transfection

PC-3, DU145, DuPro, LNCaP, RV1, C4-2 and BPH1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO₂ maintained at 37°C. RWPE-1 and PWR-1E cells were cultured in serum-free keratinocyte medium supplemented with 5 ng/ml human recombinant epidermal growth factor, and 0.05 mg/ml bovine pituitary extract. Transfection of dsRNA was carried out as previously described (13,17). RV1 and C4-2 cell lines were a gift from Dr. Benyi Li (University of Kansas). BPH1 cells were acquired from UCSF Cell Culture Facility. All other cell lines were obtained from ATCC. No further authentication of the cell lines was done by the authors.

mRNA Expression Analysis

Total RNA was isolated by using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed. The resulting cDNA samples were amplified by standard RT-PCR or real-time PCR using gene-specific primer sets in conjunction with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). All primer sequences are listed in Table S1.

Comparative Metaprofiling of cDNA Expression Data

The Oncomine Premium database (18) was accessed to perform differential expression analysis of KLF4 in existing prostate cancer microarray datasets by setting a threshold value for gene rank at 10% and *p*-value at 0.05.

Cell Viability Assay

Cells were transfected with dsRNA for ~12 hours. Following treatments, cells were transferred to 96-well microplates and seeded at a density of ~800 cells per well. Cell viability was subsequently determined every 24 hours for 6 days by using the CellTiter 96 AQueous One Solution (Promega) according to the manufacture's protocol.

Colony Formation Assay

Exponentially growing cells were plated at ~1000 cells per well in 6-well plates and transfected with dsRNA. Culture medium was changed every 3 days. Colony formation was analyzed 12 days following transfection by staining cells with 0.05% crystal violet solution for 1 hr.

Cell Cycle Analysis

Flow cytometric analysis of propidium iodide (PI) stained cells was performed as previously described (17).

Retrovirus-Based Overexpression of KLF4

A retroviral human KLF4 cDNA expression vector (pMXs-hKLF4) was obtained from Addgene (Cambridge, MA). An empty vector (pMXs-EV) was generated by removing the KLF4 gene from the retroviral sequence. Retrovirus particles were generated by transfecting 293FT cells (Invitrogen) with retroviral vectors.

Immunohistochemical (IHC) staining of tissue microarrays (TMAs)

Construction of TMAs from formalin-fixed, paraffin-embedded tissue blocks has been described previously (19). Methods for IHC staining of the TMA slides are available in Supplementary Information. The stained tissue arrays were evaluated under a light microscope by a board-certified pathologist and scored based on intensity and proportion of immunohistochemical staining as previously described (20). Intensity was scored on a scale of 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), while proportion was scored on a scale of 0 (0%), 1 (0.1–1%), 2 (2–10%), 3 (11–33%), 4 (34–66%), and 5 (67–100%). A cumulative staining score ranging from 0 to 8 was used for statistical analysis and obtained by combining the intensity and proportion scores.

Statistical analysis

Statistical significance was determined by using the StatView analysis software (SAS Institute Inc., Cary, NC). The χ^2 test was utilized to assess the significance between different proportions. Analysis of continuous variables between different groups was assessed by one-way ANOVA (analysis of variance) followed by Fisher's protected LSD (least significant

difference) test. Logistic regression analysis was used to determine correlation of KLF4 staining score, age, Gleason score, or stage to prostate cancer metastasis. All values are expressed as means $\pm$ SEM unless otherwise indicated. Significance was defined as $P < 0.05$.

Additional methods for immunoblot analysis, IHC, and invasion/migration assay are listed in Supplementary Information.

Results

KLF4 is downregulated in prostate cancer cell lines and tissue samples

We evaluated KLF4 expression levels in a panel of 9 prostate cell lines including 6 cancerous (PC-3, DuPro, DU145, LNCaP, RV-1, and C4-2) and 3 non-tumorigenic/benign (RWPE-1, PWR-1E, and BPH1) cell lines. Analysis of mRNA expression by RT-PCR revealed that KLF4 transcript levels are lower in all cancerous cell lines compared to the non-tumorigenic/benign controls (Fig. 1A). Real-time PCR confirmed that KLF4 mRNA expression was significantly down-regulated by $\geq 80\%$ in each prostate cancer cell line compared to levels in RWPE-1 and PWR-1E cells (Fig. 1B). Immunoblot analysis indicated that KLF4 protein levels correlated to that of mRNA in nearly all cell lines (Fig. 1C).

