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Mechanisms and functional consequences of PDEF protein expression loss during prostate cancer progression

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Abstract

BACKGROUND—Ets is a large family of transcriptional regulators with functions in most biological processes. While the Ets family gene, PDEF (prostate derived epithelial factor), is expressed in epithelial tissues, PDEF protein expression has been found to be reduced or lost during cancer progression. The goal of this study was to examine the mechanism for and biologic impact of altered PDEF expression in prostate cancer.

METHODS—PDEF protein expression of prostate specimens was examined by immunohistochemistry. RNA and protein expression in cell lines were measured by q-PCR and western blot, respectively. Cellular growth was determined by quantifying viable and apoptotic cells over time. Cell cycle was measured by flow cytometry. Migration and invasion were determined by transwell assays. PDEF promoter occupancy was determined by chromatin immunoprecipitation (ChIP).

RESULTS—While normal prostate epithelium expresses PDEF mRNA and protein, tumors show no or decreased PDEF protein expression. Re-expression of PDEF in prostate cancer cells inhibits cell growth. PDEF expression is inversely correlated with survivin, urokinase plasminogen activator (uPA) and slug expression and ChIP studies identify survivin and uPA as direct transcriptional targets of PDEF. This study also shows that PDEF expression is regulated via a functional microRNA-204 (miR-204) binding site within the 3'UTR. Furthermore, we demonstrate the biologic significance of miR-204 expression and that miR-204 is over-expressed in human prostate cancer specimens.

CONCLUSIONS—Collectively, the reported studies demonstrate that PDEF is a negative regulator of tumor progression and that the miR-204-PDEF regulatory axis contributes to PDEF protein loss and resultant cancer progression.

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INTRODUCTION

Ets proteins are cellular homologs of v-ets, one of the two transduced genes present in the avian E26 transforming retrovirus. Ets family members have been found in species ranging from *C. elegans* to humans and contain a conserved DNA-binding domain of 85 amino acids (1–4). The Ets domain recognizes a core sequence of 5'-GGAA-3' present in downstream target genes. Over 200 Ets target genes have been previously described (5) and currently there are over 500 genes with regulatory Ets sites (Watson, unpublished). Ets transcription factors control the expression of target genes that have critical roles in cell proliferation, differentiation, apoptosis, and oncogenesis. Altered expression of Ets genes has been observed in most human cancers, including prostate, breast and colon cancer (4,6).

Since the majority of cancers are of epithelial origin, an especially relevant subset of Ets factors are those that maintain a restricted pattern of expression limited to tissues with high epithelial content, regulating cellular proliferation and differentiation (7). Among these is Prostate Derived Epithelial Factor (PDEF), originally isolated from normal prostate tissue, with expression demonstrated in additional epithelial tissues, including ovary, breast, prostate and colon (7,8). Collective studies from our and other laboratories have shown that PDEF is a negative regulator of cell growth, migration and invasion (9–15).

Since PDEF expression is found in epithelial tissues and has regulatory roles for cell growth, migration and invasion, we decided to evaluate its expression profile in human prostate tissue. Further, we examine the biologic consequences of PDEF expression as well as define a possible mechanism that regulates PDEF protein expression.

MATERIALS AND METHODS

Patients and Tumor Specimens

Human prostate cancer paraffin blocks were obtained from the Hollings Cancer Center Tumor Bank, Medical University of South Carolina for the immunohistochemical studies. In addition, tissue microarray (TMA) slides were obtained from the NCI Tissue Array Research Program (TARP) and also stained for PDEF expression. The TARP4 TMA slide is composed of 0.6-mm tissue core samples constructed in single cores (not in duplicate) of normal tissue (n=51; normal prostate=2) as well as cancers from prostate, breast, colon, lung, ovary, lymphoma, glioblastoma, and malignant melanoma. The interpretable prostate adenocarcinoma cores analyzed in the current paper = 33. All specimens were formalin-fixed and paraffin embedded.

