[CANCER RESEARCH 63, 4626–4631, August 1, 2003]

Pdef Expression in Human Breast Cancer Is Correlated with Invasive Potential and Altered Gene Expression[1]

Ron J. Feldman, Victor I. Sementchenko, Maged Gayed, Mostafa M. Fraig, and Dennis K. Watson[2]

Laboratory of Cancer Genomics, Hollings Cancer Center [R. J. F., V. I. S., M. G., D. K. W.] and Department of Pathology [M. M. F., D. K. W.], Medical University of South Carolina, Charleston, South Carolina 29425

ABSTRACT

Ets transcription factors control multiple biological processes, including cell proliferation, differentiation, apoptosis, angiogenesis, transformation, and invasion. Pdef is an Ets transcription factor originally identified in prostate tissue. We demonstrate that human Pdef is expressed at high levels primarily in tissues with high epithelial cell content, including prostate, colon, and breast. We also determined that Pdef protein is reduced in human invasive breast cancer and is absent in invasive breast cancer cell lines. We next assessed the functional consequences of these observations. Significantly, expression of Pdef in breast cancer cells leads to inhibition of invasion, migration, and growth. Expression of Pdef also results in the down-regulation of urokinase-type plasminogen activator and activation of the promoter of the tumor suppressor gene, *Maspin*. Growth-suppressive effects of Pdef expression are mediated in part by a G_0-G_1 cell cycle arrest associated with elevated p21 levels. Collectively, these results indicate that Pdef loss may alter the expression of genes controlling progression to invasive breast cancer.

INTRODUCTION

Breast cancer is the most common malignancy diagnosed in American women and has a rate of mortality second only to lung cancer (1). Breast cancer mortality is almost invariably attributable to metastasis that is clinically untreatable despite aggressive chemical and radiation therapies (2). The proposed molecular mechanisms underlying breast cancer progression include overexpression of oncogenes such as HER-2/*neu* or *myc* (3, 4), or loss of tumor suppressor genes such as *p53* (5). Several potential metastasis modulators have also been identified (2). Furthermore, recent analyses of global changes in the transcriptome (6) and proteome (7) of breast cancer cells have yielded additional potential markers. Indeed, molecular classification of breast cancer patients by gene expression profiling has the potential to identify those tumors likely to be most aggressive (8). However, no integrated molecular and cellular mechanism for tumor invasion and subsequent metastasis has emerged. Additional studies directed toward elucidation of the factors involved in breast cancer progression should facilitate the design of molecularly based diagnostic and therapeutic approaches.

Ets transcription factors are highly conserved proteins that have a unique 85 amino acid DNA-binding domain. These proteins recognize a core 5′-GGA(A/T)-3′ sequence present in downstream target genes. The family consists of 25 members in humans, which are expressed in a variety of tissues (9). Ets-regulated pathways control normal development and cancer (9). Specifically, Ets proteins have been implicated in the progression of breast cancer. Ets2, Pea3, and Ese1 subfamilies of Ets are overexpressed in ductal carcinoma *in situ* and invasive carcinoma, and appear to act as oncogenes (10–12). Overexpression of these factors in breast cancer cell lines *versus* normal mammary epithelial cells is associated with regulation of genes involved in cell motility and invasion. Furthermore, inhibition of Ets function results in the repression of tumorigenic and invasive properties of breast cancer cells (10).

Pdef was originally identified and defined as a prostate-derived Ets factor, present in normal prostate luminal cells, and in ovary, mammary, and salivary gland (13). Subsequent investigation demonstrated that Pdef protein expression is lost in advanced prostatic cancer tissues and cell lines (14). In this study, we demonstrate that Pdef is expressed in some normal tissues with high epithelial cell content, including prostate, colon, and breast. In addition, we show that Pdef protein expression is reduced in invasive breast cancer. Furthermore, we find that expression of Pdef in invasive cancer cells inhibits their ability to invade and migrate. We demonstrate that Pdef is a negative regulator of the prometastatic gene *uPA*[3] and a positive regulator of the tumor metastasis suppressor gene *Maspin*. Moreover, expression of Pdef results in inhibition of breast cancer cell growth mediated in part by a p21-dependent G_0-G_1 cell cycle arrest. These data for the first time provide evidence for the functional significance of the observed altered Pdef expression in cancer progression.

MATERIALS AND METHODS

Cell Culture. Human breast epithelial cell lines were maintained in medium supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. MCF-7, SK-BR3, and MDA-MB-157 cells were grown in RPMI 1640, whereas MDA-MB-231, MDA-MB-361, and HeLa cells were grown in DMEM. The MDA-MB-157 cell line was generously provided by Dr. Paul Fisher (Columbia University, New York, NY); all of the other cell lines were obtained from American Type Culture Collection.

Polyclonal Antiserum Preparation. Bacterially expressed Pdef protein was used for production of rabbit polyclonal antisera (MUSC Antibody Production Facility). Affinity purified antibody was tested against lysates from Cos-1 cells transfected with Ets1, Fli1, and Pdef to determine specificity. The specificity of this antibody is also demonstrated by the absence of immunoreactive proteins in Western blots of extracts prepared from cell lines that do not express Pdef mRNA.

Immunohistochemistry of Human Breast Tumor Samples. Human breast cancer samples were obtained from the Hollings Cancer Center Tumor Bank, MUSC. All of the specimens were formalin-fixed and paraffin-embedded. Deparaffinized tissue sections were rehydrated, and endogenous peroxidase activity was blocked using 3% H_2O_2. Slides were microwaved in citrate buffer antigen retrieval solution (Vector Laboratories) twice for 5 min. Immunohistochemical staining was performed using Vector ABC (Vector Laboratories). Anti-Pdef antibodies were used at 1:1000. Sections were stained using 3,3′-diaminobenzidine and counterstained with hematoxylin. All of the sections were examined independently by a pathologist (M. M. F.). We regarded cells as immunohistochemically positive when the Pdef signal was observed in the cell nuclei. Presence of nuclear staining was semiquantitatively compared with that of normal breast tissue present in the same section using a 1–4+ scale (1–0-25% of cells with nuclear staining; 2–25-50%; 3–50-75%; and

Received 3/7/03; accepted 5/21/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by grants from the National Cancer Institute (P01 CA78582), Susan G. Komen Foundation (DISS0100709), and Department of Defense (N00014-96-1-1298).

