Prostate-Derived Ets Factor (PDEF) Inhibits Metastasis by Inducing Epithelial/Luminal Phenotype in Prostate Cancer Cells

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Abstract

Metastasis is the primary cause of prostate cancer morbidity and mortality. Our previous studies revealed that Sam pointed domain ETS transcription factor, a.k.a. prostate-derived ETS factor (SPDEF/PDEF), inhibits prostate cancer metastasis. However, the mechanism is still unclear. In this study, using microarray and gene set enrichment analysis, we discovered that PDEF upregulated epithelial/luminal differentiation-related genes while it suppressed stemness and epithelial-to-mesenchymal transition-related genes, especially Twist1. We also observed loss of PDEF and gain of Twist1 expression during prostate cancer progression in the TRAMP mouse model. Moreover, Twist1 knockdown resulted in upregulation of PDEF expression, suggesting a reciprocal regulation between PDEF and Twist1. Mechanistically, our ChIP-seq analysis revealed that PDEF directly regulated cytokeratin 18 (CK18) transcription through the GGAT motif within its putative promoter region. CK18 knockdown resulted in increased expression of Twist1, suggesting that PDEF regulated Twist1 in part via CK18. Our analysis of multiple clinical prostate cancer cohorts revealed an inverse relationship between PDEF expression and tumor grade, tumor metastasis, and poor patient survival. Furthermore, a two-gene signature of low PDEF and high Twist1 can better predict poor survival in prostate cancer patients than either gene alone. Collectively, our findings demonstrate PDEF inhibits prostate tumor progression, in part, by directly regulating transcription of CK18, and that PDEF/Twist1 expression could help distinguish between lethal and indolent prostate cancer.

Implications: This study reports the novel findings that PDEF suppresses Twist1 partly via CK18 and that PDEF/Twist1 expression could help distinguish between lethal and indolent prostate cancer.

Visual Overview: http://mcr.aacrjournals.org/content/molcanres/16/9/1430/F1.large.jpg.

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Introduction
Prostate cancer is the most commonly diagnosed cancer in men and is responsible for over 26,000 deaths in the United States each year (1). Current androgen-deprivation therapy yields a high 5-year survival rate; however, most patients relapse to metastatic castration-resistant prostate cancer (mCRPC), for which no effective therapy is available (2, 3). Epithelial-to-mesenchymal transition (EMT) plays an important role in prostate cancer metastasis (4). It has been shown to promote drug resistance, cellular plasticity, and a more invasive phenotype (5). The EMT process is orchestrated by core oncogenic transcription factors, including Twist1, Snai1, Slug and Zeb1 (6). Twist1 directly inhibits the transition (EMT) plays an important role in prostate cancer metastasis (4). It has been shown to promote drug resistance, cellular plasticity, and a more invasive phenotype (5). The EMT process is orchestrated by core oncogenic transcription factors, including Twist1, Snai1, Slug and Zeb1 (6). Twist1 directly inhibits the Twist1, Snai1, Slug and Zeb1 (6). Twist1 directly inhibits the E-cadherin–mediated cell–cell adhesion through suppression of its transcription, while it promotes the expression of N-cadherin (7, 8). Furthermore, Twist1 primes cells for prostate cancer metastasis and is correlated with disease progression (9).

Unlike the oncogenic annotation of most Ets factors, prostate-derived Ets factor (PDEF) has been acknowledged as a tumor suppressor and is highly expressed in the epithelial layer of the lumen-containing organs, especially the prostate (10–13). First identified as an activator of the androgen receptor (AR) (10), PDEF binds to DNA through a conserved central "GGG" tri-nucleotide motif (14). Mechanistic studies revealed that PDEF interacts with AR and prostate-specific transcription factor Nkx3.1 on the prostate-specific antigen (PSA) enhancer region and mediates its transcription activation (10, 15). Recently, PDEF has been characterized as one of the cell identity–related super-enhancer–driven transcription factors in the luminal prostate cancer cell line LNCaP (16). We and other groups reported that PDEF loss is associated with a higher Gleason score as well as poor patient survival (17–22). Our studies using luciferase-tagged prostate cancer cells demonstrated for the first time that PDEF suppressed prostate cancer metastasis in vivo (18). Studies from Xin-Hua Cheng and colleagues showed that loss of PDEF induced cancer progression, while expression of PDEF inhibited tumorigenesis in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, in part through the inhibition of FOXM1 (21). In the context of metastatic prostate cancer, PDEF inhibits Slug and MMPs levels (19, 22). Furthermore, oncogenes such as HOXB13 and CDK11p58 suppress PDEF expression (23, 24).