Immunohistochemistry

All of the specimens were formalin-fixed and paraffin-embedded. Deparaffinized tissue sections were rehydrated, and endogenous peroxidase activity was blocked using 3% H₂O₂. Antigen retrieval was done by heating in a microwave oven for 5 min on high power in 10 mM citrate, pH 6.0. Sections were washed and nonspecific binding was blocked with 10% horse serum in Tris-buffered saline (TBS; 50 mM Tris-HCl, 0.9% NaCl, pH 8.0) for 20 min, then incubated overnight at 4°C with a PDEF-specific primary antibody (9,12) at a 1:200 dilution in the blocking solution. Overnight incubation at 4°C was followed by three 10-min washes in TBS. ImmPRESS™ horse anti-rabbit or horse anti-mouse secondary (Vector Laboratories, Burlingame, CA) was incubated for 45 min at room temperature. Slides were counterstained with hematoxylin.

All sections were examined with Olympus BX50 microscope. The pictures were taken with Olympus DP 70 connected to DP Controller software (Olympus, center Valley, PA). For TMA, scoring of immunoreactivity was based on the intensity and distribution of positive

staining as previously described (16) with modification. TMA scoring of immunoreactivity was based on semi quantitative measurement of the intensity and distribution of positive staining examined visually under light microscopy. Strong (score +++) = dark staining that is easily demonstrated with low power objective (x4) and involves > 50% of cells. Moderate (score ++) = focal dark staining that involve 10–50% of cells or moderate staining of > 50% of cells. Weak (score +) = focal moderate staining that involve 10–50% of cells or pale staining in > 50% of cells that is not easily demonstrated with low power objective (x4). Negative (score 0) = any staining that show none of the above.

Cell culture

Prostate cancer-derived cell lines DU145, PC3, and LNCaP were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 with 10% fetal bovine serum (FBS). All cell lines were propagated at 37°C in an atmosphere containing 5% CO₂. Mycoplasma-negative cultures were ensured by PCR testing prior to the investigations. Cells were monitored throughout with consistent morphology and doubling-time.

Immunofluorescence

Cells were seeded onto sterile coverslips (18 mm in diameter) coated with 5 ug/ml fibronectin and allowed to attach overnight. Cells were fixed with 2% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked in 2% bovine serum albumin (BSA) for 1 h at room temperature. PDEF localization was examined using the antibody detailed above, nuclear staining was examined using TOPRO (Molecular Probes) and actin using phalloidin (Molecular Probes). PDEF was visualized using 480nm Alexa Fluor secondary antibody (Invitrogen, Carlsbad, CA). Immunofluorescence was examined using an Olympus IX70 confocal microscope.

Adenoviral Infection

The construction of PDEF expressing adenovirus has been previously described (9,12). Cells were infected in normal growth medium with either control virus expressing GFP (Ad-GFP), or virus expressing PDEF/GFP from a bi-cistronic promoter (Ad-PDEF). Infected cells were then incubated as normal for 16 hours. Under these conditions greater than 95% of the cells were infected as assessed by GFP expression.

Western Blot Analysis

Prostate cancer derived cell lines and tissue were used for isolation total protein. For total protein isolation, cells at 90% confluence were washed twice with ice cold PBS, and were lysed in RIPA buffer containing protease inhibitors. Equal amounts of total protein (50 µg) were resolved by 10% SDS-PAGE and subjected to Western blot analyses using ECL system (Pierce-Fisher Scientific, Rockford, IL). Frozen tissue samples (250 mg) were pulverized in LN₂ and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1% Triton X100, 0.1% SDS, 1% deoxycholate and protease inhibitor cocktail (Complete Protease Inhibitors, Roche, Nutley, NJ) for 15 minutes on ice. Total protein lysates were examined for PDEF (described above), cyclin A, cyclin D1 (Santa Cruz, Santa Cruz, CA), cyclin E, Rb (PharMingen, BD Biosciences, San Diego, CA), and p53 (DAKO, Carpinteria, CA) expression. GAPDH (Abcam, Cambridge, MA) was used as a loading control.