[2] To whom requests for reprints should be addressed, at Laboratory of Cancer Genomics, Hollings Cancer Center, Medical University of South Carolina, Charleston, SC 29425-2850. Phone: (843) 792-3962; Fax: (843) 792-3940; E-mail: watsondk@musc.edu.

[3] The abbreviations used are: uPA, urokinase-type plasminogen activator; MUSC, Medical University of South Carolina; cdk, cyclin-dependent kinase; MOI, multiplicity of infection; Ad, adenovirus; GFP, green fluorescent protein; HDAC, histone deacetylase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Rb, retinoblastoma protein; ECM, extracellular matrix.

4–75-100%). Tumors were graded using a Bloom-Richardson grading system (15). No staining was observed with negative control samples (absence of primary antibody or incubation with rabbit IgG).

Western Blot. Cells were lysed in radioimmunoprecipitation assay buffer, containing protease inhibitors (Complete Protease Inhibitors; Roche). Equal amounts of total protein were resolved by 12% SDS-PAGE and subjected to Western blot analyses using enhanced chemiluminescence system (Amersham-Pharmacia). Antibodies used were anticyclin D1 (Neomarkers), cdk4, cdk2, cyclin A, p21 (Santa Cruz), cyclin E, Rb (PharMingen), flag (M5), and β-actin (Sigma).

RNA Isolation and Northern Blot Analysis. Total RNA was isolated using TRIzol (Invitrogen) and fractionated on 1.2% agarose gels containing 0.66 M formaldehyde. RNA was transferred to nylon filters (Duralon) in 0.1 M sodium phosphate (pH 6.8), UV cross-linked, and hybridized in Quik-Hyb using random primed labeled Pdef or uPA cDNA as probe (Stratagene, La Jolla, CA).

Plasmid Construction, Transfection, and Luciferase Assays. The full-length Pdef open reading frame (GenBank accession no. NM012391) was cloned from normal colon tissue mRNA by reverse transcription-PCR. Primers [(5′-GAGAGGATCCGCCGCCAGCCCAAACAGCAGC-3′) and (5′-TAT-GCGGCCGCGGCGGGTTTCAGGCCCTGG-3′)] were used to provide *Bam*HI-*Xba*I restriction sites for cloning into the respective cloning sites of both pcDNA3 (Invitrogen) and a modified pcDNA3 vector to provide a 5′ flag epitope, pFcDNA3 (generously provided by Craig Hauser, The Burnham Institute, La Jolla, CA). Fli1 and Ets1 cDNA were also subcloned into pFcDNA3 (16). The Maspin promoter (−759 to −87; Ref. 17) was amplified from human genomic DNA and cloned into pGL2-luciferase vector (Promega). All of the constructs were verified by sequence analysis.

All of the transfections were performed using FuGENE 6 (Roche) according to the manufacturer's instructions. Ten ng of Pdef, Fli1, and Ets1 plasmid DNA were used in cotransfection experiments with the Maspin promoter luciferase construct. Increasing levels of Ets1 (10, 50, and 100 ng) plasmid were used in cotransfection studies with Pdef and the Maspin promoter luciferase construct. Luciferase assays were performed using the Luciferase Assay system as described by the manufacturer (Promega). Transfections were performed in triplicate and repeated three independent times, and values normalized to total protein, because dual reporter vectors are Ets responsive (13).

Colony Suppression Assays. MDA-MB-231 cells transfected with 1 μg of pcDNA3.1 or pFcDNA3.1-Pdef were selected in G418 (800 ug/ml) for 2–3 weeks. Cells were fixed and stained in a 0.2% crystal violet/20% methanol solution and counted. Transfections were performed in triplicate using two different plasmid preparations.

Construction of Adenovirus. Replication-deficient recombinant adenoviruses were generated by homologous recombination using a bacterial system as described previously (18). Briefly, Flag-tagged Pdef was subcloned into the shuttle vector pAdTrackCMV. For homologous recombination, the shuttle vector was digested with *Pme*I, coelectroporated with the circular adenoviral genome plasmid pAdEasy-1 (E1A deleted) into competent BJ5183 bacterial cells (Stratagene). Recombinant adenoviral DNA was purified, linearized with *Pac*I, and transfected into the 293A cells using FuGENE6 (Roche Biochemicals). Recombinant adenoviruses were plaque purified and screened for the expression of the Pdef construct by Western blot.

Adenoviral Infection and Growth Analysis. MDA-MB-231 cells were infected at 100 MOI with either control vector (Ad-GFP) or Ad-Pdef for 14 h in 2% serum (19). Under these conditions, >95% of the cells were infected, indicated by GFP expression. The virus was then removed, and medium containing 10% serum was added. At 1, 3, and 5 days, MTT assays were performed according to the manufacturer's instruction (Roche). For cell cycle analyses, infected cells were harvested 36 h after infection, fixed, and dehydrated in 70% ethanol at −20°C for at least 24 h. Cells were stained with a solution containing 0.5 mg/ml propidium iodide and 1 mg/ml RNase A (Sigma), and analyzed using flow cytometry.