A mature human prostate consists of three types of epithelial cells: luminal, basal, and neuroendocrine cells (25). Terminal differentiation secretory luminal cells form a layer surrounding the lumen. At puberty, various signals including Nkx3.1, Foxa1 and c transcription factor Nkx3.1 and 1% penicillin (5,000 u/mL)/streptomycin (500 μg/mL) at 37°C, 5% CO₂ in a humidified incubator as previously described (17). Briefly, PDEF was cloned with an amino-terminal FLAG tag and inserted into a retroviral vector pBABE. After transfection, puromycin selection was initiated and continued for 3 weeks to generate stable cell line. *Mycoplasma* test is routinely performed in the lab. Cell lines were authenticated by the STR profiling cell authentication service provided by ATCC.

Materials and Methods
Cell culture
PC3-PDEF and DU145-PDEF were generated in the lab and maintained in Dulbecco’s Modified Eagle Medium/Nutrient Mix F-12 (DMEM/F12) supplemented with 10% fetal bovine serum and 1% penicillin (5,000 u/mL)/streptomycin (500 μg/mL) at 37°C, 5% CO₂ in a humidified incubator as previously described (17). B brief, PDEF was cloned with an amino-terminal FLAG tag and inserted into a retroviral vector pBABE. After transfection, puromycin selection was initiated and continued for 3 weeks to generate stable cell line. *Mycoplasma* test is routinely performed in the lab. Cell lines were authenticated by the STR profiling cell authentication service provided by ATCC.

Animals and tumor xenografts
Transgenic mouse (Tg(TRAMP)82427Nq) in this study were obtained from The Jackson Laboratory and housed according to the Institutional Animal Care and Use Committee (IACUC) recommendations. The identity of the transgenic mouse was confirmed by the polymerase chain reaction-based DNA screening according to the instructions provided by The Jackson Laboratory. PC3-SCR and PC3-PDEF xenograft tissue samples were obtained from previous study (18).

Transfections and vectors
Transfection was performed on 4D-Nucleofactor (Lonza) using cell-specific programs carried out with 2 × 10⁶ cells suspended in transfection cuvettes with 3 μg plasmid DNA according to the manufacturer. Twist1 shRNA bacterial stock (TRC number: TRCN0000378362; sequence: CCGGATGGCAAGCTGCAGCT-ATGTGGCTGGACGACATACGCTGACGAGCTG- CAAATGAGAAGGTAGTTTTT) was purchased from MilliporeSigma. Plasmids were extracted with Plasmid Midi Kit (Qiagen).

Immunofluorescence, Immunohistochemistry and immunoblotting
For immunofluorescence, cells were seeded on the chamber slides and fixed with 3.7% formaldehyde in PBS. Then slides were incubated with cold 70% acetone and 30% methanol mixture for 5 minutes on ice for permeabilization and blocked in 5% BSA for 1 hour. Overnight primary antibody incubation and 1 hour secondary antibody were performed in a humidified chamber at 4°C and room temperature respectively. IHC and immunoblotting were performed as previously described (30). Quantitative analysis of immunoblot data was performed with Image Studio Lite software (LI-COR Biosciences). Immunoblotting was visualized using Odyssey Clx imaging system (LI-COR Biosciences). Original immunoblot data and statistical analysis can be found in Supplementary Figs. S5–S10. Antibodies used are listed in Supplementary Table S3. Quantitative real-time RT-PCR (qRT-PCR): Total RNA was extracted from cell culture with EZNA total RNA kit (OMEGA Bio-tek). cDNA was
generated using iScript cDNA synthesis kit (Bio-Rad), and qPCR was performed on LightCycler480 (Roche) as previously described (29). qPCR results were analyzed with ddCT method normalized with HPRT (internal control). Primers used are listed in Supplementary Table S4.

