Reduced PDEF Expression Increases Invasion and Expression of Mesenchymal Genes in Prostate Cancer Cells

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Abstract

The epithelium-specific Ets transcription factor, PDEF, plays a role in prostate and breast cancer, although its precise function has not been established. In prostate cancer, PDEF is involved in regulating prostate-specific antigen expression via interaction with the androgen receptor and NKX3.1, and down-regulation of PDEF by antiproliferative agents has been associated with reduced PDEF expression. We now report that reduced expression of PDEF leads to a morphologic change, increased migration and invasiveness in prostate cancer cells, reminiscent of transforming growth factor β (TGFβ) function and epithelial-to-mesenchymal transition. Indeed, inhibition of PDEF expression triggers a transcriptional program of genes involved in the TGFβ pathway, migration, invasion, adhesion, and epithelial dedifferentiation. Our results establish PDEF as a critical regulator of genes involved in cell motility, invasion, and adhesion of prostate cancer cells. [Cancer Res 2007;67(9):4219–26]

Introduction

The recent discovery of recurrent chromosomal translocations of the TMPRSS2 gene to three members of the Ets family, ERG, ETV1, and ETV4, in the majority of prostate cancer patients has highlighted the relevance of Ets transcription factors for prostate cancer (1, 2). Chromosomal translocations of multiple Ets family members are frequent in various cancer types, including FLI-1 (3, 4), ERG (3, 5), ETV1 (6), ETV4 (7), and FEV, in Ewing’s sarcomas and TEL/ETV6 in different types of leukemia, fibrosarcoma, lymphoma, and myelodysplastic syndrome (8–10), indicating a critical role for Ets factors in tumorigenesis. Furthermore, deregulated activity of Ets factors is observed in many cancers and apparently contributes not only to tumorigenesis but also to tumor progression, outcome, angiogenesis, epithelial-to-mesenchymal transition (EMT), and metastasis (11). Ets factors act as oncogenes or tumor suppressors in vitro and animal models have confirmed their oncogenic activities (11).

Most Ets genes are ubiquitously expressed, but we and others identified recently a group of Ets factors, ESE-1, ESE-2, ESE-3, and PDEF, whose expression is restricted to epithelial cells (12–14). PDEF (SPDEF) is unique among Ets factors because its expression is highly restricted to a small subset of epithelial cell types (i.e., hormone-regulated epithelium, such as prostate, mammary gland, endometrium, and ovary, as well as salivary gland and colon; ref. 12).

The precise function of PDEF in cancer development and progression remains somewhat controversial. Elevated levels of PDEF transcripts have been detected in primary human breast, prostate, and ovarian cancers, as well as breast cancer lymph node metastases, and PDEF transcripts can be detected in peripheral blood of breast cancer patients (15, 16). However, several reports indicate reduced PDEF protein expression in cancer cells relative to their normal counterparts as well as decreased protein expression in invasive, metastatic relative to early-stage breast cancer, possibly due to translational suppression (17).

A potential role for PDEF in cancer cell migration and invasion has emerged, although different investigators have come to opposite conclusions. Whereas, in one study, PDEF overexpression in MDA-MB-231 breast cancer cells inhibits cell growth and reduces invasion and migration (17), in another report, PDEF overexpression induces invasion and migration in breast cancer cells that is further enhanced by mitogen-activated protein kinase–dependent phosphorylation (18). This discrepancy may be due to differences in the cellular environment between different cell lines. A role for PDEF in prostate cancer seems likely due to its involvement in prostate-specific antigen (PSA) gene regulation and direct interaction with the androgen receptor and the prostate-specific tumor suppressor gene NKX3.1, which represses PDEF-mediated activation of the PSA promoter in prostate cancer cells (12, 19). However, the precise role of PDEF in prostate cancer has not been explored.