Invasion and Migration Assay. Infected MDA-MB-231 cells were plated at 50,000 cells/well in serum-free medium in the upper chamber of a Transwell insert (8-μm pores; Becton Dickinson) coated with Matrigel. Medium containing 10% serum was used as a chemoattractant. After 24 h, cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained using Diff-Quick (Dade Behring Inc.). The migration assay conditions were the same except that 5 μg of fibronectin (Becton Dickinson) was plated onto the upper chamber of the Transwell 12 h before the cells were added. After 8 h, cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained using Diff-Quick.

RESULTS

Human Pdef mRNA Is Expressed at High Levels Primarily in Tissues with High Epithelial Content. Northern blot analysis of total RNA isolated from human adult tissues demonstrates that Pdef mRNA is expressed in tissues with high epithelial cell content including colon, prostate, and breast (Fig. 1*A*). Pdef message is undetectable in cells of nonepithelial origin, including spleen, bone marrow, and thymus. This expression pattern is in contrast to other Ets factors, such as Ets1, Ets2, Fli1, and PU.1, which are present in tissues of hematopoietic origin. In addition, the expression of Pdef in tissues with high epithelial content is similar to that demonstrated for other epithelial-specific-Ets factors (20).

To additionally study the expression of Pdef in human breast cancer, we evaluated several breast cancer cell lines for Pdef mRNA and protein. As shown in Fig. 1*B*, Pdef mRNA is highly expressed in MCF7, SK-BR-3, and MDA-MB-361 cell lines, and expressed at markedly lower levels in the more invasive MDA-MB-231 and MDA-MB-157 cell lines. Interestingly, from the five cell lines tested, Pdef protein is present only in MCF7 cells. MCF7 cells are well-differentiated, express the estrogen receptor, and have low metastatic potential (21). Thus, loss of Pdef protein may be correlated with metastatic potential.

Pdef Protein Is Reduced in Invasive Breast Cancer Tissues. Our data suggest that Pdef protein expression may be down-regulated during the transition to invasive breast cancer. To evaluate Pdef protein expression in mammary tissue, we conducted immunohistochemical staining of human breast cancer sections. Sections contained both normal and tumor areas on the same slide. As shown in Fig. 2, *A* and *B*, Pdef protein is found predominantly in the nucleus of normal

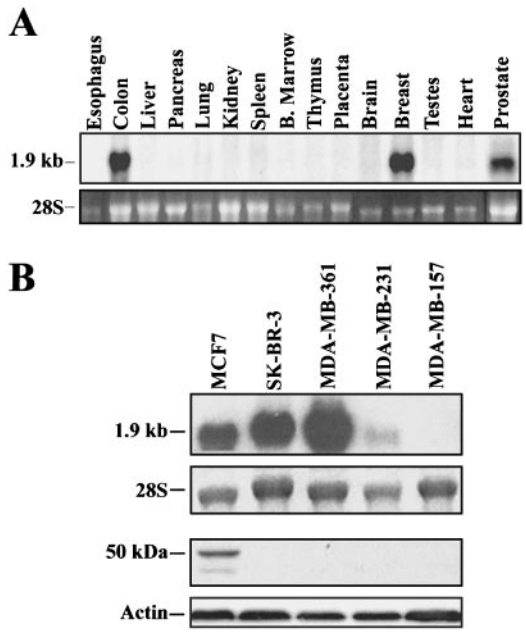

Fig. 1. Expression of Pdef in human tissues. *A, top panel,* Northern blot analysis of Pdef mRNA isolated from the indicated human tissues. *Bottom panel,* ethidium bromide staining of 28S rRNA as a loading control. *B, top panel,* Northern blot analysis of Pdef mRNA in breast cancer cell lines. Ethidium bromide staining of 28S rRNA as a loading control. *Bottom panel,* Western blot analysis of Pdef protein in the breast cancer cell lines. β-actin protein as a loading control.

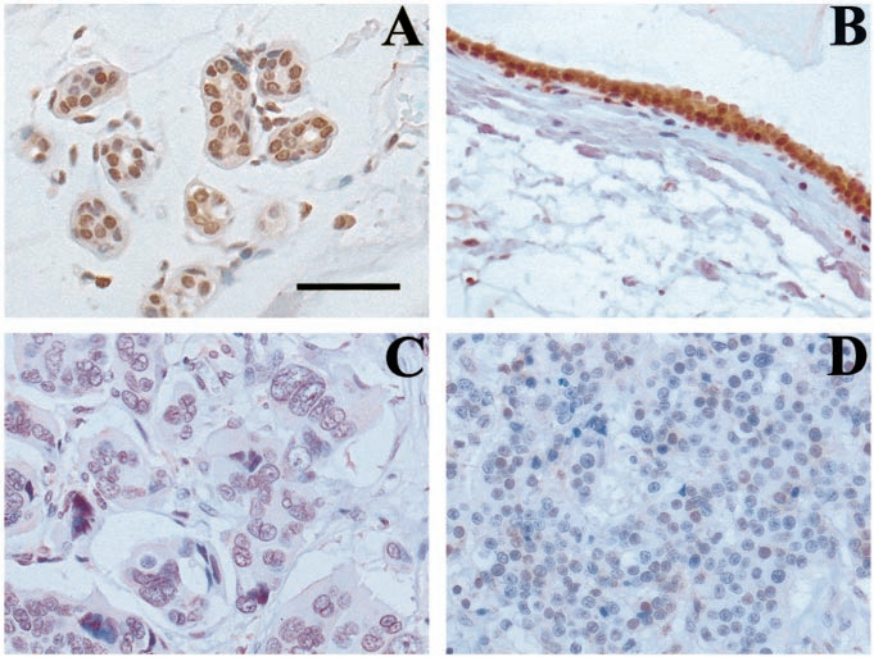

Fig. 2. Pdef is reduced in invasive breast cancer tissue. *A* and *B*, immunohistochemical staining of normal ductal epithelial cells showing nuclear staining for Pdef. *C* and *D*, reduced staining of Pdef in invasive ductal carcinoma cells compared with normal cells. *B* and *D* are from the same section. (Magnification, ×400 and the *bar* in *A* denotes 50 μm).

ductal epithelial cells. However, epithelial cells present in invasive ductal carcinomas show decreased protein expression (Fig. 2, *C* and *D*). Indeed, reduced Pdef protein expression has been observed in all seven of the invasive ductal carcinoma specimens examined. The percentage of positively stained nuclei was determined in a semiquantitative manner. Six of the seven patient samples examined showed a >50% decrease in percentage of positive nuclei compared with normal tissue (Table 1). In addition, tumor cells have significantly reduced intensity of nuclear staining (see Fig. 2, *C* and *D*).

