Identification of \textit{PDE4D} as a Proliferation Promoting Factor in Prostate Cancer Using a \textit{Sleeping Beauty} Transposon-Based Somatic Mutagenesis Screen

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Abstract

Retroviral and transposon-based mutagenesis screens in mice have been useful for identifying candidate cancer genes for some tumor types. However, many of the organs that exhibit the highest cancer rates in humans, including the prostate, have not previously been amenable to these approaches. This study shows for the first time that the \textit{Sleeping Beauty} transposon system can be used to identify candidate prostate cancer genes in mice. Somatic mobilization of a mutagenic transposon resulted in focal epithelial proliferation and hyperplasia in the prostate. Efficient methods were established to identify transposon insertion sites in these lesions, and analysis of transposon insertions identified candidate prostate cancer genes at common insertion sites, including \textit{Pde4d}. \textit{PDE4D} was also overexpressed in human prostate cancer patient samples and cell lines, and changes in \textit{PDE4D} mRNA isoform expression were observed in human prostate cancers. Furthermore, knockdown of \textit{PDE4D} reduced the growth and migration of prostate cancer cells \textit{in vitro}, and knockdown of \textit{PDE4D} reduced the growth and proliferation rate of prostate cancer xenografts \textit{in vivo}. These data indicate that \textit{PDE4D} functions as a proliferation promoting factor in prostate cancer, and the \textit{Sleeping Beauty} transposon system is a useful tool for identifying candidate prostate cancer genes. [Cancer Res 2009;69(10):4388–97]

Introduction

Forward somatic mutagenesis screens using murine retroviruses as insertional mutagens have been effective tools for cancer gene discovery in mammary tumors, hematopoietic neoplasms, and brain tumors in mice (1–3). However, many tissue types that are commonly transformed into tumors in humans are not affected by known retroviruses. Two studies have shown that genome-wide somatic mutagenesis using the \textit{Sleeping Beauty} (SB) transposon system can be used to induce tumors and identify cancer genes for hematopoietic tumors and sarcomas (4,5). Candidate cancer genes were identified in these studies by finding sites in the genome of SB-induced tumors that were recurrently mutated by transposon insertions in multiple independent tumors. Such sites are called common insertion sites (CIS), and past experience with retroviral- and transposon-based screens has shown that the genes located at CIS act as oncogenes or tumor suppressors. A major unresolved question for this approach is whether SB will be more broadly applicable for cancer gene discovery in epithelial cancers, which are responsible for 80% of human cancer deaths (6).

Prostate adenocarcinoma is the most commonly diagnosed male cancer and is one of the leading causes of male cancer death (6). Before the development of invasive adenocarcinoma, several histologic abnormalities can sometimes be observed that are considered potential precursor lesions for prostatic adenocarcinoma. These include prostatic intraepithelial neoplasia (PIN), atypical small acinar proliferation, and proliferative inflammatory atrophy (7, 8). Features associated with one or more of these candidate precursor lesions include focal areas of increased proliferation, stratification of the epithelium, loss of basal cells, and changes in nuclear morphology. Several genetic and gene expression alterations have been identified as common events in early prostate cancers. The transcription factor \textit{Nkx3.1} is commonly lost in early-stage tumor epithelial cells, including loss in 20% of high-grade PIN cases examined (9). This loss of expression is likely due to heterozygous deletions of the genomic locus and epigenetic silencing mechanisms (10). Recurrent deletions and translocations that create fusion genes involving transcription factors ERG, ETV1, or ETV4 in prostate epithelial cells have been reported to occur in a majority of prostate cancers and are also detected as early as PIN (11–13). Although these genetic alterations clearly play a role in early prostate cancers, the genetic basis for prostate cancer initiation remains poorly understood (14). In this study, transposon-based somatic mutagenesis caused foci of altered histology and elevated proliferation in the prostatic epithelium that resembled features associated with prostate cancer precursor lesions. Analysis of transposon insertion sites in these lesions identified candidate cancer genes that may play a role in prostate cancer initiation, including \textit{PDE4D}. \textit{PDE4D} was also overexpressed in human prostatic adenocarcinomas, and short hairpin RNA (shRNA) knockdown of \textit{PDE4D} reduced the proliferation of prostate cancer cells \textit{in vitro} and \textit{in vivo}, supporting its potential role as a proliferation promoting factor in human prostate cancer.

