Transcriptional Regulation of p21/CIP1 Cell Cycle Inhibitor by PDEF Controls Cell Proliferation and Mammary Tumor Progression*§

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Jeremy S. Schaefer1,1, Yamini Sabherwal1,1, Heidi Y. Shi1, Venkataraman Sriman1, JoAnne Richards1, Alex Minella1, David P. Turner1, Dennis K. Watson1, and Ming Zhang1,2,2

From the 1Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030, the 2Department of Molecular Pharmacology and Biological Chemistry and the Robert H. Lurie Cancer Center and the 3Department of Medicine, Division of Hematology and Oncology, Northwestern University Feinberg School of Medicine, Chicago, Illinois 60611, and the 4Department of Pathology and Laboratory Medicine, College of Medicine, Medical University of South Carolina, Charleston, South Carolina 29425

The Ets family of transcription factors control a myriad of cellular processes and contribute to the underlying genetic loss of cellular homeostasis resulting in cancer. PDEF (prostate-derived Ets factor) has been under investigation for its role in tumor development and progression. However, the role of PDEF in cancer development has been controversial. Some reports link PDEF to tumor promoter, and others show tumor-suppressing functions in various systems under different conditions. So far, there has been no conclusive evidence from in vivo experiments to prove the role of PDEF. We have used both in vitro and in vivo systems to provide a conclusive role of PDEF in the progression process. PDEF-expressing cells block the cell growth rate, and this retardation was reversible when PDEF expression was silenced with PDEF-specific small interfering RNA. When these PDEF-expressing cells were orthotopically implanted into the mouse mammary gland, tumor incidence and growth rate were significantly retarded. Cell cycle analysis revealed that PDEF expression partially blocked cell cycle progression at G1/S without an effect on apoptosis. PDEF overexpression resulted in an increase in p21/CIP1 at both the mRNA and protein levels, resulting in decreased Cdk2 activity. Promoter deletion analysis, electrophoresis mobility shift assays, and chromatin immunoprecipitation studies identified the functional Ets DNA binding site at −2118 bp of the p21/CIP1 gene promoter. This site is capable of binding and responding to PDEF. Furthermore, we silenced p21/CIP1 expression in PDEF-overexpressing cells by small interfering RNA. p21-silenced PDEF cells exhibited significantly increased cell growth in vitro and in vivo, demonstrating the p21 regulation by PDEF as a key player. These experiments identified PDEF as a new transcription factor that directly regulates p21/CIP1 expression under non-stressed conditions. This study conclusively proves that PDEF is a breast tumor suppressor for the first time using both in vitro and in vivo systems. PDEF can be further developed as a target for designing therapeutic intervention of breast cancer.

Gene regulation is critical to maintain the integrity of cells and ensure proper cell survival and growth. When perturbed, improper gene regulation can have dire effects on the individual cell and the larger organism, resulting in cell death or diseased states, such as cancer.

The Ets proteins are a family of transcription factors being investigated for their contributions to the underlying genetic loss of cellular homeostasis resulting in cancers. Characterized by an evolutionarily conserved DNA binding domain called the Ets domain, the Ets transcription factor family controls a myriad of cellular processes, including proliferation, differentiation, and apoptosis (1–10). One Ets family member in particular, PDEF (prostate-derived Ets factor/prostate-specific Ets or PSE), has been investigated for its role in cancer development and progression. Originally identified from the prostate epithelium, PDEF interacts with the androgen receptor to control the expression of PSA (prostate-specific antigen), which is a diagnostic marker for prostate cancer (11). Subsequently, PDEF has been found to be expressed in mammary gland epithelium as well as some other epithelium-rich tissues (12). In addition to the human and mouse genes, a homologous PDEF gene has been identified in the Drosophila genome, suggesting that PDEF may play an important and evolutionarily conserved role during cell growth and development (11–13).

Because of this, PDEF gene function has been under intense investigation to determine its role in tumor progression. Initial reports indicated that PDEF might act as an oncogene (14, 15). However, more recent studies suggest that PDEF possesses a tumor-suppressing function. A study using immunohistochemical detection of PDEF in prostate cancer specimens reported that hPDEF3-positive lesions had an average Gleason
score of 3.8, whereas hPDEF negative lesions had a Gleason score of 5.8 (16). Other studies utilizing in vitro cultured prostate and breast cell lines demonstrated that PDEF mRNA levels do not correspond to translated protein. In fact, in an examination of several human breast cancer cell lines with a range of invasive potential, PDEF mRNA was only translated into protein in the more well differentiated and less invasive MCF7 cell line (17). Likewise, in a study involving normal prostate cell lines and prostate cancer cell lines, PDEF protein was expressed only in the normal prostate cells (18). Findlay et al. (19) demonstrated that this disconnect between PDEF mRNA and protein levels is due to a microRNA-mediated inhibition of translation. Furthermore, transient adenovirus-mediated expression of PDEF in the breast cancer cells resulted in a decrease in tumor cell invasion and growth (17, 20). These results solidified PDEF as a transcription factor of interest as a potential target/tumor growth regulator of the cellular homeostasis pathways that become disrupted during cancer development and progression.