Pdef Expression Results in Distinct Changes in Cell Morphology. To determine whether the expression of Pdef leads to altered phenotypes in MDA-MB-231 cells, we generated adenovirus expressing wild-type Pdef with an NH_2-terminal flag epitope, Ad-Pdef, and Ad-GFP as a control. MDA-MB-231 cells were infected at 100 MOI, and morphological changes were observed at 36 h. Specifically, Pdef-infected cells assume a rounder shape compared with control cells (Fig. 3*A*). The number of apoptotic cells visualized morphologically was not increased by Pdef expression indicating that these cellular changes were not accompanied by widespread cell death.

Pdef Inhibits the Ability of MDA-MB-231 Cells to Invade and Migrate. We next determined the effect of Pdef expression on the *in vitro* invasiveness of MDA-MB-231 cells by measuring their ability to migrate through filters coated with reconstituted basement membrane,

Table 1 *Reduced Pdef protein expression in invasive breast cancer cells in vivo*

Breast tumor	Grade[a]	Pdef expression[b]
A (22958)	III (PD)[c]	2
B (25140)	III (PD)	1
C (B2)	II (MD)[d]	3
D (854)	II (MD)	1
E (F1)	II (MD)	1
F (844)	II (MD)	2
G (839)	III (PD)	1

[a] Tumors were graded as described previously (15).

[b] All of the tumor comparisons were made to phenotypically normal breast tissue. Normal tissue was assigned a value of 4, indicating that 75–100% of the nuclei showed positive staining. 1, <25% of the tumor nuclei exhibit positive staining compared with normal; 2, Between 25 and 50% of tumor nuclei have positive staining; 3, Between 50 and 75% of the tumor nuclei show positive staining.

[c] PD, poorly differentiated.

[d] MD, moderately differentiated.

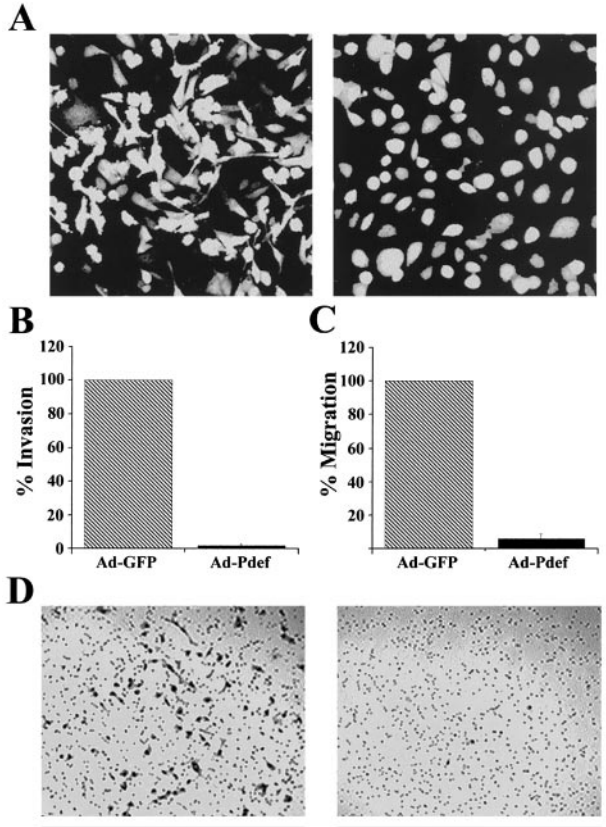

Fig. 3. Characteristics of MDA-MB-231 cells overexpressing Pdef. *A*, cells were infected with Ad-GFP (*left*) and Ad-Pdef (*right*) at 100 MOI, and examined 36 h after infection by confocal microscopy. *B*, quantitation of cells invading through Matrigel coated filters (see "Materials and Methods"). The *columns* represent average values of cells invading as a percentage of control. Cells infected with Pdef were significantly inhibited from invading, 1.8% ± 1.5, compared with control cells infected with Ad-GFP. *C*, quantitation of cells migrating through fibronectin-coated filters. The *columns* represent average values of cells migrating as a percentage of control. Cells infected with Pdef were significantly inhibited from migrating, 5.9% ± 2.8, compared with control cells infected with Ad-GFP. These assays were performed in triplicate in two independent experiments. *D*, photographs of Ad-GFP- and Ad-Pdef-infected cells, respectively, after the Matrigel invasion assay. Note the absence of Pdef-expressing cells compared with GFP-expressing cells. The *small circles* are the actual pores on the Transwell filter.

Matrigel. MDA-MB-231 cells were infected with Ad-GFP or Ad-Pdef, plated onto Matrigel-coated membranes, and invasive cells were counted. Cells expressing Ad-Pdef have a >95% reduction in the number of invading cells compared with cells infected with Ad-GFP (Fig. 3, *B* and *D*). We also examined the effect of Pdef expression on cell migration using fibronectin-coated filters. MDA-MB-231 cells expressing Pdef also show significantly reduced cell migration, less than 5–6% of the migration seen with the GFP-infected cells (Fig. 3*C*). Therefore, expression of Pdef into highly invasive cell lines results in the reduction of their *in vitro* invasive and migratory properties.