To provide insight into the function of PDEF in prostate cancer and to overcome limitations with overexpression of potentially unphysiologic concentrations of transcription factors, we have used RNA interference (RNAi) experiments to reduce endogenous PDEF expression in prostate cancer cells. We report now that inhibition of PDEF in prostate cancer cells leads to drastic physiologic and morphologic changes that are associated with the conversion of epithelial properties toward mesenchymal phenotypes with decreased epithelial markers, increased mesenchymal markers, and decreased adhesion, enhanced migration, enhanced invasion, and activation of the transforming growth factor β (TGFβ) pathway. We also show that TGFβ represses PDEF expression, indicating that PDEF suppression may mediate part of the biological effects of TGFβ. Thus, PDEF seems to play a critical role in cell motility and invasion of prostate cancer cells, steps that are critical for progression and metastasis of prostate cancer.
Materials and Methods

Cell culture. PC-3 and LNCaP prostate cancer cells and the MDA-MB-231 breast cancer cells were grown in Ham's F-12, RPMI 1640, or DMEM (all from BioWhittaker), respectively, supplemented with 10% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin (Life Technologies). Recombinant human TGFβ (7 ng/mL) was purchased from R&D Systems.

Small interfering RNA oligonucleotides and lentivirus vector generation. Three different small interfering RNA (siRNA; Dharmacon, Inc.) for human PDEF [siPDEF 1 (5'-AAGAAGGGCAUCUCCGGAAG-3'), siPDEF 2 (5'-AAGUGCUCAAGGACACUGA-3'), and siPDEF 3 (5'-AACGUGCUCAACUCCGGCA-3')] were evaluated for their abilities to reduce PDEF expression. The most effective PDEF siRNA (siPDEF 3) was inserted into a lentivirus vector using a single 83-mer oligonucleotide (5'-CTGCTCTAGACAAAGAGGATCTGACCATTCGATATC-GATGTCCTTGACACGGGATCTGTTGCTCTATACA-3'). For details, see Supplementary Data.

Generation of cell lines stably expressing PDEF siRNA. PC-3 and LNCaP cells were stably infected with the PDEF siRNA or green fluorescent protein (GFP) siRNA lentivirus and enriched as described in Supplementary Data. Protein extracts from sorted cells were analyzed by Western blot using anti-PDEF antibody. These cell lines (PC-3 siGFP, PC-3 siPDEF, LNCaP siGFP, and LNCap siPDEF) were used in all experiments.

Inverted control siRNA design and transient transfection. A scrambled control siRNA [inverted central 8 bp for siPDEF 3 (target sequence is 5'-AAGCUCAACUCCGGCA-3')] was purchased from Dharmacon. RNA (50 μmol/L) duplexes for PDEF (siPDEF 2) and scrambled control (siControl) were transfected into PC-3 cells using TKO transfection reagent (Mirus). Forty-eight hours after transfection, RNA was isolated for real-time PCR analysis.

Stable transfection. Wild-type PDEF (PDEF-wt) expression construct was made by subcloning full-length wild type PDEF cDNA into the pcDNA 5'-FLAG expression vector (Invitrogen) to generate pcDNA-PDEF-wt (FLAG-tagged; see Supplementary Data). pcDNA-PDEF-wt and scrambled control (siControl) were transfected into PC-3 cells using TKO transfection reagent (Mirus). Forty-eight hours after transfection, RNA was isolated for real-time PCR analysis.

Construction of adenovirus and adenoviral infection. The FLAG-PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data. Human PDEF–encoding adenovirus was generated as described previously and in Supplementary Data.
A PDEF siRNA or GFP siRNA were plated on top of collagen gel. Three hours after plating, cells were fixed, stained, and photographed. Adhesion was quantified by Student's t test. **, P < 0.005.

Figure 1. Generation of PC-3 cell lines stably expressing siRNA against PDEF and morphologic and adhesion effects of PDEF siRNA in PC-3 prostate cancer cells. A, Western blot analysis using a monoclonal anti-PDEF antibody showing the expression of PDEF in PC-3 cells stably expressing wild type PDEF or siRNA for PDEF compared with control parental vector cells and GFP siRNA-expressing cells. Relative expression levels normalized to tubulin are indicated. B, morphologic changes of PC-3 cells, stably infected with the PDEF siRNA or GFP siRNA lentivirus. Magnification, ×200. C, filamentous actin and cytoskeletal rearrangements visualized by TRITC-labeled phalloidin. D, PDEF knockdown decreases adhesion ability of PC-3 and LNCaP cells. PC-3 or LNCaP cells as well as the cells expressing PDEF siRNA or GFP siRNA were plated on top of collagen gel. Three hours after plating, cells were fixed, stained, and photographed. Adhesion was quantified by absorbance on 595-nm wavelength. The assays were done in triplicate in two independent experiments. Statistical analysis used is two-tailed unpaired Student’s t test. **, P < 0.005.