Despite these findings, it is not known how PDEF suppresses tumor progression. In this study, the molecular mechanism(s) underlying the effects of PDEF on tumorigenesis are examined. Through the use of in vitro and in vivo techniques, we demonstrate that PDEF actively regulates p21/CIP1 expression and therefore cyclin-dependent kinase activity to inhibit breast tumor growth.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—The mimosine, propidium iodide, and cycloheximide for the cell cycle and stability analysis were obtained from Sigma. The histone H1 substrate for the kinase assays was from Roche Applied Science. For the immunoprecipitation (IP) kinase assays, Western blots, and adhesion assays, the following primary antibodies were used: p27 (Cell Signaling), PDEF (N-14) (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), hPDEF rabbit polyclonal antibody (produced by the Watson laboratory), p21/CIP1 (BD Pharmingen), Cdk2 (D-12) and actin (Santa Cruz Biotechnology, Inc.). The following secondary antibodies were used for Westerns: horseradish peroxidase-conjugated anti-goat (Roche Applied Science), antimouse (Bio-Rad), and anti-rabbit (Santa Cruz Biotechnology, Inc.).

**Cell Culture**—The mouse breast epithelial cell line, polyoma virus middle T antigen (PyV-mT), was maintained in DMEM (Invitrogen) supplemented with 5% fetal bovine serum (HyClone Laboratories) and 1% penicillin/streptomycin (DMEM complete) at 37 °C with 5% CO₂. For the generation of the stable cell lines, PyV-mT cells were electroporated with 20 μg of the hPDEF pcDNA3.1 or empty pcDNA3.1 (vector control) plasmids. Stable clones were selected by G418 resistance and maintained in DMEM complete at 37 °C with 5% CO₂. PDEF 21 was identified as a high PDEF-expressing transfectant pool, whereas PDEF 15 was identified as a low PDEF-expressing clone line.

**Protein Stability Assays**—PDEF PyVmT and PyVmT cells were treated with cycloheximide (25 μg/ml) at various time points (2, 4, 6 h). Cells were lysed in radioimmune precipitation buffer with protease inhibitors and subjected to Western blot against p21/CIP1 polyclonal antibody.

**Isolation of RNA and RT-PCR**—Total RNA was isolated from the cell lines using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. A two-step RT-PCR was used to analyze mRNA expression of the p21/CIP1, PDEF, and L19 genes. A cDNA was created using an oligo(dT) primer and the Moloney murine leukemia virus reverse transcriptase enzyme according to manufacturer’s instructions (Invitrogen). Standard PCR techniques were then conducted with gene-specific primers (supplemental Table S1).

**shRNA Vectors and Transfection**—The PDEF shRNA vector was obtained from Dr. Fengzhi Li (SUNY at Buffalo) (21). A DNA sequence of 21 nucleotides was selected for PDEF silencing (5'-TCC CAC CTG GAC ATC TGG AAG TCA GTC AAG-3' (sense) and 5'-CA AAT ACC TGC ACA TCT GGT CAG CTC TTG ACT GAC TTC CAG ATC TGG AAG TCA GTC AAG-3' (antisense)). The fragment was directly cloned into psiRNA-hH11zeo Vector (InvivoGen) at the BbsI site to generate the hPDEF shRNA. For establishing the shRNA stable cell lines, the PDEF PyV-mT cell line was transfected with 5 μg of the hPDEF shRNA (PDEF 21 PDEF shRNA) or the shRNA vector only (PDEF 21 Cx shRNA). Stable clones were selected with Zeocin (1200 μg/ml) and maintained in DMEM complete at 37 °C with 5% CO₂. Pools of clones were selected and confirmed for the down-regulation of PDEF by Western blot. p21 was silenced using retroviral infection of a p21-specific shRNA plasmid (PDEF 21 Shp21) obtained from Dr. Alex Minella (Northwestern University, Chicago); a nonspecific SHC vector was used as a control (PDEF 21 SHC). After retroviral infection, cells were selected with hygromycin (500 μg/ml).

**Growth Assays**—For the growth assays, the stable PyV-mT clones were seeded at 1000 cells/well in 24-well dishes and allowed to grow at 37 °C with 5% CO₂. The cells were counted using a hemacytometer (see Fig. 5) or a Coulter counter (see Fig. 2). Each time point was done in triplicate.

**3-(4,5-Dimethylthiazol-2)-2,5-diphenyltetrazolium Bromide (MTT) Assay**—To measure cell viability and growth over time, an MTT assay was conducted. PDEF 21 SHC and PDEF 21 Shp21 cells were seeded in 24-well dishes at a concentration of 1000 cells/well and allowed to grow at 37 °C with 5% CO₂. MTT reagent (5 mg/ml) was added in a volume of 10 μl/well and incubated at 37 °C with 5% CO₂ for 3 h. The medium was aspirated, and 100 μl of DMSO was added and mixed until a uniform purple color formed. The cell samples were measured using a plate reader at 570 nm.