Pdef Modulates the Expression of Genes That Control Invasion and Metastasis. We sought to determine the effect of Pdef on downstream target genes that may contribute to the observed Pdef-dependent inhibition of invasion and migration. *uPA* has been shown to be an Ets target gene and is a prognostic marker for metastatic breast cancer (22). As is shown in Fig. 4*A*, expression of Pdef results in dramatic reduction in uPA mRNA level as compared with uninfected and Ad-GFP-infected controls.

Maspin is a type II tumor suppressor gene that has been shown to have antimetastatic properties when expressed in invasive breast cancer cells (23). The Maspin promoter has been shown to be regulated by murine Pdef (24). To determine whether multiple Ets factors are able to regulate Maspin, we compared the ability of Pdef, Ets1, and Fli1 to activate the Maspin promoter. Vectors expressing NH_2-terminal flag-tagged Pdef, Ets1, or Fli1 were cotransfected with a Maspin-luciferase reporter construct. Pdef expression results in a 4-fold activation of the promoter (Fig. 4*B*). Significantly, this activation appears to be specific for Pdef, because neither Fli1 nor Ets1 were able to activate this promoter. This specificity was retained even when Fli1 and Ets1 were expressed at higher levels (data not shown). Indeed, it appears that Fli1 and Ets1 expression reduces the observed baseline level, suggesting that these Ets factors may inhibit promoter activity, perhaps by competition with an endogenous regulator. To additionally examine whether Ets1 may compete with Pdef, cells were cotransfected with increasing amounts of Ets1 in the presence of constant Pdef. Ets1 expression results in the inhibition of Pdef-mediated transactivation of the Maspin promoter (Fig. 4*C*). We have determined that both Pdef and Ets1 bind to Ets consensus sites (EBS) in the Maspin promoter (electrophoretic mobility shift data not shown). Collectively, these data indicate that Ets1 can compete with Pdef for binding, and once bound, Ets1 is not able to transcriptionally activate the Maspin promoter. Taken together, these observations suggest that loss of Pdef protein in breast cancer cells may result in increased uPA and decreased Maspin expression. These two effects may contribute to increased invasiveness and metastasis.

Pdef Expression Suppresses Growth of MDA-MB-231 Cells. To determine whether Pdef expression affects cellular growth, MDA-MB-231 cells were infected, and anchorage-dependent growth was monitored by MTT assay. MDA-MB-231 cells infected with Ad-Pdef did not increase in total cell number as much as uninfected and Ad-GFP-infected control cells (Fig. 5*A*).

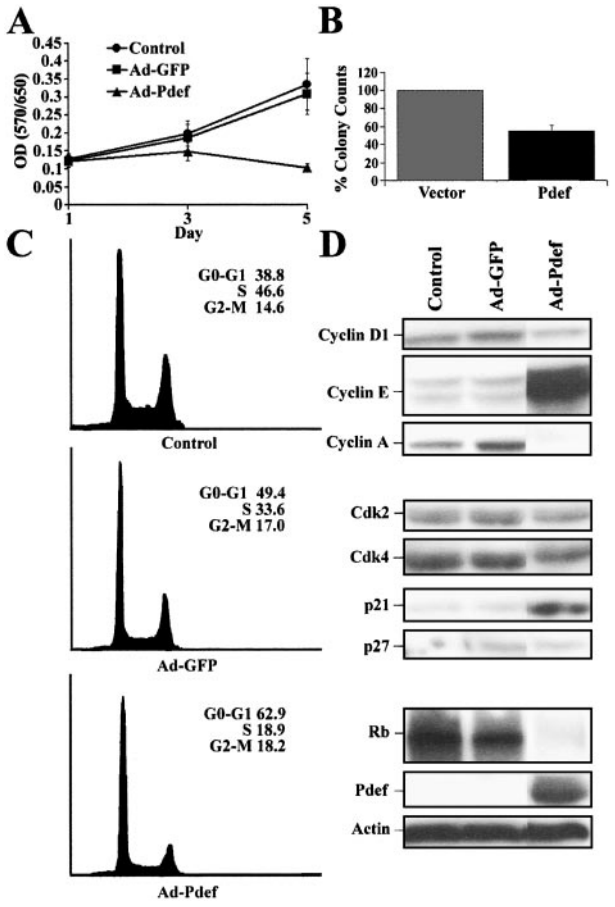

Fig. 5. Re-expression of Pdef leads to growth changes in MDA-MB-231 cells. *A*, MDA-MB-231 cells were untreated, or infected with either Ad-GFP or Ad-Pdef at 100 MOI, and cellular growth was monitored by MTT assay for 5 days. *B*, quantitation of colony numbers obtained from MDA-MB-231 cells transfected with pcDNA3.1 or pFcDNA3.1-Pdef. The *columns* represent the average values expressed as a percentage of control. *C*, cell cycle analysis of untreated cells or cells treated with Ad-GFP or Ad-Pdef at an MOI of 100 and harvested 36 h after infection. *D*, Western blot analysis of total protein isolated from cells 36 h after infection with Ad-GFP or Ad-Pdef, using antibodies against the indicated cell cycle proteins. Blots were reprobed using antibodies against Pdef and β-actin. Control lanes represent uninfected cells.