**Invasion and wound healing assay**

Invasion assay was performed as previously described (18). Wound healing assay was performed by the INLET research core facility in Louisiana State University Health Sciences Center Shreveport with IncuCyte (Essen BioScience). Cells were seeded at a density of $2 \times 10^5$ per well in a 96-well plate. On the following day, wounds were made with the IncuCyte cell migration kit, and cells were kept in 1% FBS DMEM/F12 medium to minimize cell proliferation. Images were taken by the IncuCyte system every 4 hours and analyzed with IncuCyte ZOOM software (Essen BioScience).

**Microarray and GSEA**

Total RNA for microarray gene chip was isolated using the RNeasy Mini Kit (Qiagen). The integrity of total RNA was assessed using the Bioanalyzer and Microarray was performed on Affymetrix platform with the HuGene-1.0-st-v1 chip, a service provided by Microarray core facility at the University of Colorado School of Medicine, Aurora, CO. Microarray data were analyzed using Affymetrix Transcriptome Analysis console software. GSEA was performed using default setting (31), and gene sets were extracted from MySigDB v6.0 (http://software.broadinstitute.org/gsea/mysigdb). Scatterplot was generated with R (32). Microarray data have been uploaded to GEO repository (GSE108641).

**ChiP, ChiP-qPCR, and ChiP-seq analyses**

ChiP experiments were performed with a ChiP seq kit (Chromatrap) following the instructions from the manual (https://www.chromatrap.com/uploads/2017-01-24-58-1-chromatrap-chip-seq-protocol-v.pdf). Immunoprecipitated DNA was purified with the QiAquick PCR purification kit (Qiagen). qPCR was performed as described (29), with the primers listed in Supplementary Table S4. ChiP-qPCR results were analyzed with the fold enrichment method. ChiP-seq analysis was carried out on the Galaxy platform (https://usegalaxy.org/). ChiP-seq dataset (SRP002475) was extracted from the NCBI database and aligned with Bowtie2. MACS2 was used to identify statistically significant peaks with default settings. ChiP-seq results were visualized in Integrated Genome Browser (BioViz) or Integrated Genome Viewer (Broad Institute).

**Analysis of PDEF and Twist1 in human prostate cancer**

Human prostate cancer microarray datasets GSE6919 and GSE16560 were obtained from the GEO archive (33). TCGA prostate cancer dataset was obtained from BioPortal (http://www.chiportal.org/). In GSE16560, patients were subclassified into two extreme groups: men died of prostate cancer and men who survived more than 10 years without metastasis (lethal and indolent respectively). Statistical analysis of differences in median expression levels for transcripts between the two samples groups was performed using the nonparametric Mann–Whitney test. Survival analysis was performed by stratifying patients in GSE16560 by the expression level of PDEF and Twist1 into “low” (lowest forth), “middle” (2nd and 3rd fourth), and “high” (highest fourth) expression groups. Statistical analysis of Kaplan–Meier survival curves for different strata was performed with the Gehan–Breslow–Wilcoxon test. All microarray data analysis was performed using SPSS (IBM) and GraphPad Prism.

**Analysis of transcription factors associated with poor patient survival**

Clinical data were extracted from GEO16560 and prepared with R (32). A list of human transcription factors was obtained from the Animal Transcription Factor Database (http://www.bioguo.org/AnimalTFDB/species.php?spec=Homo_sapiens). The survival analysis was carried out using Kaplan–Meier survival analysis and log-rank test while the expression of each transcription factors was stratified with interquartile into 3 levels (low: lowest quartile; high: highest quartile; middle: rest of the patients). The median survival of each patient group with the low, middle and high levels of a particular transcription factor was shown respectively in Supplementary Table S1. Known lethal prostate cancer transcription factors were found in the list such as FOXM1 whose prognostic value has been reported. For Supplementary Table S2, patients were stratified using the PDEF mRNA level with the interquartile method. The Mann–Whitney test signifies the significantly differentially expressed transcription factors in the low- and high-PDEF group in GSE16560.