We performed meta-analysis of existing cDNA microarray datasets deposited in the Oncomine database (18). We indentified 17 total datasets containing gene expression profiles of PIN (prostatic intraepithelial neoplasia) or primary prostate cancer compared to normal prostate tissue. Analysis of KLF4 levels revealed that it was significantly depleted in 3/17 of the datasets, while 2 other profiles showed a trend of KLF4 downregulation in diseased tissue (Fig. S1A) (21–24). We further identified and retrieved 11 total datasets comparing metastatic prostate cancer to localized prostate cancer. As shown in Figure 1D, KLF4 expression was significantly downregulated in metastatic samples of 5/11 datasets (22,24–27). Additional analysis of three datasets evaluating gene expression at different grades of prostate cancer also revealed a reverse correlation between KLF4 expression and Gleason score (Fig. S1B) (21,28,29). Interestingly, no datasets recorded KLF4 upregulation in diseased tissue.

We also assessed KLF4 protein levels by IHC staining of TMAs containing patient-matched normal, tumor, and/or lymph node metastases. Tissues from 29 prostate cancer patients had lymph node metastasis (LN+), while 30 prostate cancer patients had tumors without metastasis (LN–). Staining of normal tissue revealed no significant difference in KLF4 levels between LN+ and LN– patients; however, KLF4 was found to be significantly more downregulated in localized tumor tissue from LN+ patients than LN– patients (Fig. 1E, 1G–H, Table S2). Within the LN+ group, KLF4 staining intensity proceeded in the following order: normal tissue > tumor > lymph node metastases (Fig. 1F, 1I–K). However, IHC staining in the LN– group did not reveal a significance difference in KLF4 expression between normal and tumor tissue (Fig. 1F, Table S2). Logistic regression analysis within the LN+ group indicated that tumors with a KLF4 staining score < 5 had a 15-fold higher risk for developing metastatic prostate cancer ($P = 0.001$, 95% CI: 3.0–79.0) (Table S3). Among other clinicopathological parameters (e.g. age, stage, and Gleason score), KLF4 staining was the sole predictor for metastasis (Table S3). Although additional studies are required to validate and extend the IHC data in terms of clinical outcome, these results indicate that KLF4 is frequently depleted in prostate cancer with metastases and has putative prognostic value for predicting metastasis. This would suggest that KLF4 may function as an inhibitor of prostate cancer progression.

RNAa-based overexpression of KLF4 in prostate cancer cells

To explore the function of KLF4 in prostate cancer cells, we decided to activate endogenous KLF4 expression by RNAa. We designed 4 candidate dsRNAs (dsKLF4-525, dsKLF4-496, dsKLF4-261, and dsKLF4-168) according to rules derived from previous studies (13,17) that targeted the KLF4 promoter at sites ranging from -525 to -168 relative to the transcription start site (Fig. 2A). Each dsRNA was transfected into PC-3 cells and KLF4 expression was evaluated by real-time PCR three days following treatment. Compared to controls, dsKLF4-496 and dsKLF4-525 induced KLF4 expression by ~3.0- and ~1.5-fold, respectively; while dsKLF4-168 and dsKLF4-261 did not significantly alter KLF4 levels (Fig. 2B). Time-course experiments further indicated that optimal levels of KLF4 induction (~4.2-fold) were achieved by day 4 in PC-3 cells (Fig. S2).

To determine if KLF4 was susceptible to RNAa in other prostate cancer cells lines, we transfected DuPro, PC-3, DU145, and LNCaP cells with dsKLF4-496. Four days following transfection, dsKLF4-496 induced KLF4 mRNA expression by ~16, 4.6 and 3.3-fold in DuPro, PC-3, and DU145 cells, respectively (Fig. 2C and D). LNCaP cells were insensitive to dsKLF4-496 as it failed to activate KLF4 expression (data not shown). Consistent with mRNA induction, immunoblot analysis revealed that KLF4 protein levels were also elevated by dsKLF4-496 in each of the sensitive cell lines (Fig. 2E).