To additionally examine the Pdef-mediated growth suppression, we transfected MDA-MB-231 cells with a selectable mammalian vector that allows expression of Pdef and performed colony formation assays. After drug selection, we compared the number of colonies obtained from the Pdef transfection with that obtained from cells transfected with control vector alone. The number of colonies in cells transfected with Pdef was reduced by 50% (Fig. 5*B*). Some of these G418-resistant colonies were expanded for analysis of Pdef expres-

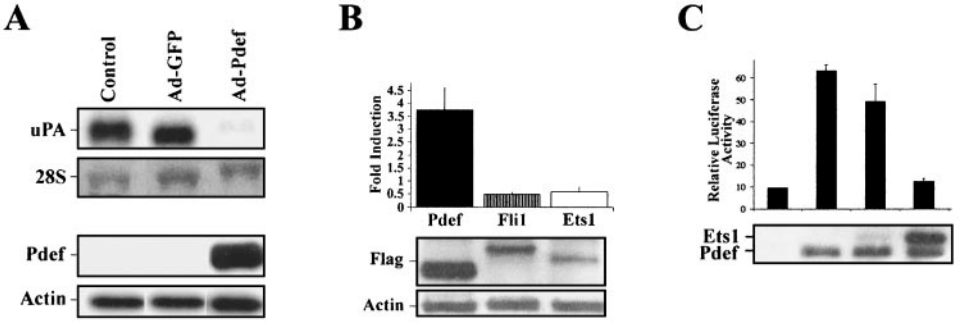

Fig. 4. Pdef can function as a repressor or activator of downstream target genes. *A*, Northern blot analysis of RNA isolated from infected MDA-MB-231 cells was performed using an uPA probe. Cell extracts were analyzed by Western blot analysis using anti-Pdef and β-actin antibodies. *B* and *C*, HeLa cells were transiently transfected with expression vectors containing the indicated Ets factor in conjunction with a luciferase construct containing the Maspin promoter. Cells were harvested at 48 h for luciferase activity, and extracts were analyzed by Western blot analysis using anti-Flag and β-actin antibodies; *bars*, ±SD.

sion. Only 1 of 19 colonies expressed Pdef protein, indicating that the majority of colonies were simply G418-resistant. These data additionally support the model that Pdef expression inhibits the growth of highly invasive breast cancer cells.

Pdef-dependent Inhibition of Cell Growth Is Mediated through Cell Cycle Changes. To begin to understand the molecular basis of the Pdef-mediated inhibition of cell growth, cell cycle analysis was performed on cells 36 h after infection with either Ad-GFP or Ad-Pdef. As shown in Fig. 5*C*, expression of Pdef in MDA-MB-231 cells leads to an increase in the percentage of cells in the G_0-G_1 phase, with a concomitant reduction in the percentage of cells in S phase, indicating a G_0-G_1 arrest. The absence of a sub-G_0 cell population indicates that Pdef expression does not induce apoptosis.

To determine the mechanism of this G_1 arrest, cell lysates prepared from infected cells were examined for expression of G_1 cell cycle regulatory proteins by Western blot analysis (Fig. 5*D*). The protein level of cdks, cdk4 and cdk2, did not significantly change. However, cyclin D1 and cyclin A levels are reduced, whereas the expression of cyclin E is significantly increased. The cdk inhibitor, p21, is also elevated, whereas Rb expression is reduced. These data indicate a broad range of Pdef effects on the cell cycle. However, the elevation in p21 could explain the observed G_1 arrest. Collectively, these data suggests that the Pdef-induced G_1 arrest may result from a p21-dependent mechanism.

DISCUSSION

In this manuscript, we show that Pdef is expressed in breast, prostate, and colon, thus displaying a wider tissue distribution than originally reported (13). We were not able to detect Pdef mRNA by Northern blot and reverse transcription-PCR analyses of total RNA isolated from other tissues, including those with high epithelial cell content, including mouse skin (data not shown). This is in agreement with the previous observation that human keratinocytes do not express Pdef mRNA (13). Immunohistochemical analysis of Pdef in breast tissue revealed predominantly nuclear expression of Pdef protein in ductal epithelial cells. Pdef protein level is greatly reduced in invasive breast cancer compared with normal tissue. These data are consistent with the recent findings of loss of Pdef expression in prostate cancer (14). Lack of detectable protein with high levels of Pdef mRNA in breast cancer cell lines implies that Pdef expression may be regulated by a post-transcriptional or post-translational mechanism.

This is also the first study to evaluate the functional significance of the observed loss of Pdef protein. Pdef expression in the highly metastatic breast cancer cell line, MDA-MB-231, resulted in inhibition of invasion, migration, and growth. Consistent with the inhibition of invasion, we observe a decrease in expression of uPA in Pdef-infected cells. The ability of cancer cells to become invasive and metastatic is believed to result from the up-regulation of enzymes responsible for normal ECM turnover. These enzymes include, but are not limited to, the plasminogen and matrix metalloprotease systems. uPA has been shown to be active on the cell surface of MDA-MB-231 cells (25) and can convert surface plasminogen into plasmin. Plasmin subsequently degrades a variety of ECM components, including fibronectin, vitronectin, and fibrin (26). In addition, plasmin can proteolytically cleave matrix metalloproteases into active enzymes, allowing for degradation of components of the basement membrane (27). Pdef-mediated down-regulation of uPA may inhibit processes required for proteolysis of the basement membrane. Furthermore, uPA binding to its receptor (uPAR) in MDA-MB-231 cells can promote association with the fibronectin receptor, $\alpha 3\beta 1$, activating focal adhesion kinase (28) and affecting cellular spreading on fibronectin. Thus, the inability of MDA-MB-231 cells to migrate on a fibronectin substrate after infection with Pdef could be because of lack of uPA expression.

Inhibition of uPA-mediated proteolysis is mediated by plasminogen activator inhibitors, PAI-1 and PAI-2, as well as other serine protease inhibitors, such as Maspin (29). Because Pdef can activate the Maspin promoter, loss of Pdef in invasive cancer cells may result in its down-regulation. Concurrently, increased transcription of uPA coupled with increased proteolytic activity could allow cancer cells to develop the ability to degrade the basement membrane and ECM, thereby becoming metastatic.