Quantitative reverse transcription PCR

Total RNA from cancer cell lines was extracted using the RNeasy Plus Mini Kit (Qiagen; Valencia, CA). Total RNA from prostate tumor and non-tumor samples was extracted using Trizol as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). 1µg total RNA was reverse transcribed in a 20µl reaction using Superscript III reverse transcriptase (Invitrogen)

for microRNA analyses and iScript (Bio-Rad; Hercules, CA) for all other studies. Real time PCR for ChIP was performed with 1 µl of a 1:10 dilution of reverse transcribed cDNA using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) in a LightCycler (Roche, Nutley, NJ), as per the manufacturer's instructions. The cycling conditions for all genes were performed as per the manufacturer's recommendations: annealing temperature was 58°C. The size and purity of the PCR products were also determined by melt curve analysis according to the Roche software. Real time PCR for gene expression was performed with 5 µl of a 1:20 dilution of reverse transcribed cDNA using the universal probe library (UPL) system (Roche) in a LightCycler 480 (Roche). The cycling conditions were performed as per the manufacturer's instructions. Primer sequences and probe numbers are described (Table 1). Real time PCR for miRNA expression was performed using TaqMan assay as per the manufacturer's instructions. Triplicate reactions were run for each cDNA sample. The relative expression of each gene was quantified on the basis of Ct value measured against an internal standard curve for each specific set of primers (Table 1) using the software provided by the instrument manufacturer (Roche). These data were normalized to GAPDH.

Cell Growth Assay

DU145 and PC3 cells were infected at low MOI with adenovirus expressing PDEF/GFP or GFP alone. Cells were trypsinized and 50,000 seeded into individual wells on a 6-well plate. At the indicated time points, cell growth medium was isolated to collect dead and unattached cells and the attached cells trypsinized and combined with the growth medium. The combined cell lysate was then centrifuged at 2500rpm to collect the cell pellet containing both dead and viable cells. Cells were resuspended in 500 µl of PBS and 10 µl was stained with trypan blue and loaded onto a counting chamber. Dead and viable cells were quantified in triplicate using a Countess cell counter (Invitrogen).

Flow cytometry

Cells were harvested 36 hours after infection with Ad-GFP or Ad-PDEF, fixed and dehydrated in 70% ethanol at -20°C for at least 24 hrs. Cells were washed twice in PBS containing 10% serum and resuspended in 500 µl of a solution containing 0.5 mg/ml propidium iodide and 1 mg/ml RNase A (Sigma-Aldrich, St. Louis, MO). This solution was kept in the dark for at least several hours before analyzed using flow cytometry.

Migration and Invasion Assays

Cell migration experiments were carried out using 8-µm pore size migration chambers (Falcon, Becton Dickinson, San Jose, CA) pre-coated at 4°C overnight with fibronectin (Becton Dickinson) at a concentration of 5 µg per square centimeter in PBS. The following day, the fibronectin solution was aspirated and the migration chambers were rinsed one time with water and allowed to air dry prior to the migration experiment. Cell invasion experiments were carried out using re-hydrated 8-µm pore size invasion chambers pre-coated with Matrigel (Becton Dickinson). Cells at 80% confluence were trypsinized, harvested, and counted. For each condition, cells were seeded at 50,000 cells/well in 500 µL serum free media, then added to each migration and invasion chamber. Medium (750 µL) containing 10% serum was used as a chemoattractant in the lower chamber. Cells were allowed to migrate for eight hours or invade for 24 hours at 37°C in the presence of 5% CO₂. Cells that did not migrate or invade were removed by wiping the top of the membrane with a cotton swab and the migrating and invading cells were fixed and stained with Diff-Quik per the manufacturers protocol (Dade Behring, Newark, DE). Migrating and invading cells in 10 high power fields in each chamber were counted and the mean cell number was calculated. Each experiment was conducted in triplicate and repeated three times.

Chromatin immunoprecipitation (ChIP)

Chromatin was prepared and immunoprecipitation performed using PC3 cells (express endogenous PDEF) in a two-step cross-linking protocol, as previously described (17). Chromatin was fragmented into 500–1000bp fragments by sonicating the cells eight times for 10s at level three in an ethanol ice bath using a Virtis Virsonic 475 sonicator (Gardiner, N.Y.). Soluble chromatin was quantified (absorbance at 260nm) and 10 absorbance units were incubated with 2µg of PDEF rabbit polyclonal antibody or IgG alone for 4 hours. Collection, washing and reverse cross-linking of immune complexes was as previously described (17). Primers (Table 1) spanning a known ETS binding site (EBS) situated 2.4 Kb upstream of the transcriptional start site of uPA (18) were used to examine PDEF occupancy. Relative enrichment was expressed as percent of total input.