Materials and Methods

\textbf{Mice.} The mouse strains used for this study have previously been described, including CAGGS-SB10 transgenic, \textit{Rosa26-SB11} knock-in, and low-copy T2 onc transgenic (4, 5).
**Immunohistochemistry.** Tissues were stained for the Ki-67 antigen as described previously (15). A modified staining protocol was used for laser capture microscopy as follows: tissues were dissected into cold PBS, embedded directly into Tissue Freezing Medium (Triangle Biomedical Sciences), and stored at −80°C. Tissue sections (5 μm) were cut at −20°C with a cryostat and immediately mounted onto PEN membrane slides (Leica) and air-dried for 30 min followed by fixation in 100% ethanol at −20°C. Slides were washed with PBS, blocked with 2% sheep serum in PBS, stained for 3 h with anti-phospho-histone-H3 (Upstate Cell Signaling Solutions) 1:150 in 2.5% sheep serum in PBS, and washed in 1× PBS-0.1% Tween 20. The primary antibody was visualized with an anti-rabbit IgG hors eradish peroxidase conjugate (Chemicon) diluted 1:500 in 2.5% sheep serum in PBS followed by 1× PBS-0.1% Tween 20 washes and staining with the 3,3′-diaminobenzidine reagent (Vector Laboratories) according to the manufacturer’s instructions. Slides were counter stained with hematoxylin, dehydrated into 100% ethanol, and air dried for 1 h before laser capture.

**Laser capture microscopy and linker-mediated PCR.** Cells of interest were captured from tissue sections using a Leica LMD6000 laser capture microdissection microscope. DNA was isolated from the captured cells using a Qiagen QIAmp DNA Micro kit according to the manufacturer’s instructions. Transposon-genomic DNA junctions were amplified as described previously (16), cloned into plasmids, and sequenced by Functional Genomic Laboratories.

**Automated quantitative analysis of PDE4D expression.** The automated quantitative analysis was conducted on a previously assembled tissue microarray of prostate tissues from patients treated at the University of Wisconsin-Madison using an established protocol (17). Epithelial compartment visualization was achieved with a 30 min incubation of a mouse anti-cytokeratin AE1/AE3 (1:200; DAKO North America) and mouse anti-E-cadherin (1:50; Abcam) cocktail followed by a 30 min incubation of Invitrogen Alexa Fluor 555 donkey anti-mouse IgG (1:200). Goat anti-PDE4D IgG (1:100; Santa Cruz Biotechnology) was preincubated with PDE4D protein (5× w/w; Santa Cruz Biotechnology) for 2 h 30 min before application. Both PDE4D antibody (1:100) and PDE4D with 5× protein were incubated for 60 min followed by a 15 min incubation with Invitrogen biotinylated swine anti-goat IgG (1:200). Staining on the tissue microarray was visualized as described previously (17). There were 336 tissue cores from 168 patients (2 cores from each patient) in the tissue microarray stained for this study. In addition to an automated image acquisition and analysis as described previously (18), all cores for this study were examined for staining and morphology by surgical pathologist (W.H.). Tissue microarray cores without sufficient epithelium (<5%) or with poor staining quality (section folding, excess trapping of fluorochrome, etc.) were excluded from analysis. In total, 282 of the 336 cores stained for PDE4D passed this quality control and were included in the final data set.

**RNA ligase-mediated 5′-Rapid Amplification of cDNA Ends.** The RNA analyzed in the RNA ligase-mediated 5′-Rapid Amplification of cDNA Ends (RLM-5′-RACE) experiment was isolated from human prostate samples obtained by the University of Minnesota Cancer Center Tissue Procurement Facility. For the study, prostate tissue samples were bisected. One piece was provided to our laboratory as a fresh sample that was used for the isolation of RNA. The adjacent piece of tissue was retained by the Tissue Procurement Facility for examination by a Tissue Procurement Facility staff pathologist who provided us with a pathology report that indicated the presence/absence and pathologic grade of prostate cancer in each tissue sample. RNAs were analyzed using the FirstChoice RLM-RACE kit (Ambion) according to the manufacturer’s instructions. The PDE4D outer and inner primers were 5′-ATGGGCCATCAGCATGTTATAATGCT-3′ and 5′-ACCAC- TAGTTCACATCTTCTAGCTTCCGTCACAGA, respectively.

**Cell culture.** DU145-derived cell lines were grown in 1× RPMI (Invitrogen/Life Technologies) supplemented with 5% activated charcoal USP (Sigma)-treated fetal bovine serum (HyClone), 100 μg/mL antibiotic/antimycotic (Invitrogen/Life Technologies), and 10−8 mol/L 5α-androsten-17β-ol-3-one (Sigma). PC3-derived cell lines were grown in 1× MEM (Invitrogen/Life Technologies) supplemented with 5% activated charcoal USP (Sigma)-treated fetal bovine serum (HyClone), 100 μg/mL antibiotic/antimycotic (Invitrogen/Life Technologies), 1× nonessential amino acids (Invitrogen/Life Technologies), and 1× sodium pyruvate (Invitrogen/Life Technologies). All cell lines were grown at 37°C in 5% CO2. Stable PDE4D shRNA knockdown and scrambled sequence shRNA control cell lines were generated using lentiviral transduction with shRNA from Open Biosystems. Cells were transduced at a multiplicity of infection of 10 following the manufacturer’s protocol and then underwent G418 selection for stable integrants. Immunoblots of cell line extracts were conducted as described previously (15) using the anti-PDE4D antibody (Abcam) diluted 1:1,500 in 2.5% milk.