**Soft Agar Colony Formation Assay**—For colony formation, 6-well dishes were plated with bottom agar (1.3 ml of 1.8% agarose and 0.3 ml of 2 × DMEM) for 20–30 min. PDEF 21 SHC and PDEF 21 Shp21 cells were mixed with the top agar (0.3 ml of 1.8% agarose and 0.7 ml of DMEM) at a concentration of 5 × 10⁴ cells/ml and plated in 6-well dishes and allowed to solidify. The cells were grown at 37 °C for 2 weeks, and the numbers of colonies were tabulated. Three independent experiments (in duplicates) were averaged for bar graph representation.

**Orthotopic Tumor Implant**—Both the left and right number 4 mammary glands of the FVB mice were injected with either stable PDEF clones or the vector control PyV-mT clones at a concentration of 5 × 10⁵ cells/10 μl of medium. Six mice were
injected with the PDEF 15 clones, 20 mice were injected with the PDEF 21 clone, and 10 mice were injected with the vector control (PyV-mT) clone. All animal experiments described herein were conducted in accordance with accepted standards of humane animal care.

The left number 4 mammary glands of nine FVB mice were injected with PDEF 21 SHC, and the right number 4 mammary glands of nine FVB mice were injected with PDEF 21 SHp21 at a concentration of 5 × 10^5 cells in 10 µl of medium. Tumor volume was measured using a Vernier caliper at the indicated time points. Tumor-free survival was defined as the percentage of mice that did not develop any detectable tumors over time. Tumor-free survivors in these studies indicate mice that did not bear palpable tumors at the conclusion of the experiment.

Cell Cycle Assays—For the cell cycle assays, 1 × 10^6 stable PDEF 21 and vector control cells were treated for 24 h with mimosine in 100-µm plates. Serum was added, and cells were collected at the indicated times for either IP kinase or flow cytometry assays. For flow cytometry analysis, cells were collected by trypsinization and washed with phosphate-buffered saline twice. The cells were fixed in 70% ethanol for 30 min at room temperature and stained with a propidium iodide (Sigma) solution containing RNase for 30 min at 37 °C prior to analysis.

Immunoprecipitation Cyclin-dependent Kinase Assay—The IP cyclin-dependent kinase assay was performed as described previously (22–25). Briefly, a whole cell extract was prepared by lysing the stable PyV-mT cells in 0.5 ml of ELB lysis buffer (on ice for 30 min). 500 µg of whole cell extract was incubated with 5 µl of anti-Cdk2 antibody and 25 µl of Protein A/G-agarose beads overnight at 4 °C with rotation. Samples were washed twice with ELB buffer, followed by a single wash with 1 × kinase buffer. The kinase reaction was set up with 7.5 µl of 2 × kinase buffer, 25 µl of 1 mg/ml histone H1 substrate, and 1 µl of [γ-32P]ATP for a total of 15 µl. Samples were incubated for 30 min at 30 °C. 6 × SDS-PAGE loading dye was added, and samples were heated for 10 min at 90 °C before running on a 12% polyacrylamide gel. Gels were transferred to polyvinylidene difluoride membrane (Bio-Rad) and exposed. The membranes were analyzed by Western blot for cell cycle proteins. Nonradioactive gels were run and transferred to polyvinylidene difluoride membranes to examine cell cycle proteins in the ELB cell lysates.

Luciferase Assay—For the luciferase assay of transiently transfected cells, 2 × 10^5 HEK-293 cells or the stable PyV-mT cells were co-transfected in 6-well plates with 500 ng of either the full-length WWPluc (p21/CIP1 promoter Luc) or one of the p21/CIP1 promoter deletion-luciferase vectors, 100 ng of the β-galactosidase vector, and 250 ng of either the PDEF or empty pcDNA3.1 vector using Fugene 6. Cells were collected and lysed 48 h later using the Promega 5 × cell culture lysis reagent (catalog number E153A) according to the manufacturer’s instructions. 20 µl of each lysate was used in the luciferase assay. Luminescence was recorded using a BD Monolight 3010 Luminometer. The luciferase activities were normalized based on β-galactosidase activity. The p21/CIP1 promoter-luciferase vectors were the gifts of Dr. Bert Vogelstein for the full-length vectors and Dr. Xiao-Fan Wang for the deletion vectors (26–29).

Expression and Purification of Glutathione S-Transferase (GST)-Recombinant hPDEF Protein—GST (Amersham Biosciences) and the hPDEF fusion protein (GST-hPDEF) were transformed in Escherichia coli BL21 cells. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside and purified according to the manufacturer’s instructions.