Oligonucleotide Transfection

The miRNA inhibitors (Ambion; Austin, TX) are single-stranded chemically enhanced oligoribonucleotides designed to inhibit the endogenous miRNAs. Cells were transfected with 100nM of the indicated oligoribonucleotide using the Oligofectamine reagent as per the manufacturer's instructions (Invitrogen). 48 hours after transfection, cells were harvested for protein or RNA extraction.

Statistical analysis

For statistical testing, two-sided paired Student's *t*-tests were calculated using an Excel spreadsheet. *p* values are given for each individual experiment, but in general, *p* < 0.05 was considered statistically significant. Error bars represent standard deviations of three independent experiments unless indicated otherwise.

RESULTS

PDEF protein expression is lost in prostate cancer

Immunohistochemical analysis was used to examine PDEF expression and distribution in paraffin embedded human prostate cancer specimens (Figure 1A). PDEF was expressed predominately in the nuclei of non-tumor tissues, while PDEF protein was lost or significantly reduced in the tumor areas. While PDEF protein can be observed in PIN lesions, it is present in the cytoplasm as well as the nucleus. In addition, a tissue microarray slide was stained for PDEF expression (Figure 1B). PDEF expression was negative (21/33), weak (11/33) or moderate (1/33) in the prostate tumor tissues present on the slide. No strong PDEF expression was observed.

PDEF localizes to the nucleus in prostate cancer derived cell lines

The cellular localization of PDEF expression was examined by immunofluorescence. Upon co-staining with the nuclear stain TOPRO and the cytoplasmic actin stain phalloidin, PDEF was found to be predominantly located within the nucleus (Fig 1C).

PDEF expression is reduced in prostate cancer-derived cell lines

We next examined several prostate cancer derived cell lines (LNCaP, PC3 and DU145) for the expression of PDEF (Figure 2A). While PDEF protein is reduced in all prostate cancer cell lines examined, PDEF mRNA and protein expression are retained in the normal prostate tissue. PDEF mRNA and protein levels are not correlated.

Re-expression of PDEF in prostate cancer derived cell lines inhibits cellular growth

To determine possible biological consequences following the expression of exogenous PDEF, we infected prostate cancer derived cell lines with low levels of endogenous PDEF protein with an adenovirus expressing PDEF and GFP (Ad-PDEF) or with an adenovirus expressing GFP (Ad-GFP) as a control. We are able to obtain greater than 95% cell infection using this adenoviral system. We selected 2 different prostate cancer cell lines for functional studies. DU145 and PC3 cells were untreated or infected with either Ad-GFP or Ad-PDEF at 5 MOI and cell viability was assessed using the Countess automated cell counter at 0, 48 and 72h (Figure 2B). Compared to Ad-GFP infected controls, cells infected with Ad-PDEF showed a reduction in total cell number, with greater than 50% reduction in viable cell number 72h after infection. In addition, we observe a significant increase in non-viable cells over time. P-values of <0.005 were calculated for changes in cell proliferation and cell death over the 24 and 72 hour time periods. These results support the model that PDEF reduces cell proliferation and causes cellular death in prostate cancer cells.

PDEF-dependent inhibition of cell growth is mediated through cell cycle changes

To further analyze the potential mechanisms for PDEF-mediated inhibition of cell growth, cell cycle analysis was performed on cells 36h after infection with either Ad-GFP or Ad-PDEF. As shown in Figure 2C, compared to Ad-GFP infected cells, expression of PDEF in DU145 and PC3 cells leads to a small increase in percentage of cells in the S and G2/M phases of the cell cycle, with a concomitant reduction in the percentage of cells in G₀–G₁. This suggests that expression of PDEF prolongs cell cycle progression through the S–G₂ transition.

To better characterize altered cell cycle progression in DU145 and PC3 cells, total protein lysates prepared from infected cells were examined for expression of cell cycle regulatory proteins by Western blot (Figure 2D). The protein levels of Cyclin A, E and D1 are decreased in both PC3 and DU145 cells infected with Ad-PDEF compared to controls. p53 levels are reduced in DU145 (PC3 cells are p53 deficient) and no change in Rb expression was observed in PC3 cells (DU145 are Rb null) expressing PDEF.