**Growth assays.** Cells were plated at an initial density of 5,000/cm2 on 4-well plates (Costar). After the growth period, cells were trypsinized and neutralized in medium, and trypsin blue was added. The number of viable cells was determined by counting the number of cells that exclude trypan blue on a hemacytometer. Alternatively, cells were plated at an initial density of 500 per well on a 96-well plate in triplicate, and cell viability was evaluated using an MTS assay (CellTiter96 Aqueous One Solution Cell Proliferation Assay; Promega) according to the manufacturer’s instructions.

**Xenograft assay.** Cultured cells were trypsinized, resuspended at 106 in 500 μL Matrigel (BD Biosciences 356230), and injected subcutaneously into CD1 nu/nu mice. After 1 month, xenografts were fixed in formalin and processed for immunohistochemistry. Following Ki-67 staining, 8 random × 20 fields were photographed and the ratio of Ki-67-positive nuclei to total nuclei was determined using a previously described computer-assisted method for image analysis (19).

**Results**

**Focal hyperplasia in the prostatic epithelium of transposon mutagenized mice.** Previous studies showed that two mouse strains with active transposition of a mutagenic transposon, T2/onc, became moribund due to the formation of tumors with a limited tissue distribution (4, 5). The two strains used in these experiments differed in the transgene driving transposase expression (CAGGS-SB10 or Rosa26-SB11). In addition, the CAGGS-SB10/T2/onc mice were null for the Arf tumor suppressor gene. We examined the morphology and histology of prostate glands from CAGGS-SB10/T2/onc low-copy mice. Unlike the previously reported high rate of embryonic lethality in Rosa26-SB11/T2/onc high-copy (≥148 copies of T2/onc) mice (4), the ratio of Rosa26-SB11/T2/onc low-copy mice to single transgenic littermates was consistent with little or no embryonic lethality. However, these mice succumbed to tumor formation, primarily leukemias, with an median latency of 6 to 7 months. Examination of the histology of prostate glands from moribund Rosa26-SB11/T2/onc low-copy mice revealed focal areas of epithelial hyperplasia in the anterior prostate (data not shown). We also evaluated Rosa26-SB11/T2/onc low-copy (~ 25 copies of T2/onc) mice. Unlike the previously reported high rate of embryonic lethality in Rosa26-SB11/T2/onc high-copy (≥148 copies of T2/onc) mice (4), the ratio of Rosa26-SB11/T2/onc low-copy mice to single transgenic littermates was consistent with little or no embryonic lethality. However, these mice succumbed to tumor formation, primarily leukemias, with a median latency of 6 to 7 months.

**Immunohistochemistry suggested that the difference in prostatic abnormalities identified were infrequent sarcomas (data not shown). We also evaluated Rosa26-SB11/T2/onc low-copy (~ 25 copies of T2/onc) mice. Unlike the previously reported high rate of embryonic lethality in Rosa26-SB11/T2/onc high-copy (≥148 copies of T2/onc) mice (4), the ratio of Rosa26-SB11/T2/onc low-copy mice to single transgenic littermates was consistent with little or no embryonic lethality. However, these mice succumbed to tumor formation, primarily leukemias, with an median latency of 6 to 7 months.4 Examination of the histology of prostate glands from moribund Rosa26-SB11/T2/onc low-copy mice revealed focal areas of epithelial hyperplasia in the anterior prostate (data not shown) and dorsolateral prostate (Supplementary Fig. S1A and B). Immunohistochemistry suggested that the difference in prostatic phenotype for the two models could be explained by differences in transposase expression in the prostate gland (Supplementary Fig. S2).

The prostate of Rosa26-SB11/T2/onc low-copy mice was also evaluated using proliferation markers Ki-67 (20) and phospho-Ser10/histone H3 (21). In single transgenic control prostates

5 L.S. Collier, unpublished observation.
(Rosa26-SB11 or T2/onc low-copy only), Ki-67-positive cells were rare and typically found as single positive cells or a pair of positive cells (Supplementary Fig. S1C and D). In contrast, Rosa26-SB11/T2/onc low-copy double transgenic mice contained focal areas with many Ki-67-positive cells (Fig. 1). A quantitative analysis of the distribution of Ki-67-positive cells confirmed the increased clustering of Ki-67-positive cells into focal areas of elevated proliferation in Rosa26-SB11/T2/onc low-copy prostates relative to controls (Fig. 2A). In addition to the change in the distribution of proliferating cells, an overall increase in the number of proliferating prostatic epithelial cells was observed in Rosa26-SB11/T2/onc low-copy mice with both Ki-67 and phospho-Ser10-histone H3 (data not shown).

**Identification of transposon insertion sites in prostatic hyperplasia.** After failed initial attempts to identify transposon insertion sites present in the prostatic lesions using laser capture microscopy, the limits of the published linker-mediated PCR (LM-PCR) method (4, 5) were evaluated using DNA from two mice with well-characterized transposon insertions inherited through the germ line (22). These mice lacked transposase expression, so all somatic cells from each animal harbored identical transposon insertions. LM-PCR on serial dilutions of high-quality DNA showed that the isolation of transposon-flanking sequences became unreliable in the low nanogram range (Fig. 2B). A modified protocol (see Materials and Methods) allowed the reliable identification of the known transposon insertion sites from ~100 laser-captured cells isolated from these animals (data not shown). When this protocol was used to identify the transposon insertion sites present in foci of proliferation (clusters of three or more phospho-Ser10-histone H3-positive cells) from Rosa26-SB11/T2/onc low-copy prostates, complex banding patterns were observed following LM-PCR that varied from sample to sample (Fig. 2C).