**Statistical analysis**

Unless otherwise stated, all samples were assayed in triplicate. All in vitro experiments were repeated with three independent biological replicates. GraphPad Prism was used to calculate SD and statistically significant differences between samples using unpaired independent sample Student t test. P values are shown in the figures.

**Results**

**Expression of PDEF decreases expression of sets of genes involved in cell migration and tumor cell metastasis**

In the present study, we expressed Flag-tagged PDEF in two metastatic prostate cancer cell lines (DU145 and PC3). We verified the expression of PDEF with two commercially available antibodies [PDEF (H1250) and PDEF (G110)] as well as with anti-FLAG antibody. Results (Fig. 1A) demonstrate that both antibodies recognize PDEF, as is confirmed by anti-Flag expression. In order to evaluate effects of PDEF on modulation of gene expression in an unbiased fashion, we evaluated global mRNA differential expression between PC3-SCR and PC3-PDEF cells using the Affymetrix platform. We confirmed that Flag-PDEF retains its biological function (as evidenced by negative enrichment of PDEF-suppressed target gene set in PC3-SCR cells (Supplementary Fig. S1B)). In a scatter-plot analysis, we observed a positive correlation for EMT-related genes in control (PC3-SCR) cells (Supplementary Fig. S1A). Hierarchical clustering analysis indicates the distinct transcription profile triggered by expression of PDEF in PC3 cells (Supplementary Fig. S1C). Using GSEA we observed that expression of PDEF in PC3 cells results in negative enrichment of migration, metastasis as well as basal phenotype-related gene sets (Fig. 1B–D). Taken together, these results indicate that PDEF transcriptionally suppresses the expression of sets of genes involved in cell migration and metastasis.

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PDEF suppresses prostate cancer cell metastasis through downregulating core EMT-related factors including Twist1

GSEA analysis demonstrated that PDEF expression negatively correlates with the expression of sets of genes involved in cell migration and tumor metastasis. We then used immunofluorescence and Western blot to verify the levels of some core EMT-related proteins. In accordance with GSEA results, expression of PDEF resulted in increased E-cadherin and decreased Twist1 protein levels (Fig. 2A and B; Supplementary Fig. S2A) in both prostate cancer cell lines. These results also showed that PDEF expression was associated with decreased vimentin protein in DU145 cells but not in PC3 cells. Transient PDEF knockdown using shRNA in LNCaP cells led to an increased abundance of Twist1 and Vimentin with decreased expression of E-Cadherin and PSA (known PDEF target gene; Fig. 2C). In order to test whether the alterations in genes associated with EMT at mRNA and protein level can lead to phenotypic changes, we evaluated the effects of PDEF expression on cell migration as well as cell invasion. Cell migration/wound healing assay was performed using IncuCyte in a real-time fashion, while cell invasion was monitored in Transwell invasion assay. We found that PDEF expression impairs the cell migration capacity, as is evidenced by the increased time required for cells to reach 50% relative wound density (~40 hours vs. ~20 hours; Fig. 2D and E). Furthermore, we observed that PDEF expression decreased the number of cells that were able to invade across Matrigel-coated membranes (Supplementary Fig. S2B).

Besides metastasis, lineage plasticity may play a role in transition to neuroendocrine phenotype in advanced prostate cancer patients. Moreover, aggressive and metastatic prostate cancer cell lines (PC3 and DU145) show altered expression of several phenotype-related genes (34). To address the extent to which PDEF expression was associated with restriction of lineage plasticity, we utilized a qPCR panel consisting of the core genes involved in EMT, prostate differentiation, neuroendocrine phenotype, and cellular stemness (qPCR panel described in ref. 34). Results presented in Fig. 2F showed that the expression of PDEF leads to the suppression of neuroendocrine phenotype-related genes, EMT-related genes, and cellular stemness-related genes while promoting the expression of prostate differentiation-related genes, suggesting a cell identity shift towards epithelial/luminal cells and away from neuroendocrine cells and stem cells phenotype.