Electrophoretic Mobility Shift Assays (EMSA)—EMSA were conducted as described previously with minor modifications (30, 31). Hybridized oligonucleotide duplexes were end-labeled with [γ-32P]dCTP. Purified GST-PDEF protein (5 µg) was incubated with the Ets binding sites of the p21/CIP1 promoter (supplemental Table S2) (300,000 cpm) for 45 min on ice in 20 µl of EMSA buffer. Supershifting and competitive inhibition were performed by adding 0.4 or 1.0 µg of anti-PDEF antibody (Santa Cruz Biotechnology, Inc.) or 100 × cold competitor oligonucleotide duplexes for 30 min on ice prior to the addition of the radiolabeled oligonucleotide duplex. Binding was analyzed on a native 5% polyacrylamide gel that was prerun for 30 min at 200 V in 1 × TBE buffer. The samples were electrophoresed at 200 V for 60 min. The gels were dried and exposed.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed using an assay kit (Upstate Biotechnology) according to the manufacturer’s instructions. Briefly, cells were cross-linked with 1% formaldehyde at 37 °C for 10 min. Cells were rinsed three times with ice-cold phosphate-buffered saline and collected into phosphate-buffered saline and centrifuged for 5 min at 1000 rpm. Crude nuclei were isolated using the protocol described for co-IP assays. Nuclei were prepared in the SDS lysis buffer provided in the kit. Lysates were sonicated at 30% power, 10 × 5 s using a Sonic Dismembrator model 300 (Fisher) to shear genomic DNA. The following antibodies were used to perform IP: polyclonal PDEF (the Watson laboratory) and polyclonal rabbit IgG as control. PCR amplification of the various Ets binding sites was performed using the following primer sets: 1) p21B, 5′-GGGGAGGAAGGGATGGAGGAG-3′ (forward) and 5′-GGTTTGCAACCATGCAC-3′ (reverse); 2) p21A, 5′-GGG GAG GAA GGG GAT GGT AGG AG-3′ (forward) and 5′-GGT GTC GCA AGG ATC CTG CTG GCA GA-3′ (reverse); 3) p21CD, 5′-GACACTTAGGTAGTC-3′ (forward) and 5′-CACTGCTGTCGCA-3′ (reverse); 4) p21EF, 5′-CAG TAG ACA CTT CC-3′ (forward) and 5′-CTCCTG-GCTGCCAGC-3′ (reverse). A total of 4 µl of extracted DNA was used and 40 rounds of amplification were performed using an iCycler iQ system, and continuous SYBR Green I fluorescence monitoring was performed according to Bio-Rad protocols.

The relative proportions of co-IP products were determined based on the threshold cycle (CT) value for each PCR. The CT value is defined as the cycle at which fluorescence rises to 10 times above the mean S.D. of the background levels in all reaction wells. A ΔCT value was calculated for each sample by subtracting the CT value for the input (sample prior to IP) from the CT obtained from the IP product. A ΔΔCT value was calculated by subtracting the ΔCT value of the sample IP with the ΔCT value of the corresponding control sample IP with rabbit IgG.
Fold enrichment was then determined by raising 2 to the $\Delta \Delta C_T$ power. The equation used in these calculations is as follows,

$$\text{Fold enrichment} = 2^{\Delta \Delta C_T}$$  \hspace{1cm} (Eq. 1)

where fold enrichment represents PDEF ChIP relative to control ChIP, and $\Delta C_T = C_T(\text{immunoprecipitated sample}) - C_T(\text{input})$.

**RESULTS**

PDEF Expression Suppresses Growth of a Mouse Mammary Tumor Cell Line in Vitro—To investigate the functional effects of PDEF expression on cell growth in vitro, stable cell lines were generated to express epitope-tagged PDEF protein. The PyV-mT (polyoma virus middle-T) mouse mammary tumor cell line was chosen as the parental cell line for this purpose. Derived from the aggressive and metastatic mammary tumors that develop in the MMTV polyoma virus middle-T antigen transgenic mice, the PyV-mT tumor cell line was selected for its tumorigenic and metastatic potential. Accordingly, these cells were transfected with either the tagged PDEF expression vector (PDEF pcDNA3.1) or an empty pcDNA3.1 plasmid (vector control). RT-PCR and Western blot analysis were used to identify the clonal lines, PDEF 21 and PDEF 15, as high and low level PDEF-expressing lines, respectively (Fig. 1, A and B). Despite differences in PDEF expression, both the PDEF 21 and 15 clones exhibited a delay in growth when compared with the vector control in an in vitro growth assay (Fig. 1C). This suggests that a threshold level of PDEF protein expression may...
exist beyond which further growth effects will be minimal. Furthermore, transfection of the PDEF 21 cells with a PDEF-specific shRNA but not a nonspecific control shRNA plasmid abrogated this growth delay effect (Fig. 1, D and E). These data indicate that PDEF expression may interfere with the life/death cycle of the mammary tumor cell either at the level of apoptosis or possibly cell cycle control to decrease or slow the growth of the cells.