To identify genes that can initiate proliferation/hyperplasia in the prostatic epithelium, 100 proliferating clusters of prostatic epithelial cells were laser captured from the prostates of 20 transposon-mutagenized mice on an otherwise wild-type genetic background (median animal age, 6 months), and 85 proliferating clusters of prostatic epithelial cells were captured from the prostates of 17 transposon mutagenized mice deficient for Arf (median animal age, 11 weeks). The laser-captured lesions were analyzed by LM-PCR, and the LM-PCR products were sequenced to determine the transposon insertion sites. Unique genomic sequences were mapped back onto the mouse genome using the mouse genome database maintained by the Wellcome Trust Sanger Institute. This analysis identified 77 unique insertions isolated from prostatic lesions generated by mutagenesis on a wild-type genetic background and 27 unique insertions isolated from prostatic lesions generated by mutagenesis on the Arf-null background. Insertions were distributed across most mouse chromosomes (Fig. 2D). The two chromosomes that harbored T2/onc concatomers in the experiment (chromosomes 1 and 15) had an increased frequency of insertions that was expected due to the tendency of transposons to mobilize at higher frequency to locations linked to the donor transposon concatomer (23), a phenomenon called local hopping. The prostate insertions were evaluated using previously proposed criteria for CIS identification (24). Using these criteria, three CIS were found in the prostate data set. The three genes at prostate CIS in the experiment were Rabgap1l, Pde4d, and Klhl13 (Supplementary Table S1). Rabgap1l is known to be located very close to the chromosome 1 T2/onc donor concatomer (5). Consequently, this gene may have been repeatedly mutated due to local hopping. The other two genes located at CIS were not linked to the T2/onc concatomers used in the experiment, so mutation of these genes likely contributed to the abnormal proliferation observed in the prostatic epithelium. Insertions in Pde4d were observed in proliferating lesions isolated from three independent mice. However, all of the insertions were in intron 5 with the MSCV promoter in T2/onc oriented toward exon 6 of Pde4d. Pde4d encodes several alternatively spliced transcripts including short-isoform transcripts that initiate translation at an in-frame AUG codon with a strong Kozak consensus located in exon 6 (Fig. 3). Thus, the T2/onc insertions would be predicted to cause overexpression of one or more endogenous short isoforms of Pde4d in prostatic epithelial cells. Transposon insertions in Klhl13 were observed in both promoter orientations.

The prostate insertion sites observed in this experiment were also compared with previously reported retroviral and transposon insertions from other cancer screens by searching the Retroviral Tagged Cancer Gene Database (25). Both Rabgap1l and Pde4d have been previously observed as CIS in other somatic mutagenesis screens for cancer genes (5, 26), whereas Klhl13 is a novel CIS.

**Figure 1.** Focal areas of elevated proliferation in the prostates of Rosa26-SB11/T2/onc mice. The possibility that the focal areas of hyperplasia in Rosa26-SB11/T2/onc low-copy double transgenic mice resulted from focal areas of increased proliferation was investigated by staining Rosa26-SB11/T2/onc low-copy double transgenic and single transgenic littermate (Rosa26-SB11 only or T2/onc low-copy only) controls with an antibody against the proliferation-associated Ki67 antigen (20, 21). Single transgenic control prostatic tissue sections contained rare Ki67-positive cells that typically appeared as individual cells or a pair of positive cells (see Supplemental Fig. 1). A and B, double transgenic Rosa26-SB11/T2/onc low-copy prostatic tissue sections contained clusters of multiple Ki67 positive cells in the dorsolateral prostate (example indicated by the arrow in A and shown at higher magnification in B); C and D, similar clusters of multiple Ki67 positive cells were observed in the anterior prostate (example indicated by the arrow in C and shown at higher magnification in D). Scale bars are shown for A and C in A, and for B and D in B.

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Cancer Res 2009; 69: (10). May 15, 2009 4390 www.aacrjournals.org

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Because many oncogenes and tumor suppressor genes function in multiple tumor types, we hypothesized that the combined prostate and Retroviral Tagged Cancer Gene Database datasets may identify new CIS. Using the criteria proposed by Mikkers and colleagues (24) for the \( f \approx 8,700 \) insertions in the combined datasets, five novel CIS were identified (Supplementary Table S2). Among the remaining insertion sites isolated from prostatic lesions, 11 were insertions into genes previously reported as CIS (Supplementary Table S3).

**Overexpression and mRNA isoform changes for PDE4D in human prostate cancer.** To investigate the possibility that PDE4D is involved in human prostate cancer, we collaborated with the laboratory of a board-certified surgical pathologist (W.H.). The Huang laboratory had previously assembled a tissue microarray of prostatic tissues from patients treated at the University of Wisconsin-Madison, including patients with benign prostatic hyperplasia and patients with prostate adenocarcinoma (17).