Overexpression of KLF4 by RNAa modulates the expression of downstream cell cycle-related genes

KLF4 regulates the expression of several cell cycle-related genes including p53, CCNB1, and members of the cyclin-dependent kinase inhibitor family p21, p27, and p57 (3,11,30,31). To determine if the RNAa-based overexpression of KLF4 modulated the expression of downstream cell cycle genes, we evaluated protein levels of p21, p27, p57, and CCNB1 in DuPro, PC-3, and DU145 cells following dsKLF4-496 transfection. Expression of p53 was not evaluated because cell lines were either null (e.g. PC-3 and DuPro) or mutant (e.g. DU145) for functional p53 (32). As shown in Figure 3A–C, dsKLF4-496 induced KLF4 levels and altered the expression of several downstream targets in DuPro, PC-3, and DU145 cells. Of interest, p21 and p27 expression was upregulated in all three cell lines, while CCNB1 was only selectively downregulated in DuPro and PC-3 cells (Fig. 3A–C). Levels of p57 protein also increased in PC-3 and DU145 cells, but markedly decreased in DuPro cells (Fig. 3A–C). In order to determine if protein levels correlated to p57 transcription, we utilized real-time PCR to quantify p57 mRNA levels. As shown in Figure S3, p57 mRNA expression increased in all three cell lines suggesting p57 is differentially regulated by post-transcriptional mechanisms in DuPro cells following KLF4 activation.

KLF4 has also been shown to downregulate genes involved in chromosome segregation and execution of the mitotic checkpoint including CENPE (centromere protein E), MAD2L1 (mitotic arrest deficient 2-like 1), and BUB1B (budding uninhibited by benzimidazoles 1 homolog B) (30). In fact, mouse embryonic fibroblasts null for KLF4 have chromosome aneuploidy and centrosome amplification (33). Therefore, we evaluated the expression of CENPE, BUB1B, and MAD2L1 in DuPro and PC-3 cells following dsKLF4-496 transfection. In DuPro cells, BUB1B and CENPE expression was downregulated by dsKLF4-496, while only CENPE significantly declined in PC-3 cells (Fig. 3D and E). Taken together, these results indicate that the RNAa-based overexpression of KLF4 modulates the expression of several downstream genes that may have functional significance in regulating cell cycle progression in prostate cancer cells.

KLF4 inhibits growth and survival in prostate cancer cells

Prostate cancer cells transfected with dsKLF4-496 displayed altered morphology and decreased cell density characteristic of impeded growth (Fig. S4). Quantitative analysis by MTS assay indicated that PC-3 and DuPro cell viability steadily decreased following dsKLF4-496 transfection (Fig. 4A). By day 6, growth was inhibited by ~40% and ~70% in PC-3 and DuPro cells, respectively. Clonogenicity assays also revealed that KLF4 overexpression reduced the number and size of colonies formed by PC-3 and DuPro cells (Fig. 4B).

To evaluate the effect of KLF4 activation on cell cycle distribution, DNA content was analyzed by flow cytometry in cells stained with PI following dsKLF4-496 transfection. In PC-3 cells, dsKLF4-496 caused a significant increase in G1/G0 populations with concurrent declines in S and G2/M populations as compared to control treatments (Fig. 5A and C). Arrest in G2/M phase was associated with KLF4 activation in DuPro cells with proportional declines in S and G1/G0 populations (Fig. 5B and D). KLF4 overexpression by dsKLF4-496 also led to an increase in cells with sub-diploid (<2C) DNA content; a marker for DNA fragmentation/apoptosis (Fig. 5A–D). Interestingly, we also observed cell populations in dsKLF4-496 transfected cells with DNA content exceeding levels found in mitotic cells (>4C) (Fig. 5A–D). Cell populations with increased ploidy may reflect defective cytokinesis resulting from the downregulation of CENPE and/or BUB1B (Fig. 5D and E). In support, multinucleated cells were routinely observed in images taken of cells transfected with dsKLF4-496 (Fig. S5). Taken together, these results indicate that KLF4 overexpression by dsKLF4-496 reduced cell growth and manipulated prostate cancer phenotypes in a manner indicative of altered expression of downstream KLF4-regulated genes.

Vector-based overexpression of KLF4 recapitulates RNAi results

To validate the results obtained through RNAi-mediated activation of KLF4, we utilized a retroviral transduction system to overexpress KLF4 cDNA (pMXs-hKLF4) in prostate cancer cell lines. Infection of KLF4 viral particles caused robust induction of KLF4 protein levels and modulation of several downstream target genes (i.e. p21, p27, p57, and CCNB1) in a pattern similar to RNAi-mediated overexpression including (i) increased levels of p21 and p27, (ii) declines in CCNB1 levels, and (iii) differential regulation of p57 protein between DuPro and PC-3 cells (Fig. S6A and B). The subtle variation in downstream gene sensitivity to KLF4 may result from the inherent differences between RNAi-mediated and viral-based overexpression techniques. Morphologically, viral transduction of KLF4 caused changes consistent with growth inhibition, as well as a noticeable presence of multinucleated cells (Fig. S7). Functional studies in PC-3 and DuPro cells revealed that KLF4 transduction decreased cell viability and colony forming potential as compared to control treatments (Fig. S8). Vector-based overexpression of KLF4 recapitulated the results obtained by RNAi-mediated KLF4 induction. This data validates RNAi as a technique to study KLF4 overexpression and confirms its function as a putative tumor suppressor in prostate cancer cells.