PDEF knockdown causes morphologic changes of PC-3 cells. We investigated whether altering PDEF expression induces morphologic changes. Whereas overexpression of PDEF did not significantly affect morphology of PC-3 cells, loss of PDEF expression by RNAi induced a shape change from rounded epithelial-like cells to fibroblast-like cells with elongated morphologies, a phenotype reminiscent of EMT (Fig. 1B). Immunofluorescence with phalloidin revealed significant redistribution of actin cytoskeleton from a cortical ring at cell junction into stress fibers in PC-3 cells expressing PDEF siRNA (Fig. 1C).

PDEF knockdown reduces prostate cancer cell adhesion. To explore whether inhibition of PDEF expression affects cellular adhesion, an in vitro adhesion assay of siPDEF- or siGFP-expressing PC-3 and LNCaP cells on a matrix of fibronectin or Matrigel was done. PDEF knockdown decreased adhesion of PC-3 cells on Matrigel by 80% (P < 0.001) and of LNCaP cells by 70% compared with either control siGFP cells or parental cells (P = 0.002; Fig. 1D; see representative image in Supplementary Fig. S1). These results show that down-regulation of endogenous PDEF reduces prostate cancer adhesion to extracellular matrix.

PDEF knockdown enhances prostate cancer cell migration and invasion. Because the morphologic changes induced on interference with PDEF expression suggested that those cells had undergone EMT, we evaluated the effect of PDEF knockdown on serum-induced migration of PC-3 cells in a Transwell chamber assay. siPDEF increased the ability of PC-3 cells to migrate through the pores by >50% compared with siGFP-treated cells (P = 0.041; Fig. 2A). Because cell migration is a process that promotes tumor invasion, we tested the effect of PDEF knockdown on cell invasion using Matrigel-coated Transwell chambers. siPDEF compared with siGFP strongly increased PC-3 cell invasion by 60% after 24-h incubation (P = 0.042; Fig. 2B). Representative images are shown in Supplementary Fig. S2. Under the same conditions, overexpression of PDEF decreased PC-3 cells invasive ability by 40% (P = 0.081; Fig. 2C). Similar inhibition of migration and invasion by PDEF overexpression was seen in MDA-MB-231 breast cancer cells. Cells expressing AdPDEF have >60% reduced migration and invasion of cells compared with Ad-FLAG–infected cells (Fig. 2A and B). We have not used MDA-MB-231 breast cancer cells for siRNA experiments because this cell line does not express endogenous PDEF. These results show that PDEF is an inhibitor of prostate and breast cancer cell migration and invasion.

PDEF regulates genes involved in cell adhesion, integrin signaling, EMT, and vascular endothelial growth factor signaling. To identify PDEF target genes associated with the biological phenotypes, RNAs from siPDEF- and siGFP-expressing PC-3 cells were hybridized to Affymetrix HT U133AAofAv2 GeneChips, which contain >22,000 probe sets. One thousand five hundred eighty-four genes were up-regulated and 1,299 were down-regulated by at least 1.2 LCB of fold change in PC-3 cells expressing siRNA against PDEF. A set of genes shown previously to reflect a transition of epithelial to mesenchymal characteristics was identified. Whereas epithelial markers, E-cadherin and keratins 3, 6a, 6b, 7, 8, 18, and 19, were down-regulated, mesenchymal markers, vimentin, N-cadherin, mesenchymal stem cell antigen, and CTGF, as well as inducers of mesenchymal transition, TGFβ1 and Snail2, were up-regulated in PDEF knockdown cells (Fig. 3; Supplementary Table S1). Multiple genes associated with cell adhesion and migration, such as collagens COL1A1, COL4A1,
COL4A2, COL4A5, COL4A6, COL5A1, COL5A2, COL6A1, COL6A2, COL6A3, COL13A1, and COL16A1; syndecan-1 and syndecan-2; tenascin C; laminin β2 and γ1; integrins αv, α5, and α6; and CDH11, were up-regulated in PDEF knockdown cells, whereas cytoskeletal and intermediate filament genes as well as integrins β3, β5, and β8 were down-regulated, indicating cytoskeletal rearrangements and changes in cell adhesion and migration (Fig. 3; Supplementary Table S1). Modeling of differentially regulated genes identified the integrin signaling pathway as a major target for PDEF (Fig. 3; Supplementary Fig. S3) because, in addition to effects of PDEF on expression of extracellular matrix proteins and integrins, several genes downstream of integrins in the signaling pathway, such as tyrosine kinase focal adhesion kinase (FAK)/PTK, DOCK 1 and 4, RAC2, WASPIP, and phosphoinositide 3-kinases PIK3C3 and PIK3CD, were up-regulated in PDEF knockdown cells (Supplementary Table S1). These results indicate that inhibition of PDEF expression may lead to integrin receptor aggregation induced by extracellular matrix interaction, which could result in stimulation of signal transduction cascades resulting in cytoskeletal rearrangements and enhanced prostate cancer cell migration. Further, detailed analysis of these signaling pathways on the protein level is needed to support this notion.