**PDEF Expression Suppresses Growth of PyV-mT Cells in Vivo**—To further investigate the role of PDEF expression on tumorigenic incidence and growth, the vector control and the PDEF-expressing stable PyV-mT cell clones were implanted orthotopically in the left and right number four mammary glands of FVB mice. The Kaplan-Meier curve of Fig. 2B demonstrates that the implanted PDEF 21 clones had a decreased incidence of tumors in relation to the vector control cells. At the study’s conclusion (at 120 days postimplantation), ~35% of the PDEF 21 cell line implants were still tumor-free in comparison with the PDEF 15 and vector control cell line implants which had 100% tumor incidence. As expected, the decreased tumor incidence translated into a decreased tumor size in the PDEF 21 cell implants (Fig. 2A). The tumors that developed from the PDEF 21 cell implants grew much more slowly and were smaller than the vector control cell implants. The average number of days for the vector control cell implants to reach 750 cubic millimeters was 34.5, whereas for the PDEF 21 cell implants it was 73.9 (Student’s t test, p < 0.05). The PDEF 15 clone, which exhibits lower PDEF protein expression compared with PDEF 21, also affected cell growth in vitro, although with this reduced PDEF protein expression, a reduced inhibition of tumor growth was observed (Fig. 2A). Ki67 staining of tumor sections confirmed reduced proliferation in the PDEF tumors (Fig. 2C). These data indicate that the observed in vitro growth delay caused by PDEF expression could be replicated in an in vivo orthotopic tumor implantation model. Fluorogenic caspase

![FIGURE 2. Expression of PDEF retards in vivo cell growth of PyV-mT cells in an orthotopic mouse mammary tumor implant model. A, in vivo tumor growth curve of the stable PDEF 15 (p > 0.05), PDEF 21 (p < 0.05), and vector control cells. The error bars for each graph represent S.D. B, tumor incidence curves for the PDEF 15 (p > 0.05), PDEF 21 (p < 0.05), and vector control PyV-mT cells. C, Ki67 proliferation staining of tumor sections from PDEF 21 and vector cells. The proliferation index is percentage of Ki-67-positive cells in tumors in PDEF 21 and vector cells (p value < 0.02). D, caspase 3 fluorogenic assay to measure apoptosis of PDEF 21 and vector control cells. No statistically significant difference was observed between the PDEF 21 and vector control cells grown with or without serum for 48 h. Caspase 3 activity was normalized to protein concentration.](image-url)
FIGURE 3. Cell cycle profile of PDEF 21 and vector control cells reveals a cell cycle inhibition by PDEF in G_{1}/S in synchronized PDEF21 compared with that in vector control cells, and the growth retardation results from increased p21 protein and decreased Cdk2 activity. A, flow cytometric analysis of PDEF 21 and vector control cells after synchronization and release at 0, 15, and 24 h time points with different cell cycle profiles. B, Western blot of the PDEF 21 and vector control cells using antibodies against the indicated proteins. Note the increased p21 cell cycle inhibitor in PDEF 21 cells at 15 h and non-synchronized cells; the relative intensities have been quantitated using Image J analysis. C, cycloheximide protein stability assay with p21/CIP1 antibody revealed that degradation of p21/CIP1 protein was not impeded in the PDEF 21 cells in comparison with PyV-mT cells. The degradation kinetics showing relative intensity has been quantitated with Image J. D, Cdk2 IP kinase assay of the PDEF 21 and vector control cells using histone H1 as the substrate.
3 assays using tumor cell extracts did not reveal a significant difference in apoptosis in the presence and absence of serum (Fig. 2).

**PDEF Expression Alters the Cell Cycle Profile**—Given that PDEF expression resulted in a cell growth delay in the mammary tumor cell lines, cell cycle analysis was performed at a number of time points following cell cycle synchronization with mimosine. As shown in Fig. 3A, 24-h mimosine treatment effectively synchronized both the PDEF 21 and the vector control cells at the G0/G1 phase of the cell cycle with over 85% of each group being assigned to the G0/G1 phase. A number of reports have successfully shown the reversibility of this treatment in the examination of cell cycle events (33–36). Although the vector control cells had progressed out of the G0/G1 phase and into the other phases of the cycle after 15 h, a significant portion (78%) of the PDEF 21 cells remained in the G0/G1 phase at this time point. The PDEF 21 cells did eventually recover and continue to progress through the cell cycle by 24 h, confirming the non-lethal and reversible effects of mimosine.

To further analyze the cause of the observed cell cycle delay, cell lysates from the PDEF 21 and the vector control PyV-mT cells were collected and analyzed via Western blot analysis for known cell cycle regulatory proteins (Fig. 3B). Although the protein levels of p27/KIP1 and Cdk2 remained relatively unchanged and equal between the PDEF 21 and the vector control PyV-mT cells during the time course studied, p21/CIP1 was increased in the PDEF 21 PyV-mT cells in comparison with the vector control PyV-mT cells, particularly at the 15 h time point. RT-PCR analysis of these cells at this time point confirmed the protein expression results (data not shown). This mRNA and protein data suggest that the observed PDEF growth delay may be dependent upon a p21/CIP1-mediated growth delay.