KLF4 overexpression inhibits prostate cancer cell migration and invasion

Downregulation in metastatic prostate tissue suggests that KLF4 may also play a role in cell migration and invasion. To assess changes in cell migration, PC-3 cells were transduced with KLF4 (pMXs-hKLF4) and allowed to migrate through a transwell membrane into complete media. In order to control for cell number and limit the antiproliferative effects of KLF4, equal quantities of cells were transferred to the membrane surface and cell migration was assessed within 20 hours [i.e. when differences in cell viability were minimal (Fig. S9)]. Compared to the empty vector control, overexpression of KLF4 inhibited cell migration; ~44% reduction in migratory cells was detected in the KLF4-infected (pMXs-

hKLF4) cell population (Fig. 6A and B). Additional analysis *via* scratch assay also revealed slower cell migration following KLF4 overexpression in PC-3 cells (Fig. S10). To evaluate cell invasion, PC-3 cells were transduced with KLF4 (pMXs-hKLF4) and plated on the matrigel surface. As shown in Figure 6C and D, KLF4 overexpression significantly reduced PC-3 cell invasion; ~65% reduction of invading cells was detected in the KLF4-infected (pMXs-hKLF4) cell population as compared to the empty vector control.

Migration/invasion-related genes (e.g. E-cadherin, CXCR4, CTNNB1, MMP11, and TIMP2) have been implicated in prostate cancer metastasis and/or regulated by KLF4. Among the five genes, TIMP2 (TIMP metalloproteinase inhibitor 2) was the only gene consistently altered by KLF4 overexpression (data not shown). As shown in Figure 6E, both dsKLF4-496 and KLF4 viral particle (pMXs-hKLF4) increased TIMP2 mRNA levels by ~2.5- and ~1.8-fold, respectively. Knockdown of KLF4 by siRNA (siKLF4) also correlated with a reduction in TIMP2 levels to suggest that KLF4 may directly regulate TIMP2 expression (Fig. 6F). Taken together, our findings indicate that KLF4 inhibits prostate cancer cell migration/invasion and associates with elevated TIMP2 expression.

Discussion

The role of KLF4 in prostate cancer has never been investigated. Within this study, we reveal that KLF4 is downregulated in prostate cancer cell lines and prostate cancer tissue with metastases. Interestingly, all 6 prostate cancer cell lines evaluated in this study, with exception to RV1, are derived from metastatic prostate tissue (32). Analysis of 47 total datasets from the Oncomine database revealed that no dataset recorded KLF4 upregulation in diseased tissue. Rather, the only significant changes in KLF4 expression were downregulation with increased frequency in metastatic sample datasets. Analysis of patient-matched tissues also revealed that low KLF4 immunostaining may have prognostic value for prostate cancer metastasis. Collectively, this data suggests that KLF4 functions as an inhibitor of prostate cancer progression. In support, restoration of KLF4 expression significantly reduced prostate cancer cell viability, clonogenicity, growth, migration, and invasion – features indicative of tumor suppressor function. Such phenotypes also associated with changes in gene expression known to negatively impact cell growth and migration. For instance, KLF4 induced p27 expression in prostate cancer cell lines; low p27 levels associate with aggressive/recurrent prostate cancer, while its overexpression correlates with growth inhibition in cell culture (34).

KLF4 has also been shown to inhibit migration and invasion in several cancer models (3,35,36). Interestingly, forced expression of KLF4 in prostate cancer cells elevated expression of TIMP2; a metalloproteinase thought to function as an inhibitor of metastasis. TIMP2 has been reported to be inactivated *via* DNA hypermethylation in metastatic prostate cancer tissue and inhibit cancer cell migration (37,38). In this regards, TIMP2 may function as a downstream factor of KLF4 to inhibit cell migration/invasion in prostate cancer.