Another pathway possibly contributing to enhanced invasion and migration in PDEF knockdown prostate cancer cells is the vascular endothelial growth factor (VEGF) signaling pathway. A transcriptional activator of VEGF, hypoxia-inducible factor-1α, as well as VEGF and VEGFB are up-regulated in PDEF knockdown cells. The VEGF receptor as well as the downstream signaling genes PI3K, FAK, PLCγ1, and PKCα are up-regulated, indicating potential activation (Fig. 3; Supplementary Table S1).

Many biological effects elicited by PDEF knockdown in PC-3 cells and the changes in epithelial and mesenchymal marker
expression are reminiscent of TGFβ signaling. Indeed, the TGFβ-activated Smad1, Smad2, Smad3, and Smad4 transcription factors are up-regulated in PDEF knockdown cells as well as several TGFβ-inducible genes, such as Snail2 and plasminogen activator inhibitor-1 (PAI-1), a key regulator of tumor invasion and metastasis (Fig 3; Supplementary Table S1 and Fig. S3). Strikingly, the Wnt/β-catenin pathway, which plays a central role in adhesion and migration, is significantly modulated in PDEF knockdown cells, particularly a strong induction of the downstream transcriptional mediators LEF-1 and TCF8, up-regulation of several catenins, frizzled homologue 8, dickkopf homologue 3, and Wnt5a, and down-regulation of E-cadherin (Fig. 3; Supplementary Table S1). These results show that PDEF suppresses tumor cell invasion and migration via modulating multiple signaling pathways.

**Functional annotation of differentially expressed genes.** To investigate the biological processes deregulated in PDEF knockdown PC-3 cells, we implemented an enrichment analysis of all differentially expressed genes (1,584 up and 1,299 down) using the database for annotation, visualization, and integrated discovery (DAVID; ref. 25). DAVID is useful to identify enriched biological themes, functional clusters of genes. Clusters of biological processes, keywords, terms, functions, and pathways with enrichment scores of \( >2 \) against all human genes as background are listed in Supplementary Table S2. One of the top categories identified includes signaling pathways relevant to cell invasion, adhesion, migration, and invasion (see colorgrams in Supplementary Fig. S4). The colorgrams include enriched genes involved in the Wnt, VEGF, integrin, and TGFβ pathways (Supplementary Fig. S5).

**Blocking PDEF expression in PC-3 cells results in loss of epithelial markers and induction of mesenchymal markers.** Various genes linked to mesenchymal/migratory cells as described above were induced on PDEF depletion. Furthermore, genes associated with epithelial cells and known or suspected to be lost

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**Figure 4.** EMT markers regulated by PDEF in PC-3 cells. A, real-time PCR analysis of Snail2, vimentin, cytokeratin 18, and E-cadherin in PC-3 cells with different PDEF expression levels. Total RNA was collected from PC-3 cell stably expressing siRNA for GFP or PDEF, or wild type PDEF, and control vector. Each RNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, expression of PDEF, vimentin, Snail2, and cytokeratin 18 by real-time PCR was detected in PC-3 cells transiently transfected with second siRNA for PDEF (siPDEF2) or an inverted control siRNA. Each RNA expression was normalized to GAPDH. C, Western blot analysis for cytokeratin 18 and vimentin in PC-3 and LNCaP cells (24 h in culture) stably expressing siRNA against PDEF or GFP. Tubulin levels were analyzed as a control protein, the levels of which were not expected to change during EMT. D, immunostaining of vimentin and cytokeratin 18 in PC-3 stably expressing siPDEF, PDEF-wt, or parental cells.
overexpression in PC-3 cells inhibit the TGFβ action and modulated expression of TGFβ target genes, we evaluated whether PDEF may be downstream of TGFβ. Northern blot analysis of TGFβ-stimulated LNCaP (Supplementary Fig. S6) and PC-3 (Fig. 5A) cells indicates decreased PDEF expression in TGFβ-treated cells in a time-dependent manner, starting 8 h after stimulation (Fig. 5A). Real-time PCR confirmed down-regulation of PDEF expression by TGFβ stimulation (Fig. 5B).