Decreased PDEF expression and increased EMT- and NEPC-related proteins during tumor progression in TRAMP mice

We observed that expression of PDEF inhibited EMT- and NEPC-related genes. These observations prompted us to evaluate the expression of PDEF, Twist1 and CHGA during tumor progression in vivo. We used a TRAMP model, a spontaneous model of prostate cancer development and progression. Prostate sections from different stages of TRAMP mouse were tested for expression of PDEF, Twist1, and CHGA by IHC. We found that PDEF...
expression is limited to the luminal cells of the prostate in wild-type mice as well as in the early-stage (12 weeks old) of TRAMP mice (Fig. 3). We also noted that PDEF expression is completely lost at aggressive disease stage (30 weeks old; Fig. 3). In contrast to PDEF, Twist1 and CHGA expression was undetected in similar sections from wild-type mice as well as in early stages of prostate cancer from TRAMP mice; however, we noted high levels of Twist1 and CHGA expression in prostate sections from late-stage cancer (Fig. 3). These observations paralleled with loss of E-cadherin and gain of Vimentin during prostate cancer progression in the TRAMP mouse model (Fig. 3; Supplementary Fig. S2C), indicate that PDEF expression is inhibited, and Twist1 and CHGA expression is turned on during prostate cancer progression in the TRAMP model.

Figure 2.
PDEF suppresses prostate cancer cell metastasis through downregulating core EMT-related factors including Twist1. A, Immunofluorescence for E-Cadherin and DAPI in PC3-SCR and PC3-PDEF cells. Images were taken under 40×. Scale bar, 50 μm. B, Immunoblot analysis of EMT-related protein. Corresponding GAPDH bands are shown for each blot. C, Western blot analysis comparing probing for indicated proteins expression in LNCaP cells when treated with shRNA toward PDEF. D, Analysis of relative wound density in wound healing assay comparing the migration rate of PC3-SCR and PC3-PDEF cells. E, Wound healing assay comparing PC3-SCR and PC3-PDEF. Snapshots at specific time points (0 and 24 hours) from microscope were used as representative images. F, qRT-PCR analyses for key prostate epithelial differentiation– (PD), neuroendocrine differentiation– (NE), EMT– and stem cell (SC)–related markers on PC3-SCR and PC3-PDEF. The Y-axis represents log2-transformed fold change in HPRT-normalized mRNA expression when PDEF was overexpressed.
Reciprocal regulation between PDEF and Twist 1

Our results suggest a negative correlation between PDEF and Twist1. In order to study the mechanism behind the interplay between PDEF and Twist1, we used shRNA to knockdown Twist1 in PC3-SCR cells. Western blots showed a significant restoration of E-cadherin, a known downstream target suppressed by Twist1 (Fig. 4A). We also observed an increased PDEF and decreased Vimentin levels after Twist1 knockdown (Fig. 4A). Next, we analyzed the promoter sequence of PDEF from mouse and human genome for Twist1 binding motifs. However, we could not identify any Twist1 binding sites according to multiple algorithms available on TRANSFAC. These considerations rule out direct regulation of PDEF by Twist1. We also extracted a raw data set of PDEF ChIP-seq experiments performed in VCaP cells (14) and reanalyzed these data with model-based analysis for the ChIP-Seq (MACS; ref. 35). Our analysis pipeline was confirmed with the significant PDEF binding peak in the PSA enhancer (ARE3) region colocalized with AR and pol II binding peak (Fig. 4B; Supplementary Fig. S3A). These data indicated AR and PDEF co-occupy PSA enhancer (ARE3). However, we could not detect any PDEF binding peak near Twist1 gene (Fig. 4C; Supplementary Fig. S3C). These data point to indirect regulation of Twist1 by PDEF and suggest that PDEF may regulate Twist1 indirectly. Taken together, our data suggest that indirect reciprocal regulation between PDEF and Twist1.

PDEF inhibits Twist1 in part through transcriptional regulation of CK18

CK18 along with CK8 has been widely acknowledged as the marker(s) of prostate luminal cells. The molecular mechanism of the transcriptional regulation of CK18 in luminal epithelial cells in the prostate is unknown. Our analysis of the ChIP-seq

Figure 3.
Decreased PDEF expression and increased EMT- and NEPC-related proteins during tumor progression in TRAMP mice. Representative IHC staining for (from left to right) Twist1, PDEF, E-Cadherin, Vimentin, and Chromogranin A in prostate tissue sections from 12-week (top) and 30-week (bottom) old TRAMP mouse. Twist1 was stained using alkaline phosphate while other probes were stained with DAB. Images were taken under 40×. Scale bar, 50 μm.