Interestingly, several reports have indicated that uPA and Maspin may also be regulated epigenetically (30, 31). MCF7 cells do not express uPA, attributable, at least in part, to promoter methylation (30, 32). In contrast, MDA-MB-231 cells express high levels of uPA and exhibit a hypomethylated promoter. Maspin is not expressed in MCF7, SK-BR-3, and MDA-MB-231 cells, yet can be reactivated by addition of a DNA methylase inhibitor alone (33) or in combination with a histone deacetylase inhibitor (34). A critical Ets site in the Maspin promoter has also been found to be methylated in breast cancer cells, resulting in absence of Maspin expression (33, 35).

Cell cycle analysis of MDA-MB-231 cells infected with Ad-Pdef indicates a G_0-G_1 arrest. In mid-G_1, cyclin D1 is normally up-regulated, and associates with cdk4 and cdk6 to phosphorylate Rb (36). During late G_1, cyclin E expression increases and associates with cdk2 to additionally phosphorylate Rb. Interestingly, we find a large increase in cyclin E, whereas cyclin D1 levels decrease. Thus, cyclin E may compensate for decreased levels of cyclin D1. In addition, it has been shown that dephosphorylated Rb, in association with E2F, can recruit histone deacetylase to mediate transcriptional repression of genes such as cyclin E (37). It is possible that reduction in levels of Rb would allow for inappropriate expression of cyclin E. Increases in cyclin E may result in a compensatory up-regulation of p21 that is thought to provide a protective barrier against deregulation of cyclin E expression (38). In early S phase, cyclin A becomes induced, and binds to cdk2 to phosphorylate Rb and allow for progression through the cell cycle. Expression of Pdef results in reduced levels of cyclin A, consistent with a G_0-G_1 arrest. It has been shown previously that cyclin D1, Rb, and p21 are Ets target genes (9).

In summary, Pdef is an epithelial tissue-specific Ets transcription factor that is down-regulated during mammary carcinogenesis. Expression of this protein into invasive breast cancer cells results in inhibition of cell invasiveness, motility, and growth. It will be of interest to determine whether Pdef expression in additional cancer-derived cell lines has similar inhibitory effects. Future studies will assess tumor-inhibiting properties in an *in vivo* tumor mouse model to evaluate the therapeutic potential of expressing Pdef in breast cancer cells.

ACKNOWLEDGMENTS

We thank Drs. Robin Muise-Helmericks and Patricia Watson for critical review of the manuscript. We also acknowledge the technical assistance of Margaret Romano (Hollings Cancer Center Tumor Bank), John Zhang (MUSC Antibody Facility), and Rick Peppler (Flow Cytometry Facility).

REFERENCES

1. Duffy, M. J. Biochemical markers in breast cancer: which ones are clinically useful? Clin. Biochem., *34:* 347–352, 2001.
2. Debies, M. T., and Welch, D. R. Genetic basis of human breast cancer metastasis. J. Mammary Gland Biol. Neoplasia, *6:* 441–451, 2001.
3. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer, Science (Wash. DC), *244:* 707–712, 1989.