![Figure 2](image)

**Figure 2.** Analysis of proliferating prostatic lesions in Rosa26-SB11;T2/onc low-copy mice. To quantify the altered pattern of proliferation in the prostates of Rosa26-SB11;T2/onc low-copy mice, Ki67 positive cells were photographed and additional Ki67 positive cells within 25 \( \mu \text{m} \) were counted (\( n = 61 \) for single transgenic controls, \( n = 133 \) for double transgenic experimental mice). A, plot of the distribution of Ki67 positive cells relative to the number of additional Ki67 positive cells within 25 \( \mu \text{m} \) is shown. In single transgenic control prostates, there was an average of 0.62 additional Ki67 positive cells within 25 \( \mu \text{m} \) of a Ki67 positive cell. In Rosa26-SB11;T2/onc low-copy prostates, there was an average of 7.3 additional Ki67 positive cells within 25 \( \mu \text{m} \) of a Ki67 positive cell. The altered distribution of Ki67 positive cells for Rosa26-SB11;T2/onc low-copy prostates relative to single transgenic controls was highly significant (\( P < 0.0001 \) by t-test). B, when initial attempts to isolate transposon-genomic DNA junctions from laser captured proliferating prostatic lesions failed, the limitations of published linker mediated PCR protocols for cloning transposon-genomic DNA junctions were evaluated using serial dilutions of genomic DNA isolated from two mice with defined transposon insertions inherited through the germline. Animal #1 (left 4 lanes) harbored a single insertion while animal #2 (right 4 lanes) harbored 5 insertions. All insertions were reproducibly amplified. C, modified protocols for linker-mediated PCR and immunohistochemistry were used to achieve efficient amplification of transposon-genome junction fragments from laser captured cells isolated from tissue sections stained with an antibody against proliferation-associated marker phosphorylated Histone H3. When laser-captured proliferating lesions from the prostates of Rosa26-SB11;T2/onc low-copy double transgenic mice were analyzed by linker-mediated PCR, a variety of flanking genomic sequences were amplified and the pattern of amplified genomic fragments varied from lesion-to-lesion (samples 1-8). D, the chromosome distribution of unique genomic insertion sites (\( n = 104 \)) isolated from laser-captured proliferating lesions is shown with the asterisks indicating chromosomes harboring the T2/onc transgenic concatemers used in the experiment. Approximately 34% of the insertions were linked to the donor T2/onc concatemers while the remaining insertions were distributed across 17 of the 18 other chromosomes.

Overexpression and mRNA isoform changes for PDE4D in human prostate cancer. To investigate the possibility that PDE4D is involved in human prostate cancer, we collaborated with the laboratory of a board-certified surgical pathologist (W.H.). The Huang laboratory had previously assembled a tissue microarray of prostatic tissues from patients treated at the University of Wisconsin-Madison, including patients with benign prostatic hyperplasia and patients with prostate adenocarcinoma (17).

The approach used for analyzing PDE4D protein expression on the tissue microarray was a previously described method (18) for automated quantitative analysis. For the automated quantitative analysis approach, proteins were detected using fluorescence-based immunohistochemistry. The tissue microarray was co-stained with the antibody against PDE4D, an epithelial cytoplasm-specific antibody cocktail (E-cadherin and anti-cytokeratin antibodies), and a nuclear stain (4',6-diamidino-2-phenylindole). The stained tissue microarray was then imaged using a series of fluorescent filters corresponding to each stain, and the images were analyzed using...
previously developed algorithms (18) to determine relative expression levels of PDE4D. A parallel tissue microarray was analyzed in which the anti-PDE4D antibody was blocked with a PDE4D peptide to confirm specificity of the PDE4D signal. A strong PDE4D-specific signal was observed for the PDE4D antibody that colocalized with the E-cadherin/cytokeratin stains in human prostatic adenocarcinomas (Fig. 4A). The prostate tissue microarray cores from patients with prostatic adenocarcinoma varied in pathologic grade (as scored by surgical pathologist, W.H.) and included cores that were primarily composed of noninvasive epithelial cells (normal or PIN), cores that were primarily invasive cancer, and cores from sites of metastasis. When the automated quantitative analysis data were quantified and stratified by these pathologic features, all prostate tissues from patients with adenocarcinomas had higher PDE4D expression than tissues from patients with benign prostatic hyperplasia (Fig. 4B). No significant differences in PDE4D expression level were observed among the different pathologic grades for tissue cores from prostate cancer patients. PDE4D expression in prostate cancer was also evaluated by immunoblotting in normal primary prostatic epithelial cells and five common prostate cancer epithelial cell lines. PDE4D protein expression was increased in the human prostate cancer cell lines compared with normal primary prostatic epithelial cells (Supplementary Fig. S3).