Figure 4.
Interplay between PDEF and Twist1. A, Immunoblot analysis of Twist1, E-Cadherin, Vimentin and PDEF protein level in PC3-SCR cells with knockdown control (shNT) or Twist1 knockdown (shTwist1). Statistical analysis showed significant downregulation of Twist1 protein and a significant increase of PDEF protein. ChIP-seq analysis was performed on Galaxy platform, data were extracted from SRP002475 and aligned with Bowtie2. MACS2 was used to identify peaks. Gene tracks view of PDEF ChIP-seq and IgG control at the KLK3 (PSA) locus (B) and Twist1 locus (C).
data revealed for the first time that PDEF occupies the putative promoter region of CK18 (gene name KRT18; Fig. 5A). The binding is independent of AR (Supplementary Fig. S3B). Further analysis with the pol II ChIP-seq from the same cell line demonstrated the colocalized of pol II and PDEF in the proximate region within the putative promoter region of CK18.

Figure 5.
PDEF binds to the promoter of CK18 and induces its expression in vitro and in vivo. A, Gene tracks view of PDEF ChIP-seq and IgG control at the CK18 locus. B, ChIP-qPCR analysis of Flag-PDEF binding enrichment on the PSA enhancer region (ARE3) and the CK18 promoter region (PBCK18). C, qPCR and immunoblot analysis of CK18 mRNA and protein level in PC3-SCR/PDEF and DU145-SCR/PDEF cells. D, The positive correlation between PDEF and CK18 in TCGA cohorts. Spearman r test was used and P values are shown in the graph. E, Representative IHC staining for CK 18 in PC3-SCR and PC3-PDEF xenograft. F, Immunoblot analysis of E-cadherin and Twist1 levels after CK18 knockdown by two independent siRNAs in LNCaP. G, Immunoblot evaluation of PDEF and CK18 levels after CK18 knockdown by two independent siRNAs.
Discussion

Our results showed that PDEF downregulated Twist1 and upregulated CK18 expression in prostate cancer cells, suggesting a potential regulatory role for PDEF in modulating Twist1 expression. Results presented herein show that PDEF inhibits Twist1 expression in part by directly modulating CK18. We also provide the first direct evidence of decreased PDEF and increased Twist1 during prostate cancer progression in the TRAMP mouse model. Twist1, a basic helix–loop–helix (bHLH) transcription factor, plays key roles in embryonic development, cell lineage determination, and differentiation, but is lost in adult tissues (36). However, the Twist1 expression is reported in several human cancer tissues including prostate cancer and invariably correlates with tumor progression as well as metastasis (9, 37). Mechanisms leading to the reactivation of Twist1 in human cancers are not completely understood. It is tempting to speculate that PDEF directly regulates Twist1 transcription, but our analysis of PDEF-ChIP-seq data revealed that no significant PDEF binding peaks were found within the Twist1 promoter region. Taken together, these results suggest that PDEF modulates Twist1 indirectly. We also observed that decreased Twist1 results in upregulation of PDEF expression, suggesting for the first time a reciprocal regulation between PDEF and Twist1. However, our in silico analysis identified no conserved Twist1 binding motif (CANNTG) either, showing that Twist1 regulates PDEF indirectly.

Analysis of PDEF expression in several prostate cancer clinical cohorts revealed that PDEF expression inversely associated with tumor progression, tumor metastasis, and poor survival. In contrast, Twist1 expression positively correlated with poor clinical outcome. Integrating PDEF and Twist1 expressions could better predict survival in prostate cancer patients as compared with PDEF or Twist1 alone. These observations raise the possibility that integrated PDEF and Twist1 expressions might be useful markers to distinguish between an indolent versus lethal prostate cancer. Although previous studies reported that PDEF or Twist1 expression in limited samples is an independent indicator of aggressive prostate cancer, results presented are the first direct demonstration that integrated PDEF and Twist1 expressions can distinguish lethal prostate cancer from an indolent disease (9, 17, 38). Our results presented are the first direct demonstration that integrated PDEF and Twist1 expressions can distinguish lethal PCa from an indolent disease. These results are similar to the observations of Cheng et al. where another oncogenic transcription factor FOXM1 was integrated with PDEF (GSE16560; ref. 22). However, we identified no significant PDEF binding site near the FOXM1 promoter region in our ChIP-seq analysis.