**FIGURE 4.** Determination of the responsiveness of the p21 promoter (WWP) to PDEF transcription factor using promoter luciferase reporter assays. Identification of the candidate Ets binding site(s) on the p21 promoter is shown. A, p21 promoter luciferase assay in vector control and PDEF 21 cells. A full-length p21 promoter (WWP) was transiently transfected to PDEF 21 and vector control cells (p < 0.05). Error bars, S.E. Each transient transfection was repeated at least three times. The -fold change was normalized to β-galactosidase activity. B, co-transfection of p21 promoter (WWP) and PDEF expression vector (pEF-PDEF) or pEF control vector in HEK-293 cells, which does not have endogenous PDEF expression (p < 0.05). There is a >2.5-fold induction in HEK-293 cells co-transfected with WWP and pEF-PDEF compared with that with WWP and pEF control vector. C, illustration of the 2.4-kb p21 promoter with candidate Ets binding sites indicated as p21A to p21F. Note the location of the p21A and p21B Ets binding sites at -2191 and -2118 bp on the p21 promoter. Transient transfections of HEK-293 cells with (+) or without (−) PDEF expression vector are indicated (vector control is used for transfection without PDEF expression vector). Error bars, S.E. (three repeated experiments). The promoter deletion analysis clearly showed that the Δ-400 WWP p21 promoter missing the Ets binding sites p21A and p21B significantly decreased its promoter activity in comparison with full-length p21 promoter (p < 0.05). D, promoter luciferase activity in MCF7 breast cells of p21 promoter (WWP) and Δ-400 (p21A and p21B sites deleted).
growth inhibition. Protein stability assays revealed that degradation of p21/CIP1 protein was not impeded in the PDEF 21 cells, thus indicating that the increased p21/CIP1 protein levels are due to transcriptional regulation by PDEF (Fig. 3C).

To verify the biological significance of the observed up-regulation of p21/CIP1 mRNA and protein in the PDEF 21 PyV-mT cells, immunoprecipitation kinase assays were conducted using an antibody against the late G1/S cyclin-dependent kinase, Cdk2 (Fig. 3D). The kinase activity of Cdk2 was decreased in the PDEF 21 PyV-mT cells in comparison with the vector control PyV-mT cells at both the random/unsynchronized and 15 h time points. Immunoprecipitation with an isotype control antibody had no effect on the kinase activity of either cell line. These data indicate that the elevated levels of p21/CIP1 protein are functionally active and sufficient to inhibit the Cdk2 activity, thus contributing to the apparent delay in progression through the G1 phase of the cell cycle.

**PDEF Up-regulates p21/CIP1 Gene Expression**—To examine whether PDEF could directly up-regulate the p21/CIP1 gene, transient co-transfections were conducted. A p21/CIP1 reporter construct, WWP-Luc (from Dr. Bert Vogelstein), which contained ~2.4 kb of the human p21/CIP1 promoter sequence fused to a luciferase reporter was transiently transfected into stable PDEF 21 and PyV-mT vector control cells. A ~20-fold change in induction was seen in the promoter activity of PDEF 21 cells over that in the PyV-mT vector control cells (Fig. 4A). Furthermore, co-transfection of the p21 promoter (WWP) construct and the PDEF expression control vector in HEK-393 (human embryonic kidney epithelial cell line) cells, which have no endogenous PDEF expression level showed a ~2.5-fold induction in luciferase activity (Fig. 4B). Thus, PDEF probably acts on the p21 promoter (WWP) to regulate its transcription. Transcription factor binding site analysis and comparison of promoter DNA sequences among humans, mice, and rats revealed various Ets binding sites (EBSs) as potential binding sites for PDEF. To identify the exact site of the functional EBS on the p21/CIP1 promoter, a series of progressive 5′-promoter deletions were obtained from Dr. Xiao-Fan Wang. Tran-
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FIGURE 6. p21 silencing abrogates the PDEF growth inhibition in an in vitro two-dimensional cell culture and in a three-dimensional soft agar colony formation assay. A, Western blot of PDEF expression in PDEF 21 cells retrovirally infected with the control (PDEF 21 SHC) and p21 shRNA silencing vector (PDEF 21 SHp21). PDEF 21 SHp21 cells show significant p21 reduction compared with the PDEF 21SHC cells, with actin as loading control. B, MTT assay of PDEF 21 SHC and PDEF 21 SHp21 cells over a 5-day period. PDEF 21 SHp21 cells have a 2.5-fold increase in proliferation rate in vitro in comparison with PDEF 21 SHC cells with a p value of <0.017. C, soft agar colony formation assay shows increased colony-forming potential in PDEF 21 SHp21 cells in comparison with PDEF 21 SHC cells with p < 0.024. Colonies were counted for a 2-week period, and experiments were repeated three times.