To further understand the expression of PDE4D in human prostate cancer, pooled RNA from samples of pathologically normal prostate and prostatic adenocarcinomas were evaluated by RLM-5'-RACE (27). The predominant RLM-5'-RACE products from normal prostate and prostate cancer RNAs were distinct (Fig. 4C), suggesting altered PDE4D isoform expression in human prostate cancer. Sequencing of the predominant RLM-5'-RACE product obtained from the pooled prostatic adenocarcinoma RNA identified a cDNA with a first exon that began at base pair 349 of the Genbank deposited sequence for the PDE4D5 isoform (accession no. AF12073.1) and was spliced to exon 2 of PDE4D in a manner identical to the other PDE4D long isoform transcripts.

**PDE4D knockdown reduces the growth rate of human prostate cancer cells and xenografts.** As an initial test of the functional role of PDE4D in prostate cancer cells, PDE4D protein expression was reduced in DU145 and PC3 cells using three different shRNAs targeting sequences in PDE4D (Fig. 5). Based on densitometry of immunoblots with an anti-PDE4D antibody, these shRNAs achieved 70% to 97% knockdown of PDE4D protein expression (data not shown). In all cases, the knockdown cell populations had significantly reduced in vitro growth rates compared with control cells with stable transfection of scrambled sequence shRNAs (Fig. 5A-C). The effect of PDE4D knockdown on cell migration was also evaluated using an in vitro wound healing assay, and PDE4D knockdown significantly decreased the migration rate of DU145 cells (Supplementary Fig. S4).

To further evaluate the role of PDE4D in prostate cancer cells, we used the exon 14/15 PDE4D knockdown and scrambled shRNA control DU145 cell lines in a xenograft model. PDE4D knockdown resulted in smaller xenografts compared with control tumors (Fig. 6A; Supplementary Fig. S5A). The reduction in wet weight of the PDE4D knockdown xenografts was statistically significant, but there was considerable variability in wet weight among the xenografts in each group due to the presence of large cysts lacking viable tumor cells in many of the xenografts (Supplementary Fig. S5B-D). Consequently, we also assayed tumor cell proliferation using immunostaining for the Ki-67 antigen. Analysis of the Ki-67 labeling index for PDE4D knockdown and control xenografts found a statistically significant 39.0% reduction (P < 0.01, t test) in the Ki-67 labeling index for knockdown tumors relative to controls (Fig. 6B-D), indicating a reduced proliferation rate in response to PDE4D knockdown. We also examined apoptosis in the xenografts using a TUNEL assay. Whereas moderate rates of apoptosis were observed in all xenografts, no significant differences were observed between the control shRNA and PDE4D knockdown tumors (data not shown).
Figure 4. PDE4D is overexpressed and has altered mRNA isoform expression in human prostate cancer patient samples. A, fluorescent images from a prostate cancer core on a human prostate tissue microarray (TMA). The TMA was co-stained with an anti-E-cadherin/anti-cytokeratin cocktail (green channel) to identify the epithelial cell cytoplasm, DAPI (blue channel) to identify nuclei, and anti-PDE4D (red channel). PDE4D was detected in prostate cancer and co-localized with the epithelial cytoplasm-specific stain (yellow signal in top right panel). A parallel TMA was stained in the presence of a PDE4D peptide (lower row) and the anti-PDE4D signal was blocked, indicating that the anti-PDE4D signal reflected specific staining for PDE4D. B, graph showing the results of automated quantitative analysis (18) for PDE4D staining on the TMA. The higher PDE4D expression level in patients with prostatic adenocarcinoma relative to patients with benign prostatic hyperplasia (BPH) was statistically significant (P < 0.001 by t-test). Error bars show the 95% confidence interval. When the tissues from adenocarcinoma patients were stratified by the histopathology of the tissue core into cores that contained primarily non-invasive epithelium (normal ducts or PIN), primarily invasive cancer, or cores from sites of metastasis, the PDE4D expression level differences among the different samples from adenocarcinoma patients were not statistically significant. C, RNA pools made from 10 prostatic adenocarcinoma samples (left lane) or 10 histologically normal prostate patient samples (right lane) were analyzed by RLM-5'-RACE using primers in exon 10 of PDE4D that would allow all known PDE4D isoforms to be amplified (location of the primers shown in Fig. 3B), and the resulting pattern of amplified 5'-cDNA ends was distinct for the two pools. D, sequencing data are shown for the major RLM-5'-RACE product amplified from human prostatic adenocarcinoma RNA. This data showed that the first exon of the amplified transcripts began at base pair 349 of the Genbank deposited sequence for the PDE4D5 isoform (accession AF12073.1) and was spliced to exon 2 of PDE4D in a manner identical to the other PDE4D long isoforms. The location of the inferred transcriptional start site is indicated in the diagram in Fig. 3B.

not shown), suggesting that the reduction in tumor size was primarily due to decreased proliferation in response to PDE4D knockdown.