Repression of PDEF expression is required for induction of mesenchymal genes by TGFβ. To determine whether PDEF repression is required for TGFβ induction of mesenchymal genes, PC-3 cells overexpressing PDEF were stimulated with TGFβ for 3 days. Real-time PCR showed that TGFβ induced expression of vimentin and Snail2 in control PC-3 cells transfected with the parental vector, whereas induction of these genes by TGFβ was completely inhibited in PDEF-overexpressing cells (Fig. 5B). These data most vividly show that PDEF repression is mediating at least some of the biological effects of TGFβ.

PDEF and TGFβ regulate transactivation of the Snail2 promoter. Snail2, in addition to being a mesenchymal marker, represses expression of E-cadherin and is a critical regulator of EMT (26). To determine whether PDEF and TGFβ affect Snail2 transcription, we inserted the 1.2-kb human Snail2 promoter upstream of the luciferase reporter gene into the pGL3 vector. Analysis of the Snail2 promoter sequence identified several potential PDEF binding sites (consensus sequence: GGAA/T) and Smad binding sites (consensus sequence: AGAC) in the 1.2-kb promoter.

Figure 5. PDEF is a downstream target of TGFβ. A, Northern blot analysis of PDEF expression in LNCaP cells cultured in serum-free medium with TGFβ for different times. Northern blots were probed with [32P]UTP-labeled PDEF cDNA. A GAPDH probe was used for equal loading. B, PDEF overexpression in PC-3 cells inhibits the TGFβ effects on EMT markers. PC-3 cells overexpressing PDEF and control cells were starved in serum-free medium overnight and then stimulated with 7 ng/mL TGFβ for 3 d. Real-time PCR for vimentin, fibronectin, and Snail2, in the absence or presence of TGFβ.

during EMT, such as E-cadherin, plakoglobin, and cytokeratins, were down-regulated in PC-3 siPDEF cells. We validated expression of several classic markers of EMT, including vimentin, Snail2, cytokeratin 18, and E-cadherin, by real-time PCR in PDEF knockdown and PDEF-overexpressing PC-3 cells. Whereas Snail2 and vimentin were induced by siRNA against PDEF, overexpression of PDEF reduced expression of these genes (Fig. 4A). In contrast, cytokeratin 18 and E-cadherin were down-regulated in PDEF knockdown cells, and expression increased in PDEF-overexpressing cells (Fig. 4A), further supporting the conclusions from the previous experiments. Similar effects of PDEF on vimentin and E-cadherin expression were seen in another prostate cancer cell line, LNCaP, as well as in two breast cancer cell lines, SKBr-3 and MCF-7. The specificity of PDEF siRNA oligonucleotides and of the effect of knocking down PDEF was validated by transient transfection of scrambled oligonucleotides (siControl, inverted central 8 bp of the specific siRNA) and a second siRNA against PDEF (siPDEF2) into PC-3 cells. RT-PCR analysis 48 h after transfection (Fig. 4B) confirmed that expression of PDEF and cytokeratin 18 was decreased by siPDEF2 only, whereas Snail2 and vimentin were induced.

PDEF-dependent changes in mRNA expression led to equivalent changes in protein expression as analyzed by Western blotting. Cytokeratin 18 protein levels decreased and vimentin protein levels increased in PDEF knockdown PC-3 and LNCaP cells (Fig. 4C). Immunofluorescence analysis confirmed a loss of the epithelial marker cytokeratin 18 in PDEF knockdown cells and an increase of the mesenchymal marker vimentin, whereas PDEF-overexpressing cells did not stain for vimentin and stained stronger for cytokeratin 18 (Fig. 4D). Moreover, control PC-3 cells expressing siGFP and parental PC-3 cells stained faintly for vimentin and intermediate for cytokeratin 18.