PDEF expression levels alter cell migration and invasion

The effect of PDEF re-expression on chemokinetic (movement towards a stimulant) migration and invasion was examined in transwell migration assays using serum as a chemoattractant (Figure 3A). Compared to control cells, adenoviral mediated expression of PDEF in DU145 or PC3 cells reduced the number of cells able to migrate across fibronectin-coated and invade across matrigel-coated membranes between 60–70%.

PDEF loss alters the expression of genes associated with aggressive prostate cancer

Since PDEF is a transcription factor, it is likely that its loss in tumors would alter the expression of many genes and that this altered transcriptome would contribute to new phenotypes during cancer progression. Based on our microarray studies ((14) and data not shown), we selected three candidate PDEF responsive genes which have been reported to be associated with cellular apoptosis and migratory/invasive behavior of prostate cancer cells: Survivin, urokinase plasminogen activator (uPA), and slug. qPCR analysis was used to quantify survivin, uPA, and slug mRNA expression (Figure 3B). We found that re-expression of PDEF in PC3 and DU145 cells led to a decrease in survivin, uPA and slug mRNA expression (Figure 3B). To test the possibility that PDEF may be a direct negatively regulator of survivin and uPA, we performed chromatin immunoprecipitation (ChIP) analysis using PC3 cells which expresses endogenous PDEF as the source of chromatin. qPCR analysis indicated that survivin and uPA promoter fragments were enriched following

immunoprecipitation with a PDEF-specific antibody, compared to IgG control antibody (Figure 3C). The survivin and uPA promoters have previously been shown to have functional Ets binding sites and the current data supports the model that these sites are bound by PDEF *in vivo* and thus, that survivin and uPA are direct PDEF transcriptional targets (Figure 3C). Together, these and other data support the model that PDEF target genes control several aspects of the multi-step metastatic process and specifically, loss of PDEF regulatory networks is a key event in the development of invasive cancer (2).

PDEF protein expression is modulated by miR-204 expression

We next wanted to identify pathways involved in the post-transcriptional regulation of PDEF that ultimately results in protein loss, providing novel mechanistic insight into the cellular events leading to a more aggressive phenotype. Our studies in breast cancer identified a microRNA, miR-204, involved in the negative regulation of PDEF expression (19). Therefore, we performed qPCR to assess the levels of miR-204 in a panel of prostate cell lines and demonstrated that miR-204 is elevated in PC3 and DU145 cells when compared to normal prostate cells (Figure 4A). To assess whether miR-204 has a functional role in the down-regulation of endogenous PDEF expression in prostate cancer, we over-expressed miR-204 in PC3 and DU145 cells. This resulted in a loss of PDEF protein, without significantly affecting the levels of PDEF mRNA (Figure 4A–4B & data not shown). Reciprocal knockdown of endogenous miR-204 levels was performed using antisense oligoribonucleotides (ASO) targeted against miR-204 in PC3 and DU145 cells and resulted in an increase in PDEF protein levels, while the PDEF mRNA levels remained unchanged (Figure 4C & data not shown). These results demonstrate that miR-204 regulates endogenous PDEF mRNA at the post-transcriptional level, most likely through a mechanism of translation inhibition.

Functional consequences of miR-204-mediated negative regulation of PDEF

We and others have shown that PDEF is a negative transcriptional regulator of survivin, uPA and slug expression. Therefore, these genes were selected for examination in our gain-of-function and loss-of-function miR-204 studies as described above. Consistent with negative regulation by PDEF, miR-204-mediated reduction in PDEF protein expression resulted in an increase in the levels of survivin, uPA, and slug by qPCR analysis (Figure 4D). Reciprocal to that inhibition of miR-204 leading to an up-regulation of PDEF protein expression resulted in a decrease in the levels of survivin, uPA, and slug, as shown by qPCR analysis (Figure 4D).

miR-204 levels are elevated in human prostate tumor samples

Based upon the impact of miR-204 on PDEF protein expression and PDEF-dependent phenotypes, we evaluated the levels of miR-204 in RNA prepared from human prostate tumor and normal prostate samples by qPCR. Relative to that found in normal tissue, the levels of miR-204 were found to be significantly elevated in 4/5 tumor samples (Figure 4E; Table 2). These data support miR-204 function as an ‘oncomiR’ and further support the model that elevated expression of miRNAs contribute to the loss of PDEF protein expression in prostate cancer.