Discussion

Genetic screens in model organisms have been an important strategy for understanding the molecular genetics of several cancers. For many years, one of the most productive screening approaches has been the use of slow transforming retroviruses as insertional mutagens to tag and identify endogenous oncogenes and tumor suppressor genes in mice (reviewed in ref. 28). Screens using retroviruses have investigated several tumor types including mammary tumors, hematopoietic neoplasms, and brain tumors. These screens have identified genes and genetic interactions that have proven to be directly relevant for understanding the equivalent human cancers. One of the major limitations of retroviruses as a screening tool is their cell type specificity that
defines the types of tumors they can induce. This has prevented retroviral-based screens for many of the most common tumor types in humans.

Recently, the SB transposon system has been shown to function as an insertional mutagen to discover cancer genes for hematopoietic tumors, medulloblastomas, and sarcomas in genetically modified mice (4, 5). These initial proof-of-principle studies showed that mobilization of transposons engineered to have gain- and loss-of-function elements could accelerate tumor formation, and cloning transposon insertion sites identified candidate cancer genes at CIS. Transposon-based screening has great potential because mobilization of the transposon is not cell type restricted, so screens are possible in tissues not amenable to retroviral screens such as the sarcomas investigated by Collier and colleagues. However, it has previously not been determined if transposon-based screens can be used to investigate epithelial tumors that constitute the majority of human cancers. In addition, although the candidate cancer genes at the CIS observed by Dupuy and colleagues and Collier and colleagues included genes not previously implicated in cancer, it is not yet clear whether these candidate cancer genes will prove to be relevant for understanding disease processes in humans.

The current study addresses these issues by investigating transposon-mutagenized mice for prostate cancer-related phenotypes. The prostates of Rosa26-SB11;T2/onc low-copy mice had focal areas of epithelial hyperplasia and elevated epithelial proliferation that resembled features of prostate cancer precursor lesions. Cloning transposon insertion sites from these lesions identified candidate cancer genes at transposon CIS. We conducted our screen using both wild-type and Arf-null genetic backgrounds. Insertions were observed at newly identified and previously known CIS on both genetic backgrounds. Based on the CIS in the Rettoviral Tagged Cancer Gene Database (25), ~2.2% of all genes have been identified as CIS in one or more cancer screens. For our screen, 19% of insertions on a wild-type genetic background were into CIS genes reflecting a 9-fold enrichment over what would be predicted by chance. For insertions on the Arf-null background, 41% of insertions were into CIS genes, reflecting a 19-fold enrichment over what would be predicted by chance. These data suggest that most of the insertions into CIS genes in our screen were likely to be selected insertions that contributed to the focal prostate epithelial hyperplasia and proliferation observed in Rosa26-SB11;T2/onc low-copy mice. Further investigation of one
mRNA expression observed in some expression profiling studies may reflect post-transcriptional regulation of PDE4D. Comprehensive side-by-side analyses of mRNA and protein expression for multiple PDE4D isoforms in the rat have found a poor correlation between mRNA and protein expression levels for multiple PDE4D isoforms. This has led to the hypothesis that these PDE4D isoforms are post-transcriptionally regulated (31). A more dramatic finding in our study was a shift in PDE4D isoform usage in prostate cancer that we identified in RLM-5'-RACE experiments (Fig. 4C and D). The RLM-5'-RACE protocol includes ligation of a RNA adaptor to mRNAs before reverse transcription in a reaction that requires the presence of a 7-methylguanosine cap at the 5'-terminus of the mRNA (27). This allows RLM-5'-RACE to selectively amplify full-length cDNA 5'-ends. We infer from our data that the predominant PDE4D transcript in prostatic adenocarcinomas is initiated at base pair 349 of the Genbank deposited sequence for the PDE4D5 isoform (accession no. AF12073.1). Transcripts initiated at this location would not include the normal translation initiation codon for the published PDE4D5 transcript and would presumably initiate translation at one of the in frame AUG codons present in the truncated mRNA transcript. This possibility is consistent with the size of the PDE4D protein bands observed by immunoblot in prostate cancer cell lines (Supplementary Fig. S3).

Collectively, the increased expression of PDE4D and altered isoform usage in prostate cancer cells indicate that there will be lower cAMP levels in at least some subcellular compartments of prostate cancer cells relative to normal prostatic epithelial cells. This correlates with previous in vitro studies in prostate cancer cell lines that have found that increasing levels of cAMP by stimulating adenylate cyclase with forskolin, using cAMP analogues, or treatment with general phosphodiesterase inhibitors led to growth arrest and apoptosis of prostate cancer cell lines (37–39). Our data that selectively targeting PDE4D with shRNAs also limits prostate cancer cell growth suggest that future studies with PDE4D selective inhibitors as potential anti-prostate cancer agents are warranted.

Considered more broadly, pharmacologic inhibition of the PDE4 family of enzymes induces apoptosis or inhibits growth in a variety of cancer cell types. However, the role of PDE4D specifically in human cancer has not been widely investigated. PDE4 family inhibition induces apoptosis in chronic lymphocytic leukemia cells of the CIS genes, Pde4d, showed that it is a mitogenic factor for prostatic epithelial cells that is also overexpressed in human prostate cancers. Thus, transposon-based screens in mice can discover novel genes that are directly relevant for understanding human prostate cancer.