Subgrouping all the patients from the same clinical cohorts using PDEF expression level revealed that Twist1 but not FOXM1 is the most differentially expressed transcription factor with statistical significance (Supplementary Table S2). Twist1 has been reported to bind to the promoter region of FOXM1 and induce its transcription in gastric cancer cells (39). Further studies are required to explain the interplay between PDEF, Twist1 and FOXM1.

Metastasis is a complex process and is the leading cause of death in prostate cancer (40). Our current studies using GSEA analysis to compare the global transcription pattern between PC3-SCR and PC3-PDEF revealed a negative correlation between PDEF and migration and metastasis-related gene sets. We observed that PDEF expression results in negative enrichment of genes involved in not only the EMT process but also neuroendocrine phenotype, basal

**PDEF Promotes Prostate Luminal Differentiation**

**Decreased expression of PDEF is associated with prostate cancer progression, metastasis and poor patient survival**

Previously, we observed that PDEF expression was decreased in high Gleason grade prostate cancer using IHC and limited sample size on a prostate tissue array (18). Moreover, PDEF has been identified as one of the super-enhancer–related transcription factors in LNCaP cells due to the high abundance of H3K27ac within its genomic proximity region (16). We also recognized that transcription factors, by controlling the expression of diverse sets of genes, are essential drivers of cellular phenotype. These observations prompted us to probe for the expression of PDEF and other transcription factors in publicly available prostate cancer databases. As such, in order to find the transcription factor(s) that drives lethal prostate cancer in an unbiased approach, we probed for the expression of all known transcription factors in a clinical cohort (GSE16560) for their ability to predict patient survival. The results from Supplementary Table S1 showed Twist1 is among the top transcription factors positively associated with poor survival significantly. The median survival time in patients with high Twist1 expression level is only 77 months, while those in patients with middle and low Twist1 expression levels, it is 102 or 131 months, respectively (Fig. 6H). However, other core EMT-related transcription factors failed to predict poor patient survival. Moreover, our analysis revealed a significant loss of PDEF and gain of Twist1 comparing metastasis versus primary tumor samples (Fig. 6A and D) as well as when comparing lethal versus indolent tumor samples (Fig. 6B and E). Gene expression data obtained from The Cancer Genome Atlas (TCGA) also showed a graded loss of PDEF with increasing Gleason score and a significant loss in high Gleason score patient versus low Gleason score patient (Fig. 6C). However, the Twist1 expression level was not associated with Gleason grade (Fig. 6F). In contrast to Twist1, we observed a negative correlation between PDEF expression and poor survival (low PDEF vs. high PDEF; 72 vs. 121 months; Fig. 6G). Furthermore, Kaplan–Meier survival analysis showed that the low PDEF or high Twist1 expression are associated with poor survival (Fig. 6H). A combined two-gene signature of low PDEF and high Twist1 has a much stronger prognostic effect on survival (Fig. 6I).

**Our ChIP-qPCR results verified the occupancy of PDEF in the promoter region of CK18 with significant enrichment in the putative binding region as well as in the ARE3 region (Fig. 5B). In accordance with our hypothesis, expression of PDEF in PC3 and DU145 cells significantly increases CK18 expression in mRNA and the protein level (Fig. 5C). Results from the analysis of the TCGA database showed PDEF expression is positively correlated with the expression of CK18 and a significant negative correlation between CK8/18 expression and disease progression (Fig. 5D; Supplementary Fig. S4). Furthermore, we observed the downregulation of Twist1 and upregulation of CK18 in PC3-PDEF xenograft tissues comparing with PC3-SCR xenograft tissues (Fig. 5E; Supplementary Fig. S4). Moreover, knockdown of CK18 in PC3-PDEF and DU145-PDEF results in the reduced expression of E-cadherin with increased expression of Twist1 in the protein level (Fig. 5F), while knockdown of CK18 in LNCaP cells does not affect the expression of PDEF, suggesting a unidirectional regulation of PDEF on CK18 (Fig. 5G).