4. Escot, C., Theillet, C., Lidereau, R., Spyratos, F., Champeme, M. H., Gest, J., and Callahan, R. Genetic alteration of the c-myc protooncogene (MYC) in human primary breast carcinomas, Proc. Natl. Acad. Sci. USA, *83:* 4834–4838, 1986.
5. Davidoff, A. M., Kerns, B. J., Pence, J. C., Marks, J. R., and Iglehart, J. D. p53 alterations in all stages of breast cancer. J. Surg. Oncol., *48:* 260–267, 1991.
6. Porter, D. A., Krop, I. E., Nasser, S., Sgroi, D., Kaelin, C. M., Marks, J. R., Riggins, G., and Polyak, K. A SAGE (serial analysis of gene expression) view of breast tumor progression. Cancer Res., *61:* 5697–702, 2001.
7. Wulfkuhle, J. D., McLean, K. C., Paweletz, C. P., Sgroi, D. C., Trock, B. J., Steeg, P. S., and Petricoin, E. F., III. New approaches to proteomic analysis of breast cancer. Proteomics, *1:* 1205–1215, 2001.
8. van't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R., and Friend, S. H. Gene expression profiling predicts clinical outcome of breast cancer. Nature (Lond.), *415:* 530–536, 2002.
9. Sementchenko, V. I., and Watson, D. K. Ets target genes: past, present and future. Oncogene, *19:* 6533–6548, 2000.
10. Sapi, E., Flick, M. B., Rodov, S., and Kacinski, B. M. Ets-2 transdominant mutant abolishes anchorage-independent growth and macrophage colony-stimulating factor-stimulated invasion by BT20 breast carcinoma cells. Cancer Res., *58:* 1027–1033, 1998.
11. Chen, J. H., Vercamer, C., Li, Z., Paulin, D., Vandenbunder, B., and Stehelin, D. PEA3 transactivates vimentin promoter in mammary epithelial and tumor cells. Oncogene, *13:* 1667–1675, 1996.
12. Chang, C. H., Scott, G. K., Kuo, W. L., Xiong, X., Suzdaltseva, Y., Park, J. W., Sayre, P., Erny, K., Collins, C., Gray, J. W., and Benz, C. C. ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. Oncogene, *14:* 1617–1622, 1997.
13. Oettgen, P., Finger, E., Sun, Z., Akbarali, Y., Thamrongsak, U., Boltax, J., Grall, F., Dube, A., Weiss, A., Brown, L., Quinn, G., Kas, K., Endress, G., Kunsch, C., and Libermann, T. A. PDEF, a novel prostate epithelium-specific ets transcription factor, interacts with the androgen receptor and activates prostate-specific antigen gene expression, J. Biol. Chem., *275:* 1216–1225, 2000.
14. Nozawa, M., Yomogida, K., Kanno, N., Nonomura, N., Miki, T., Okuyama, A., Nishimune, Y., and Nozaki, M. Prostate-specific transcription factor hPSE is translated only in normal prostate epithelial cells. Cancer Res., *60:* 1348–1352, 2000.
15. Elston, C. W., and Ellis, I. O. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology, *19:* 403–410, 1991.
16. Czuwara-Ladykowska, J., Sementchenko, V. I., Watson, D. K., and Trojanowska, M. Ets1 is an effector of the transforming growth factor β (TGF-β) signaling pathway and an antagonist of the profibrotic effects of TGF-β, J. Biol. Chem., *277:* 20399–408, 2002.
17. Zhang, M., Maass, N., Magit, D., and Sager, R. Transactivation through Ets and Ap1 transcription sites determines the expression of the tumor-suppressing gene maspin. Cell Growth Diff., *8:* 179–186, 1997.
18. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. A simplified system for generating recombinant adenoviruses, Proc. Natl. Acad. Sci. USA, *95:* 2509–2514, 1998.
19. Fujii, T., Garcia-Bermejo, M. L., Bernabo, J. L., Caamano, J., Ohba, M., Kuroki, T., Li, L., Yuspa, S. H., and Kazanietz, M. G. Involvement of protein kinase C δ (PKCδ) in phorbol ester- induced apoptosis in LNCaP prostate cancer cells. Lack of proteolytic cleavage of PKCδ, J. Biol. Chem., *275:* 7574–7582, 2000.
20. Feldman, R. J., Sementchenko, V. I., and Watson, D. K. The epithelial-specific Ets factors occupy a unique position in defining epithelial proliferation, differentiation, and carcinogenesis. Anticancer Res., *23:* 2125–2132, 2003.
21. Zajchowski, D. A., Bartholdi, M. F., Gong, Y., Webster, L., Liu, H. L., Munishkin, A., Beauheim, C., Harvey, S., Ethier, S. P., and Johnson, P. H. Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells. Cancer Res., *61:* 5168–5178, 2001.
22. Duffy, M. J. Urokinase plasminogen activator and its inhibitor, PAI-1, as prognostic markers in breast cancer: from pilot to level 1 evidence studies, Clin. Chem., *48:* 1194–1197, 2002.
23. Zou, Z., Anisowicz, A., Hendrix, M. J., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science (Wash. DC), *263:* 526–529, 1994.
24. Yamada, N., Tamai, Y., Miyamoto, H., and Nozaki, M. Cloning and expression of the mouse Pse gene encoding a novel Ets family member. Gene, *241:* 267–274, 2000.
25. Andronicos, N. M., and Ranson, M. The topology of plasminogen binding and activation on the surface of human breast cancer cells. Br. J. Cancer, *85:* 909–916, 2001.
26. Andreasen, P. A., Egelund, R., and Petersen, H. H. The plasminogen activation system in tumor growth, invasion, and metastasis. Cell Mol. Life Sci., *57:* 25–40, 2000.
27. Ramos-DeSimone, N., Hahn-Dantona, E., Sipley, J., Nagase, H., French, D. L., and Quigley, J. P. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. J. Biol. Chem., *274:* 13066–76, 1999.
28. Wei, Y., Eble, J. A., Wang, Z., Kreidberg, J. A., and Chapman, H. A. Urokinase receptors promote β1 integrin function through interactions with integrin $\alpha 3\beta 1$. Mol. Biol. Cell, *12:* 2975–2986, 2001.
29. McGowen, R., Biliran, H., Jr., Sager, R., and Sheng, S. The surface of prostate carcinoma DU145 cells mediates the inhibition of urokinase-type plasminogen activator by maspin. Cancer Res., *60:* 4771–4778, 2000.
30. Guo, Y., Pakneshan, P., Gladu, J., Slack, A., Szyf, M., and Rabbani, S. A. Regulation of DNA methylation in human breast cancer. Effect on the urokinase-type plasminogen activator gene production and tumor invasion. J. Biol. Chem., *277:* 41571–9, 2002.
31. Futscher, B. W., Oshiro, M. M., Wozniak, R. J., Holtan, N., Hanigan, C. L., Duan, H., and Domann, F. E. Role for DNA methylation in the control of cell type specific maspin expression. Nat. Genet., *31:* 175–179, 2002.
32. Xing, R. H., and Rabbani, S. A. Transcriptional regulation of urokinase (uPA) gene expression in breast cancer cells: role of DNA methylation. Int. J. Cancer, *81:* 443–450, 1999.
33. Domann, F. E., Rice, J. C., Hendrix, M. J., and Futscher, B. W. Epigenetic silencing of maspin gene expression in human breast cancers. Int. J. Cancer, *85:* 805–810, 2000.
34. Maass, N., Biallek, M., Rosel, F., Schem, C., Ohike, N., Zhang, M., Jonat, W., and Nagasaki, K. Hypermethylation and histone deacetylation lead to silencing of the maspin gene in human breast cancer, Biochem. Biophys. Res. Commun., *297:* 125–128, 2002.
35. Zhang, M., Magit, D., and Sager, R. Expression of maspin in prostate cells is regulated by a positive ets element and a negative hormonal responsive element site recognized by androgen receptor. Proc. Natl. Acad. Sci. USA, *94:* 5673–5678, 1997.
36. Ho, A., and Dowdy, S. F. Regulation of G(1) cell-cycle progression by oncogenes and tumor suppressor genes. Curr. Opin. Genet. Dev., *12:* 47–52, 2002.
37. Zhang, H. S., Gavin, M., Dahiya, A., Postigo, A. A., Ma, D., Luo, R. X., Harbour, J. W., and Dean, D. C. Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell, *101:* 79–89, 2000.
38. Minella, A. C., Swanger, J., Bryant, E., Welcker, M., Hwang, H., and Clurman, B. E. p53 and p21 form an inducible barrier that protects cells against cyclin E-cdk2 deregulation. Curr. Biol., *12:* 1817–1827, 2002.