PDE4D is a gene encoding several phosphodiesterase enzymes that cleave cAMP and are expressed in multiple tissues (29–34). PDE4D encodes at least nine protein isoforms that all possess the phosphodiesterase catalytic domain in the COOH terminus but differ at the NH2 terminus. The nine isoforms are categorized into two groups: long isoforms (3-5 and 7-9) and short isoforms (1, 2, and 6). In our study, we identified multiple transposon insertions into the mouse Pde4d gene that are predicted to cause over-expression of one or more short isoforms. A previous investigation of rats that had increased susceptibility to prostate carcinogenesis due to developmental exposure to estrogens found increased expression of PDE4D, one of the long isoforms, in the prostates of susceptible rats (35). This led the authors to hypothesize that PDE4D overexpression had a causal role in prostate cancer susceptibility in the rat model. Our finding that PDE4D protein is overexpressed in the prostatic epithelial cells of patients with prostatic adenocarcinoma relative to patients with the benign disease benign prostatic hyperplasia regardless of the histopathology of the epithelial cells in the adenocarcinoma patients (normal, PIN, invasive cancer, and metastasis) suggests that PDE4D overexpression in the aging human prostate may also be a permissive event that increases susceptibility to prostate cancer.

The status of PDE4D in human prostate cancer has not previously been specifically investigated, but genome-wide expression profiling studies of human prostate cancer often included probes against PDE4D transcripts. We reviewed PDE4D for expression changes in the available prostate cancer expression profiling studies using the Oncomine Database (36). The available expression profiling studies generally observed no change for PDE4D mRNA expression or a decrease in expression with increasing pathologic grade of prostate cancer. The potential discrepancy between our finding of an increase in PDE4D protein expression in prostate cancer patients and the decreased PDE4D mRNA expression observed in some expression profiling studies...
Prostate cancer is the most common cancer in men (6) and there is considerable genetic heterogeneity among individual prostate cancers (14). Transposon-based somatic mutagenesis is a promising strategy for uncovering the genetically diverse ways that prostate tumors can evolve. This study refined the methodology for transposon-based screening to allow investigation of small lesions that resembled the initial steps of prostate cancer progression. This identified Pde4d as a proliferation promoting factor in the prostate of mice and humans. In addition to Pde4d, this study identified 18 other genes as candidate prostate cancer genes including genes at CIS in the prostate data set (Supplementary Table S1), genes at CIS for the combined prostate + Retroviral Tagged Cancer Gene Database data sets (Supplementary Table S2), and prostate insertions at genes that have previously been identified as CIS (Supplementary Table S3). Among the 19 candidate prostate cancer genes identified in our study were 5 genes that have previously been investigated for their potential roles in prostate cancer including Dpt, Notch1, Runx3, Igf1r, and Ghr (representative studies: refs. 45–49). The remaining 14 candidate prostate cancer genes have not previously been investigated for their potential roles in prostate cancer. Future studies are anticipated to include evaluation of these genes in human prostate cancers and additional screening for candidate cancer genes using the refined methods developed for this study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 10/7/08; revised 2/25/09; accepted 3/3/09; published OnlineFirst 4/28/09.

Grant support: Department of DefenseCongressionally Mandated Research Program award W81XWH-05-1-P006017 and NIH/National Institute on Aging grant AG04247 (P.C. Marker), National Cancer Institute/NIH grant CA118600 (D.A. Largaespada), American Cancer Society postdoctoral fellowship PF-05-153-01 (MGO) and National Cancer Institute/NIH grant CA122183 (L.S. Collier), and National Cancer Institute/NIH training grant T32CA936 (E.P. Bahrmann and S.L. Kudack). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank M. Roussel for the Aaf-null mice, A. Dupuy, N. Copeland, and N. Jenkins for the Rosa26-SB11 mice, and Erin Riley for technical assistance on the project.

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(40). However, this effect may not be due to PDE4D specifically, as PDE4D mRNA expression was found to be lower in chronic lymphocytic leukemia cells than in normal peripheral blood mononuclear cells (41). Analysis of genomic copy number changes in human lung adenocarcinoma has detected homozygous deletions at the PDE4D locus (42). Similar studies on esophageal adenocarcinoma identified both homozygous deletions and loss of heterozygosity at the PDE4D locus (43), implicating PDE4D as a tumor suppressor gene in these two tumor types. As regulation of PDE4D activity is complex, close examination of its genomic sequence, transcriptional regulation, alternative splicing, and other post-transcriptional/translational modifications will be necessary to address the contribution of PDE4D to tumor formation in humans. In addition, it is possible that the ability of PDE4D to enhance or limit tumor growth may be cell type dependent, a phenomenon that has been observed for other cancer genes such as NOTCH1 (44).


Transposon Screen Implicates PDE4D in Prostate Cancer
Identification of \textit{PDE4D} as a Proliferation Promoting Factor in Prostate Cancer Using a \textit{Sleeping Beauty} Transposon-Based Somatic Mutagenesis Screen