Figure 6. TGFβ and knockdown of PDEF enhance Snail2 transcription at the promoter level. A, Luciferase reporter assays of siGFP- and siPDEF-expressing PC-3 cells transfected with 600 ng pGL3 or pGL3-Snail2 promoter were done as described. B, 600 ng pGL3 or pGL3-Snail2 promoter was transfected into PC-3 cells. Sixteen hours following transfection, cells were switched to serum-free medium with or without 2 ng/mL TGFβ and incubated for an additional 8 h. Luciferase activity was determined as described. Statistical analysis used is two-tailed unpaired Student’s t test. *, P < 0.05.
Snail2 promoter (Supplementary Fig. S7). PC-3 cells containing either siPDEF or siGFP were transiently transfected with either the Snail2 promoter luciferase vector or the parental pGL3 vector, and luciferase activity was read 24 h after transfection. Activity of the Snail2 promoter increased ~2.0-fold ($P < 0.05$) in PC-3 with siPDEF compared with siGFP (Fig. 6A), correlating with the Snail2 expression data. Similarly, TGFβ treatment of PC-3 cells transfected with the luciferase vectors increased Snail2 promoter activity ~1.5-fold ($P < 0.05$; Fig. 6B).

**Discussion**

Our original discovery of PDEF as an epithelial-specific Ets transcription factor involved in prostate cancer–specific PSA gene expression indicated a critical role for PDEF in prostate cancer (12). Further publications confirmed the role of PDEF and showed that the prostate cancer–specific tumor suppressor NKX3.1 directly interacts with PDEF and thereby inhibits PDEF-mediated PSA promoter transactivation (19). Additional reports suggest that PDEF protein expression, in contrast to mRNA expression, is reduced in prostate cancer cells relative to normal prostate epithelium. Similarly, the role of PDEF in breast cancer remains controversial. Whereas several publications suggest that PDEF is a marker for metastatic breast cancer, PDEF mRNA is up-regulated in breast cancer epithelium relative to normal mammary epithymium throughout tumor progression, and PDEF overexpression in MCF-10A breast epithelial cells coexpressing the receptor tyrosine kinases (RTK) ErbB2 and colony-stimulating factor-1 (CSF-1) receptor/CSF-1 induces migration and invasion of breast cancer cells (18), other reports indicate reduced PDEF protein expression in advanced breast cancer and inhibition of migration and invasion in breast cancer cells overexpressing PDEF (17).

Whereas previous publications relied on overexpression experiments to determine biological function of PDEF, we have focused our effort on reducing expression of endogenous PDEF by RNAi to evaluate the role of PDEF in prostate cancer and only apply overexpression when necessary to show the opposite effect of reducing PDEF expression. Our results show that inhibition of endogenous PDEF expression in PC-3 cells results in alterations of cell morphology with increased motile-invasive activities concomitant with changes in epithelial and mesenchymal marker gene expression as well as pleiotropic responses in multiple signaling pathways associated with adhesion, migration, and invasion of cancer cells. Thus, our data indicate that PDEF expression reduces motility and invasion and enhances adhesion of prostate cancer cells. Our data are consistent with published reports by Feldman et al. (17) on the inhibitory role of overexpressed PDEF in breast cancer cell migration and the role of the PDEF Drosophila homologue D-Ets4 as an inhibitor of migration of primordial germ cells (27). Our results, nevertheless, do not rule out that PDEF in response to RTKs and extracellular signal-regulated kinase activation switches from an inhibitor of migration into an activator of migration as suggested by the results from Gunawardane et al. (18), suggesting a bimodal function of PDEF that may be modulated both by regulation on the protein expression level and by phosphorylation.