The fused luciferase reporter of the p21/CIP1 gene promoter, and other p21 EBS sites (p21C, -D, and -E) were used in EMSA to determine whether PDEF could bind to any of these EBS sites. As expected, the GST-PDEF protein specifically bound the PSA EBS probe (Fig. 5). PDEF bound to p21B probe with high specificity, and the binding was efficiently competed by 100× unlabeled p21B probes and the PSA EBS probe but not the random oligonucleotide (Fig. 5A). The GST-PDEF protein bound only to the −2181 bp region (p21B) and did not bind to p21A, p21F (Fig. 5A), or any other p21 EBS sites (p21C, -D, -E; data not shown). Furthermore, we used nuclear extracts isolated from PDEF 21 cells and p21B probe for the EMSA. Endogenous PDEF bound specifically to p21B EBS probe, as does purified recombinant PDEF (Fig. 5B).

However, in order to confirm this PDEF interaction with the p21 promoter in cell culture, ChIPs were conducted using the PDEF antibody, and the extracts were isolated from MCF7 cells endogenously expressing PDEF. As shown in Fig. 5C, a DNA fragment for PDEF binding was specifically amplified with the primers for p21B. The -fold change for PDEF binding to p21B versus control was significantly greater than that to p21A, p21CD, and p21EF by a quantitative RT-PCR assay, confirming the preferred binding of PDEF to the p21B site in the p21 promoter in vivo. This study for the first time reports the direct binding interaction of PDEF on the p21 promoter and identifies a specific site that is involved in the binding interaction required for gene regulation.

p21 Silencing Abrogates the PDEF Growth Inhibition in Vitro—To confirm that p21 was the mediator responsible for the PDEF-induced growth inhibition, we knocked down p21/CIP expression in PDEF-expressing PyV-mT tumor cells by RNA interference. To do so, the PDEF 21 cells were transfected with a p21-specific shRNA plasmid. p21 expression was effectively reduced in PDEF 21 transfected with the p21-specific shRNA plasmid (PDEF 21 SHp21) in comparison with the nonspecific shRNA plasmid (PDEF 21 SHC) (Fig. 6A). MTT and soft agar assays were conducted to determine the proliferative and transformed phenotypes of these cells. As shown in Fig. 6B, PDEF 21 SHp21 increased the growth of the PDEF PyV-mT cells compared with the PDEF 21 SHC cells. Likewise, the number of colonies formed in the soft agar assay was increased in PDEF 21 SHp21 cells (Fig. 6C). These data point to p21 as the key mediator of the PDEF-induced inhibition of anchorage-dependent and -independent cell growth.
Silencing Abrogates the PDEF Growth Suppression of PyV-mT Cells in Vivo—To further investigate the relationship between PDEF and p21 expression on tumorigenic potential in mouse model in vivo, the PDEF 21 SHp21 and PDEF 21 SHC cell clones were implanted in the number 4 mammary glands of FVB mice, and tumor growth was monitored. A, the in vivo tumor growth curve demonstrated that there was a significant increase in tumor growth in the PDEF 21 SHp21 cells in comparison with the PDEF 21 SHC cells with a p value of 0.002. B, the Kaplan-Meier curve demonstrated that the PDEF 21 SHp21 cells had decreased survival relative to the PDEF 21 SHC cells.

DISCUSSION

Following the initial discovery of PDEF in 2000 (12), much work has been done to assess its role in normal growth and development as well as diseased states, including cancer. PDEF has been shown to play the role of promoter of tumor progression and suppressor in the hands of many researchers using different systems. It is noted that most evidence reported the use of an in vitro cell culture system or correlative expression data for histological tissue studies, without direct evidence from an in vivo animal model for PDEF function. For this reason, we performed both in vitro and in vivo experiments to identify the role of PDEF in breast tumor progression. In this study, we take the approaches of PDEF overexpression and gene silencing techniques. We have not only demonstrated the tumor suppressor function of PDEF but also identified the mechanism by which PDEF suppresses tumor progression. In particular, the data reported here demonstrate a direct involvement of the PDEF transcription factor in the regulation of the p21/CIP1 cell cycle inhibitor. Under stress or DNA damage conditions, p21/CIP1 is a critical mediator of p53-dependent cell cycle arrest. In this case, p53 activates p21, thereby inducing cell cycle arrest, allowing the cell to repair DNA damage and preserving the genomic stability of cells, which frequently becomes destabilized during tumor development (38). Therefore, it is not surprising that p21 expression must be tightly controlled to ensure that the p21-induced cell cycle arrest functions properly to prevent the genomic instability that could lead to tumor formation. However, under non-stress/damaged conditions, p21 also acts as a key cell cycle regulator at G1/S phase (39, 40). How p21 gene expression is regulated during cell proliferation and differentiation under normal, non-