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cell phenotype, and cellular stemness while promoting prostate differentiation. Considering the current understanding that cellular plasticity (loss of epithelial phenotype and gain of mesenchymal, neuroendocrine and/or stem-cell-like phenotypes) is associated with prostate cancer progression and metastasis, our results present a novel mechanism in which PDEF by restricting cellular plasticity (by suppressing mesenchymal, neuroendocrine, basal, and stem-like phenotype and inducing luminal/epithelial differentiation) might inhibit tumor metastasis in vivo as observed in our previous studies and by others (19, 20, 41–43).

The tumor-suppressive role of Ets factors has been neglected by the oncogenic property of ERG and TMPRSS2 fusion in prostate cancer (44). Increased expression of PDEF was observed in benign and prostate intraepithelial neoplasia tissues compared with adjacent normal prostate tissues while the loss of PDEF was also reported during tumor progression (17, 18, 22, 45–48). The role of PDEF in prostate cancer remained controversial until we and others confirmed that PDEF is a tumor and metastasis suppressor in prostate cancer (20, 41). Based on the observations from this study, we speculate that elevated PDEF level in the early-stage prostate cancer is likely due to the epithelial hyperplasia nature, while the loss of PDEF leads to the loss of luminal cell phenotype and contributes to cellular plasticity and tumor metastasis. Recent papers have shown emerging association of PDEF with terminal differentiation in the breast, colon and lung (11, 12, 49). Our analysis of TCGA data presented in this study revealed that loss of CK18 along with its filament partner CK8 is associated with prostate cancer progression. Reanalyzing a publicly available PDEF ChIP-seq experiment revealed for the first time that CK18 is directly regulated by PDEF. Loss of CK18 is associated with a more dedifferentiated cell state in vitro and poor survival of breast and colorectal cancer patients (50–52). Loss of CK18 and CK8 also promotes cell migration through increased MMP2 and MMP9 in the HepG2 cell (53). Our previous study also showed that PDEF inhibits prostate cancer metastasis through downregulation of MMP9 (17). Thus, it is possible that CK18 modulation by PDEF may be one mechanism by which PDEF restricts cell motility and impairs metastasis. Overall, expression of PDEF in metastatic prostate cancer inhibits Twist1 and promotes luminal differentiation, in part through direct regulation of CK18.

Figure 6.
PDEF expression is inversely correlated with Twist1 in human prostate cancer and of prognostic value for the prostate carcinoma patient survival. Human prostate cancer datasets GSE6999 (54) and GSE16560 (55) were extracted from the GEO archive. TCGA data were downloaded from cBioportal. Expression levels were compared between primary and metastasis tissues (A and D), indolent and lethal prostate cancers (B and E), and among patient with different Gleason scores (C and F). Two-gene expression signature predicts poor patient survival (G–I). Kaplan-Meier survival analysis of prostate cancer patients using dataset GSE16560. Patients were stratified by the expression level of PDEF or Twist1 or both together.
Disclosure of Potential Conflicts of Interest

H.K. Koul has ownership interest in a patent application filed. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Wang, S. Koul, H.K. Koul
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Wang, H.K. Koul
Writing, review, and/or revision of the manuscript: F. Wang, S. Koul, H.K. Koul

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.S.T. Shannagam
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Acknowledgments

This work was supported in part by financial support from Carroll W. Feist Endowed Chair Funds (to H.K. Koul) and PWCC-Hormone Related Malignancies Program Support (to H.K. Koul). H.K. Koul was supported in part by VA Merit Award 1BX001258 (to H.K. Koul: PI) and NC1 RO1-CA161880 (to H.K. Koul: PI).

The authors acknowledge the technical assistance and support from the Genomics and Microarray Core at the University of Colorado Anschutz Medical Campus with Affymetrix Microarray work. We thank for Prof R. Shi, MD PhD, for the suggestions and help with statistical analysis.

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Received January 3, 2018; revised March 15, 2018; accepted May 15, 2018; published first May 30, 2018.

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Mol Cancer Res; 16(9) September 2018 1439


Molecular Cancer Research

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