Discussion

Dysregulation of multiple pathways allows for the development of prostate cancer. The ability of transcription factors to regulate hundreds of genes means their altered expression provides a mechanism to elicit many cancer associated phenotypes. We have previously described a model that a conversion of tumor suppressor ETS factor towards oncogenic ETS factor expression occurs during cancer progression (1,3,4). ETS1, ETS2, ERG, and ETV1

are over-expressed in prostate cancer and their pro-proliferative and pro-invasive activities place them among the oncogenic ETS factors. In contrast, ESE3 and PDEF show reduced expression in cancer cells. The available data supports the model that PDEF plays a role in regulating processes related to proliferation, differentiation, and/or apoptosis in the prostate. Dysregulation of these processes occurs in cancer whereby cells can assume a more undifferentiated state and become resistant to apoptosis.

We show that PDEF protein is lost or significantly reduced in the majority of prostate cancer tissue specimens examined. These data are consistent with the previous findings showing loss of PDEF protein expression in breast (9), ovarian (13), and colon (15) cancers. Some controversy remains regarding the expression of PDEF in prostate cancer. Initial studies demonstrated loss or reduced PDEF protein in prostate cancer (20,21), with no staining in high Gleason grade lesions (21). Subsequent studies reported that PDEF is expressed in 27% of benign prostate tumors and 40% of prostate adenocarcinomas specimens. This study also reported that PDEF was more highly expressed in tumors with intermediate or high Gleason score compared with low-grade tumors (22). In contrast, a recent publication reported that PDEF was lost in prostate cancer, with least expression in high grade prostate cancers (23).

Based upon the observed loss of PDEF in prostate cancer and cancer-derived cell lines, we next evaluated the functional significance of the observed loss of PDEF protein. Expression of PDEF in prostate cancer cell lines with low endogenous PDEF expression resulted in inhibition of cellular growth due to reduced proliferation and increased apoptosis. In PC3 and DU145 cells, PDEF expression resulted in slight accumulation of cells in S and G₂/M phases of the cell cycle. This observation is different than the G₁ arrest noted following PDEF expression in MDA-MB-231 breast (9) or DLD-1 colon (15) cancer cells. In breast and colon cells, the G₁ arrest was associated with reduced Rb, cdk2 and cyclin A levels and increased p21 and cyclin E levels. In contrast, prostate cancer cells expressing PDEF did not show significant reduction in Rb (DU145 is Rb null), and cyclin A, E and D1 levels were reduced.

Expression of PDEF in prostate cancer cells resulted in significant cell death. ETS factors have also been implicated in the regulation of apoptosis (24). Survivin (BIRC5) is an anti-apoptotic protein that is over-expressed in many cancers (25). In prostate cancer, survivin expression has been associated with high grade (Gleason score 8 or 9), supporting a role in prostate cancer cell proliferation and aggressive phenotypes (26,27). Recently, it has been shown that shRNA mediated silencing of PDEF expression resulted in the upregulation of survivin expression in MCF-7 cells, as well as increased *in vitro* and *in vivo* cell growth and resistance to drug-induced apoptosis (10). Furthermore, re-expression of PDEF in PDEF-negative ovarian tumor cells inhibited tumor cell growth, induced apoptosis, down-regulated survivin expression and its promoter activity (13). We find that PDEF expression decreased survivin expression, and expression of miR-204, which reduces PDEF protein expression, increases survivin expression in prostate cancer cells. Thus, the collective results support an inverse correlation between PDEF and survivin expression. In addition, the ChIP studies described here demonstrate for the first time that PDEF directly binds to the survivin promoter.