Some biological responses elicited on interference with PDEF expression are reminiscent of EMT and TGFβ function (28). TGFβ plays a major role in human cancer, including prostate cancer, and functions as both a tumor suppressor in early tumorigenesis and a tumor promoter during tumor progression (29). TGFβ is frequently overexpressed in hormone-refractory prostate cancer and a risk factor for tumor progression and poor clinical outcome (30, 31). During tumor progression, TGFβ induces EMT, tumor cell migration, and invasion by enhancing expression of mesenchymal genes, extracellular matrix proteins, cell adhesion proteins, and proteases, thus promoting the metastatic potential of cancer cells (32–34). TGFβ function is critically involved in epithelial dedifferentiation toward mesenchymal cells by activating transcriptional repressors of the Snail family that inhibit epithelial-specific E-cadherin expression (35, 36). Our data show that TGFβ reduces PDEF expression and reduced PDEF expression affects expression of various TGFβ target genes, including Smads, which mediate TGFβ responses, vimentin, Snail2, fibronectin, and various extracellular matrix and adhesion proteins. Because PDEF overexpression blocks the TGFβ effect on expression of several critical target genes, PDEF may play an important role in TGFβ signaling and cancer cell migration.

Although EMT plays a critical role in progression and metastasis of various cancer types, EMT per se has not been typically observed in prostate cancer. Nevertheless, evidence of EMT-like changes exists in prostate cancer cell lines and during prostate cancer progression. Overexpression of PSA or kallikrein 4 in PC-3 cells induces cell migration through Matrigel and a morphologic change from rounded epithelial-like to spindle-shaped, mesenchymal-like cells concomitant with a decrease in adhesion (37). This EMT-like phenotype is associated with decreased E-cadherin and increased vimentin expression (37). In contrast, overexpression of the Wnt inhibitor Fzrb/secreted Frizzled-related protein 3 in PC-3 cells leads to induction of epithelial markers, such as E-cadherin, and decrease in mesenchymal markers vimentin and fibronectin resulting in reduced invasive capacity (38). These results are highly reminiscent of our own observations with regard to PDEF knockdown, and our transcriptional profiling data implicate Wnt and TGFβ pathways in PDEF function. Our transcriptional profiling analysis shows up-regulation of several positive regulators or mediators of the Wnt signaling pathway, such as WNT5A, TGFβ1, LEF1, TCF3, TCF4, FZD1, FZD4, and FZD8, and down-regulation of negative regulators, such as SFRP1, in PDEF knockdown cells, indicating that PDEF expression may inhibit Wnt signaling. Nevertheless, some positive regulators are down-regulated rather than up-regulated, showing the complexity of effects and the caution to overinterpret microarray data without further protein analysis. Nevertheless, an indication of activation of Wnt signaling is the high up-regulation of the downstream target LEF1. Motility and metastatic potential of prostate cancer cell lines directly correlates with vimentin expression levels and high vimentin expression in tissue sections correlates with poorly differentiated prostate cancers and bone metastases (39, 40). E-cadherin loss correlates with increased Gleason scores, increased invasive capacity, extracapsular dissemination, and bone metastasis (41, 42). Thus, there is a clear shift on prostate cancer progression and metastasis from a well-differentiated, epithelial phenotype expressing E-cadherin to a poorly differentiated, mesenchymal, invasive phenotype expressing vimentin. Our data implicate PDEF in these processes. Additionally, our results indicate involvement of PDEF in multiple biological pathways implicated in cancer development and progression. In the integrin signaling pathway, genes associated with cell adhesion and migration, such as the collagens COL1A1, COL4A1, and COL1A2 and integrins ITGα6, ITGα5, and ITGα3, as well as the positive signaling regulators FAK (PTK2), MYLK, WASPPIP, RAC2, RHOQ, and RRAS, are up-regulated in...
PDEF knockdown cells, suggesting that PDEF expression leads to inhibition of the integrin pathway resulting in reduced cancer cell motility.

In summary, our data indicate that PDEF is a downstream target of TGFβ and repression of PDEF expression is a critical step for TGFβ to elicit its biological effects in prostate cancer cells, apparently via activation of several signaling pathways (Wnt, VEGF, and integrin) linked to adhesion, migration, invasion, and EMT.

Acknowledgments

Received 10/6/2006; revised 1/5/2007; accepted 2/22/2007.

Grant support: NIH grant ROI CA84674 and Department of Defense grant PC001420 (T.A. Libermann), HMS Fund for Women’s Health (X. Gu), and Department of Defense grant PC051217 (L.F. Zerbini).

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We thank Dr. Inder M. Verma for help with generation of LV-siRNA PDEF.
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