FIGURE 7. p21 mediates the anti-tumor function of PDEF in an in vivo animal experiment. Silencing of p21 in PDEF 21 cells abrogates the PDEF growth suppression function in vivo. The PDEF 21 SHp21 and PDEF 21 SHC cell clones were implanted in the number 4 mammary glands of FVB mice, and tumor growth was monitored. A, the in vivo tumor growth curve demonstrated that there was a significant increase in tumor growth in the PDEF 21 SHp21 cells in comparison with the PDEF 21 SHC cells with a p value of 0.002. B, the Kaplan-Meier curve demonstrated that the PDEF 21 SHp21 cells had decreased survival relative to the PDEF 21 SHC cells.
stressed conditions is an important question? Our data clearly addressed this question. This is the first report showing that a cell cycle inhibitor is regulated by a member of the Ets transcription factor family.

The PyV-mT mouse breast cancer cell line represents an excellent mouse model of breast tumorigenesis. We showed that overexpression of PDEF in PyV-mT cells reduced cell growth in in vitro cultures; orthotopic implantation of these cells in mice mirrored the in vitro findings in that tumor growth was reduced. We further demonstrated that this growth deficiency was the result of a perturbation of the cell cycle proteins and activities and not due to an increase in apoptosis. In particular, the activity of the cyclin-dependent kinase Cdk2 was decreased in the PDEF 21 PyV-mT cells in comparison with the control cells. Furthermore, it was demonstrated that this alteration of activity was due to the up-regulated expression of the cyclin-dependent kinase inhibitor p21/CIP1, which is consistent with published reports documenting the inhibitory effects of the p21/CIP1 gene (29, 41–43). Specifically, we have provided novel evidence that PDEF acts at the transcriptional level to positively regulate p21/CIP1 gene expression and that disruption of p21 both in vitro and in vivo abrogates the PDEF effects on cell growth. Degradation of p21 was not impaired in PDEF PyV-mT cells. In fact, p21 was seen to be degraded faster in the presence of PDEF, which could possibly be due to increased transcription/synthesis of p21 in the PDEF PyV-mT cells. This increase of p21 is so significant that it accounts for the increased steady state abundance of p21, regardless of its being degraded faster in these cells. This does raise the question of why p21 seems to degrade so quickly in the PDEF PyV-mT cells. One explanation could be that there is so much more p21 due to increased synthesis in the PDEF-overexpressing cells that it is being rapidly degraded by the proteasome, especially when p21 is free (i.e. not bound to cyclin/Cdk1), and it is misfolded and quite rapidly degraded. On the other hand, in cyclin-Cdk1 complexes, p21 is more stable. Furthermore, silencing p21 expression in PDEF-PyV-mT cells by retroviral infection elicited increased growth and colony-forming potential in comparison with control (PDEF 21 SHC). Likewise, in vivo injection of PDEF 21 SHp21 resulted in increased tumor growth. Through EMSAs and ChIP PCR analysis, we have shown that the proximal promoter of the p21/CIP1 gene is a direct target of the PDEF transcription factor and have identified the Ets DNA binding element located within the p21/CIP1 gene promoter (p21B at −2118 bp) responsible for p21 induction in vitro and in vivo.

Several PDEF transcriptional targets have been identified recently. For example, survivin, an IAP (inhibitor of apoptosis) gene family member, was identified as a potential mediator of the PDEF growth effect (21). Considering that survivin inhibits apoptosis while promoting tumorigenesis and that its expression has been shown to be negatively associated with tumor severity and aggression, this has profound implications for PDEF (44–50). With this discovery, the list of PDEF target genes expands to five to include p21/CIP1, maspin, survivivin, p62, and PSA (11, 17, 21, 37, 51, 52). Collectively, these observations show that PDEF participates in the regulation of a key cell cycle regulator (p21/CIP1), a regulator of the ubiquitin-proteasome pathway (p62), a tumor suppressor (maspin), a proposed oncogene (survivin), and a tissue-specific cancer marker (PSA). To date, only our study for the first time shows p21/CIP1 by ChIP to be a direct target of PDEF. In terms of cancer progression, PDEF up-regulates the expression of the tumor suppressor and cell cycle regulator but down-regulates the expression of the proposed oncogene. In addition, PDEF has been found to physically associate with the suspected tumor suppressor NKKX3.1 as well as the androgen receptor and Runx1 (AML-1) transcription factor (11, 32, 53, 54). Given these results, it is becoming apparent that PDEF exerts its effects through multiple gene targets, depending on the internal cell milieu and co-activators present. In our study with the breast epithelial cells, PDEF acts as an inhibitor of cell proliferation under non-stressed conditions.

Finally, this study for the first time has identified the specific site that PDEF binds to on the p21 promoter and regulates its inhibitory activity. p21 is a general cell cycle inhibitor, which makes it difficult to develop therapeutic interventions against p21. Our study opens the possibility of developing novel strategies to target PDEF for cancer therapeutic interventions.

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