PDEF is a negative regulator of cancer cell migration and invasion (9,11,12,15). The reduced expression of slug and uPA observed upon PDEF re-expression may contribute to the inhibition of both migration and invasion. Slug is a well characterized and critical developmental regulator of the epithelial to mesenchymal transition (EMT). EMT is characterized by a series of molecular changes resulting in the ability of epithelial cancer cells to acquire properties of mesenchymal cells such as increased motility, and invasion (28). Significantly, many metastatic cancers recapitulate the EMT resulting in enhanced cell

motility and invasiveness. Slug is upregulated by DHT and EGF, providing a possible molecular mechanism by which epithelial cell-specific genes are silenced during prostate cancer development and progression (29). Knockdown of slug by specific siRNA altered expression of EMT markers and inhibited invasion of PC-3 and PC3-16 prostate cancer cell lines (30). *In vitro* studies using PC3 and LNCaP prostate cancer cells have demonstrated that the observed anti-migratory and anti-invasive properties of PDEF may be mediated in part by its negative regulation of mesenchymal genes and support the model that PDEF is a negative regulator of EMT (11). Our functional studies support the model that loss of PDEF protein contributes to prostate cancer progression. More recently, EMT has been associated with resistance from apoptosis (31,32) and thus the reduced expression of slug and increased cell death observed following PDEF re-expression may be causally linked.

Over-expression of uPA is associated with higher risk of overall and aggressive disease recurrence in men treated with radical prostatectomy for clinically localized prostate cancer (33,34). Studies have shown that direct silencing of uPA by siRNA results in inhibition of PC3 cell growth and motility (35). We demonstrate that PDEF re-expression inhibits migration and invasion of both DU145 cells and PC3 cells. PC3 cells contain amplification of the uPA gene and are significantly more migratory and invasive than DU145 cells (36). While previous studies have defined Ets regulatory pathways that activate transcription of uPA, our studies demonstrate that PDEF is a negative regulator of uPA expression and that uPA is a direct transcriptional target gene for PDEF. Pro-proliferative effects are the result of protease-mediated release of sequestered growth factors and cytokines. Other proteases are also negatively regulated by PDEF, including MMP7 (15) and MMP9 (23) and these may also contribute to the release of growth stimulatory factors.

Comparative *in situ* hybridization and IHC demonstrated that PDEF protein is lost in prostate cancer cells that retain mRNA expression (20). The rapidly evolving field of miRNAs has established close correlations between their altered expression and many types of cancer (37–39). Our previous study identified two miRNAs (miR-204 and miR-510) that are involved in the negative regulation of PDEF mRNA translation during breast cancer progression (19). Although no previous studies have examined expression or functional role of miR-204 in prostate cancer, miR-204 has been found to be both up-regulated (19,40–42) and down-regulated (43–45) in other cancers. Significantly, amplification of a non-coding region mapped to chromosome 9q21 (the genomic location of miR-204) has been associated with prostate cancer (46). We have shown that PDEF is a direct target for miR-204 (19) and we show here that modulation of the miR-204-PDEF regulatory axis in prostate cancer cells affects survivin, slug and uPA expression. Furthermore, we show that miR-204 levels are elevated in prostate tumor compared to normal tissue.

In summary, PDEF is an epithelial specific ETS transcription factor that is down-regulated during prostate carcinogenesis. Recent studies have demonstrated an inverse correlation between PDEF expression and ovarian cancer patient survival (13) and a possible association between the absence of PDEF and breast cancer death (10). The potential prognostic value of PDEF expression loss in prostate and colon patients remains to be elucidated. Our functional studies support the model that loss of PDEF expression occurs during prostate cancer progression and leads to enhanced cellular growth, migration and invasion. Taken together, our data suggest the possibility that the elevation of miR-204 in the prostate may be the initiating step for PDEF loss and prostate cancer progression. Hence, future studies directed towards the elucidation of this relationship may provide insight into the design of novel therapies for prostate cancer, and more importantly the inhibition of prostate cancer progression and subsequent poor overall survival.

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Figure 1. PDEF protein expression is reduced in prostate cancer tissue and cell lines
(A) Immunohistochemical staining of normal prostatic epithelial cells showing nuclear staining for PDEF. Black arrowheads and arrows highlight representative nuclear vs. cytoplasmic staining, respectively. Panels are from the same section. (Magnification, X400).
(B) Quantitation of IHC scores of prostate cancers from TMA slide (n=33 cases). **(C)** Immunofluorescent staining of PDEF (green), nuclei (TOPRO, blue) and actin (phalloidin, red) in DU145, PC-3 and LNCaP prostate cancer cell lines (Magnification, X400; bars=50µM).