RNAa-mediated overexpression of KLF4 inhibited cell proliferation/survival and arrested cell cycle progression. In DuPro cells, KLF4 overexpression resulted in G2/M arrest, while accumulation in G1/G0 phases of the cell cycle was detected in PC-3 cells. KLF4 has been shown to cause either G1 or G2 arrest in different cell types based on modulation and function of downstream genes (10,31,39). Differential regulation of p57 protein levels between PC-3 and DuPro cells may play a role in delineating alternate phases of cell cycle arrest; elevated levels of p57 associate with G1 arrest (40). Declines in CCNB1 may contribute to G2 arrest as KLF4 has been previously shown to prevent entry into mitosis through reductions in CCNB1 levels (10). Based on combination and susceptibility of downstream genes to KLF4 reactivation, DuPro and PC-3 cells were inhibited at different

phases of the cell cycle. The ability of KLF4 to inhibit cell cycle progression at multiple checkpoints is indicative of a gene with potent tumor suppressor-like function. Transfection of dsKLF4-496 also led to a small population of cells with sub-diploid DNA content in both DuPro and PC-3 cells. Enhanced cell death may also contribute to the tumor suppressor-like activity of KLF4 in prostate cancer cells.

Vector-based overexpression is the traditional approach to evaluate the function of tumor suppressor genes or oncogenes in cancer cells. However, all vector-based systems require ectopic expression from an exogenous construct. Ectopic expression vectors do not typically resemble natural genes (41,42). They are frequently created from cDNA libraries or amplicons, which do not contain introns or UTR elements. Such regions can have pivotal effects on endogenous gene function in cancer biology (41). As a laboratory tool, RNAa has the unique ability to enhance endogenous transcription of a targeted gene. Such a technique may allow for a more natural approach at analyzing gene function. In support, RNAa appeared to facilitate a greater measurable effect on downstream gene expression compared to viral transduction (Fig. 4 and Fig. S6).

In conclusion, both cDNA and protein expression profiling demonstrated that KLF4 is significantly downregulated in prostate cancer tissue with metastases. Furthermore, decreased KLF4 expression has potential to serve as a strong and independent predictor of prostate cancer metastasis. By using RNAa as a laboratory technique, we revealed that KLF4 inhibits prostate cancer cell growth/survival and arrests cell cycle progression *via* modulating the expression of key downstream genes. KLF4 also suppressed prostate cell migration/invasion and upregulated TIMP2. This study reveals that KLF4 functions as a putative tumor suppressor in prostate cancer, as well as demonstrates the applicability of RNAa to study gene function. Much like RNAi, RNAa offers a new therapeutic approach for combating disease at the genetic level. As such, the ability to re-activate KLF4 by RNAa may have therapeutic potential in the treatment of metastatic prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. KLF4 expression is downregulated in prostate cancer cell lines and tissues samples

A. KLF4 mRNA expression levels were evaluated by RT-PCR in non-tumorigenic prostate epithelial (RWPE-1 and PWR-1E), benign prostate hyperplasia (BPH1), and prostate cancer (PC-3, DuPro, DU145, LNCaP, RV-1, and C4-2) cell lines. **B.** Relative expression of KLF4 was determined by real-time PCR (mean $\pm$ SEM from two independent experiments). Expression values of KLF4 were normalized to β -actin levels. **C.** KLF4 and β -actin protein levels were detected by immunoblot analysis. β -actin served as a loading control. **D.** Analysis of only KLF4 from catalogued expression arrays retrieved from the Oncomine database. Significant downregulation of KLF4 was noted in 5/11 datasets comparing metastatic (MPCa) to localized prostate cancer (PCa). **E.** Tissue microarrays (TMA) containing specimens from prostate cancer patients with (LN+) or without (LN-) lymph node metastasis were immunostained for KLF4 and scored by a board-certified pathologist. Staining scores were compared between LN+ and LN- groups in tumor and normal tissue. **F.** Staining scores in lymph node metastases (LN) and/or primary tumors were compared to normal tissue in the LN+ and LN- groups. **G-H.** Depicted are representative microphotographs of KLF4 staining in localized LN- (G) and LN+ (H) tumor tissue. Insets are enlarged images showing nuclear staining of KLF4. **I-K.** Depicted are representative microphotographs of patient-matched tissue. Denote stronger staining in normal tissue (I) compared to localized cancer (J) and lymph node metastases (K).