Figure 2. Re-expression of PDEF leads to growth changes in prostate cancer cells
(A) Top panel: Western blot analysis of endogenous PDEF protein expression in normal prostate and prostate cancer cell lines LNCaP, PC-3 and DU145. GAPDH was used as a loading control. Bottom panel: Quantitative real-time PCR analysis for PDEF mRNA prepared from normal prostate tissue and the indicated prostate cancer cell lines. **(B)** Quantitation of viable and dead PC3 and DU145 cells either untreated, or infected with Ad-GFP or Ad-PDEF at 5 MOI. Cellular growth was monitored by direct cell counting for 48 and 72 hrs. **(C)** Cell cycle analysis of untreated DU145 and PC3 cells or cells treated with Ad-GFP or Ad-PDEF at a MOI of 5 and harvested 36 hours after infection. **(D)** Western blot

analysis of endogenous Cyclin A, E, D1, p53, Rb, PDEF and GAPDH (as loading control) in PC3 and DU145 cells infected with Ad-GFP or Ad-PDEF virus.

Figure 3. PDEF expression inhibits cell migration, invasion and the expression of urokinase plasminogen activator (uPA), survivin and slug

(A) DU145 and PC3 cells were untreated or infected with either Ad-GFP or Ad-PDEF and migration and invasion measured by transwell assays. Data is representative of duplicate experiments performed in triplicate. (B) Quantitative real-time PCR of uPA, survivin, and slug levels normalized relative to the amount of GAPDH in PC3 and DU145 cells infected with Ad-GFP or Ad-PDEF. (C) Chromatin immunoprecipitation (ChIP) analysis using chromatin prepared from the PC3 cell line. qPCR analysis was performed using primers for the uPA or survivin promoters after ChIP with a PDEF-specific antibody, compared to IgG control antibody.

Figure 4. miR-204 negatively regulates PDEF protein expression and is elevated in human patient prostate tumor samples

(A) qPCR of miR-204 levels in LNCaP, PC3 and DU145 compared to normal prostate epithelial cells (PrEC). (B) Representative western blot (upper panel) and semi-quantitative analysis (lower panel) of endogenous PDEF protein expression in PC3 and DU145 cells transfected with pSupp vector alone (control) or pSupp/miR-204 (miR204). (C) Representative western blot (upper panel) and semi-quantitative analysis (lower panel) of endogenous PDEF in PC3 and DU145 cells transfected with anti-sense oligonucleotide (ASO) against miR-204 (ASO204) or without ASO (control); MCF7 is shown as an endogenous PDEF control. (D) Quantitative real-time PCR of survivin, slug and uPA levels normalized relative to the amount of GAPDH target in PC3 and DU145 cells either over-expressing miR-204 (miR204) or transfected with the ASO 204 (ASO204) compared to

control cells (control). **(E)** Quantitative real-time PCR of miR-204 levels in human prostate tumor samples when compared to normal human prostate control. * $p < 0.05$ by students t-test.

TABLE 1

PCR primers and probes for realtime PCR.

	Forward	Reverse	Probe #
uPA ChIP	GAAGAGTGGAAGTGCTAGGTGAAGA	TAGCAGCTTTCATGACTCCAGATTAC	
Survivin ChIP	TTTCGGGGTAGTATGGTAATGC	TCCTCCCTTTCTCTCCTCCT	
uPA	TTGCTCACCACAACGACATT	GGCAGGCAGATGGTCTGTAT	46
Survivin	GCCCAGTGTTTCTTCTGCTT	CCGGACGAATGCTTTTATG	11
Slug	TGGTTGCTTCAAGGACACAT	GTTGCAGTGAGGGCAAGAA	7
Gapdh	AGCCACATCGCTCAGACAC	GCCCAATATCGACCAAATCC	60

TABLE 2

x-fold increase in miR-204 levels in human prostate tumor samples compared to normal prostate normalized to GAPDH.

Tumor #	x-fold increase
Normal	0.97 ± 0.10
95-02-A160D	37.4 ± 4.75 *
94-11-B004F	2.20 ± 0.97
97-10-A051E	4.35 ± 0.25 *
97-04-A110A	18.2 ± 2.26 *
93-12-A202b	5.29 ± 0.50 *

*
p < 0.001 by students t-test.