Figure 2. KLF4 overexpression by dsRNA in prostate cancer cell lines

A. A schematic representation of the KLF4 promoter. Indicated are the locations of each dsRNA target site relative to the transcription start site (+1) and the sense sequence of the corresponding dsRNA. **B.** PC-3 cells were transfected at 50 nM concentrations of the indicated dsRNAs for 72 hours. Mock samples were transfected in the absence of dsRNA. KLF4 expression was assessed by real-time PCR. Results are plotted as fold change relative to mock transfections (mean $\pm$ SEM of two independent experiments). **C.** DuPro, PC-3, and DU145 cells were transfected at 50 nM dsControl or dsKLF4-496 for 96 hours. KLF4 and GAPDH expression levels were assessed by RT-PCR. **D.** Cells were transfected as in C. Relative KLF4 expression was quantified by real-time PCR in each cell line (mean $\pm$ SEM from three independent experiments). KLF4 expression was normalized to β -actin levels. **E.** Cells were transfected as in C. KLF4 and β -actin protein levels were detected by immunoblot analysis using protein-specific antibodies. β -actin served as a loading control. * $p < 0.05$, ** $p < 0.01$ vs. Mock.

Figure 3. KLF4 activation alters the expression of cell cycle-related genes in prostate cancer cells
A–C. DuPro, PC-3, and DU145 cells were transfected with 50 nM of the indicated dsRNA for 96 hours. Protein levels of KLF4, p21, CCNB1, p27, p57, and β-actin were determined by immunoblot analysis. **D** and **E.** DuPro (**D**) and PC-3 (**E**) cells were transfected as in **A**. Relative expression levels of BUB1B, CENPE, and MAD2L1 were quantified by real-time PCR (mean ± SEM from three independent experiments). Expression values were normalized to β-actin levels. * $p<0.05$, ** $p<0.01$ vs. Mock.

Figure 4. Overexpression of KLF4 by RNAi inhibits prostate cancer cell viability and clonogenicity

A. PC-3 and DuPro cells were transfected at 50 nM dsRNA for the indicated lengths of time. MTS reagent was utilized to quantify cell viability at each time point. Data is plotted as the mean $\pm$ SEM of two independent experiments relative to mock treatments. **B.** PC-3 and DuPro cells were plated at 1000 cells/well in 6-well tissue culture plates and transfected with mock, dsControl, or dsKLF4-496. Cells were grown for 12 days and analyzed for colony formation by staining with crystal violet. Shown are representative photographs taken of tissue culture plates from each dsRNA treatment group following staining for colony formation. * $p < 0.05$, ** $p < 0.01$ vs. dsControl.

Figure 5. KLF4 inhibits cell cycle progression in prostate cancer cells

A and B. PC-3 (A) and DuPro (B) cells were transfected with mock, dsControl, or dsKLF4-496 for 72 hours. Floating and attached cells were collected, stained with PI, and processed for analysis by flow cytometry to measure DNA content. Shown are representative FL2A histograms. **C and D.** Flow cytometry data from PC-3 (C) and DuPro (D) cells was analyzed to determine cell cycle distribution. Percentages of sub-diploid/apoptotic (<2C) and polyploid (>4C) cells were calculated from entire gated whole-cell populations, while cell cycle distribution (G0/G1, S, and G2/M) was determined from only surviving cells. Data is represented as the mean values $\pm$ SEM of two independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. Mock.

Figure 6. Overexpression of KLF4 inhibits PC-3 cell invasion and migration

A. PC-3 cells were infected with retroviral particles generated from an empty control vector (pMXs-EV) or a vector expressing KLF4 (pMXs-hKLF4). Equal numbers of infected cells were plated in the upper chamber of a transwell plate and allowed to migrate through a membrane for 20 hours. Cells that passed through the membrane were stained with crystal violet. Representative photographs were taken at 40 $\times$ magnification. **B.** The number of migrated cells was quantified in four random images from each treatment group. Results are the means $\pm$ SEM from two independent experiments plotted as percent (%) migrating cells relative to pMXs-EV. **C.** Transwell membranes were coated with matrigel. Equal numbers of infected cells were plated in the upper chamber of the Transwell plate and cultured for 20 hours. Cells that invaded the lower chamber were stained with crystal violet. Representative photographs were taken at 40 $\times$ magnification. **D.** The number of invading cells was quantified and presented as in B. **E.** TIMP2 mRNA expression was assessed by real-time

PCR in PC-3 cells following transfection at 50 nM dsRNA or transduction with retroviral particles. Expression data was normalized to β -actin. **F.** PC-3 cells were transfected with the indicated siRNAs for 72 hrs. KLF4 and TIMP2 levels were assessed by real-time PCR. Results are presented as means $\pm$ SEM of two independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. pMXs-EV (B and D) or Mock (E and F).