Global Gene Expression Analysis Identifies PDEF Transcriptional Networks Regulating Cell Migration during Cancer Progression

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Submitted February 15, 2008; Revised May 19, 2008; Accepted June 16, 2008
Monitoring Editor: Carl-Henrik Heldin

Prostate derived ETS factor (PDEF) is an ETS (epithelial-specific E26 transforming sequence) family member that has been identified as a potential tumor suppressor. In multiple invasive breast cancer cells, PDEF expression inhibits cell migration by preventing the acquisition of directional morphological polarity conferred by changes in cytoskeleton organization. In this study, microarray analysis was used to identify >200 human genes that displayed a common differential expression pattern in three invasive breast cancer cell lines after expression of exogenous PDEF protein. Gene ontology associations and data mining analysis identified focal adhesion, adherens junctions, cell adhesion, and actin cytoskeleton regulation as cell migration-associated interaction pathways significantly impacted by PDEF expression. Validation experiments confirmed the differential expression of four cytoskeleton-associated genes with known functional associations with these pathways: *uPA*, *uPAR*, *LASP1*, and *VASP*. Significantly, chromatin immunoprecipitation studies identified PDEF as a direct negative regulator of the metastasis-associated gene *uPA* and phenotypic rescue experiments demonstrate that exogenous urokinase plasminogen activator (uPA) expression can restore the migratory ability of invasive breast cancer cells expressing PDEF. Furthermore, immunofluorescence studies identify the subcellular relocalization of urokinase plasminogen activator receptor (uPAR), LIM and SH3 protein (LASP1), and vasodilator-stimulated protein (VASP) as a possible mechanism accounting for the loss of morphological polarity observed upon PDEF expression.

INTRODUCTION

In patients with solid tumor malignancies, metastasis accounts for the majority of cancer-related deaths (Sporn, 1996). Acquisition of the metastatic phenotype initiates a series of distinct molecular mechanisms that result in the formation of secondary tumors that in turn cause clinical complications and mortality (Sporn, 1996; Loberg *et al.*, 2007). It is believed that only a limited subset of primary tumor cells (1 in 10 million) have the capacity to undergo somatic mutation and acquire the metastatic phenotype. Metastatic-related processes therefore may be a rate-limiting step for cancer progression and represent an important target for therapeutic intervention (Compagni and Christofori, 2000; Fidler, 2003). Two of the initial defining properties of a metastatic cancer cell lie in its ability to dissociate

from intracellular adhesions and become motile (Friedl and Wolf, 2003; Yamazaki *et al.*, 2005). Such processes are driven by complex regulatory signaling cascades that transiently and/or permanently alter the expression of a multitude of genes that act to reorganize the cytoskeletal network (Yamazaki *et al.*, 2005; Kedrin *et al.*, 2007). Such altered expression patterns are largely due to the aberrant activity of transcription factors.

The E26 transforming sequence (ETS) family of transcription factors is associated with functional roles in many cancer-related processes, including cell proliferation, differentiation, apoptosis, angiogenesis, and transformation as well as cell migration and invasion (Seth and Watson, 2005). Significantly, ETS factors are known to both positively and negatively regulate the migratory phenotype, depending on the family member expressed and the context of expression (Turner *et al.*, 2007a; Turner and Watson, 2008). In breast and other solid tumors, the increased expression of ETS1 and ETS2 (Watabe *et al.*, 1998; Behrens *et al.*, 2001; Buggy *et al.*, 2004, 2006) as well as PEA3 (Benz *et al.*, 1997; Bosc *et al.*, 2001; Bieche *et al.*, 2004) is associated with increased metastatic potential and an increased ability for motile function through the altered expression of regulatory target genes known to mediate cytoskeletal changes. Prostate derived ETS factor (PDEF) is an epithelial specific ETS family member that is associated with the negative regulation of metastatic potential (Feldman *et al.*, 2003; Ghadersohi *et al.*, 2006; Gu *et al.*, 2007; Turner *et al.*, 2007b). Although PDEF message is sometimes found to be expressed in primary breast and

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-02-0154>) on June 25, 2008.

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Abbreviations used: ChIP, chromatin immunoprecipitation; EBS, ETS binding site; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; ETS, E26 transforming sequence; ITGA5(6), integrin α 5(6); LASP1, LIM and SH3 protein; PDEF, prostate-derived epithelial factor; pFAK, phosphorylated focal adhesion kinase; uPA, urokinase plasminogen activator (PLAU); uPAR, urokinase plasminogen activator receptor (PLAUR); VASP, vasodilator-stimulated phosphoprotein.

prostate cancer and has been proposed to be a poor prognosis marker (Nozawa *et al.*, 2000; Ghadersohi and Sood, 2001; Tsujimoto *et al.*, 2002; Ghadersohi *et al.*, 2004), this often does not correlate with protein expression (Nozawa *et al.*, 2000; Tsujimoto *et al.*, 2002; Feldman *et al.*, 2003; Ghadersohi *et al.*, 2006). In contrast to message, studies demonstrate that PDEF protein is expressed in normal breast tissue, reduced in well-differentiated ductal carcinoma and lost in poorly differentiated ductal carcinoma (Feldman *et al.*, 2003; Ghadersohi *et al.*, 2006). Interestingly, both RNA and protein expression are lost in invasive tissue and cell-lines displaying a metastatic phenotype (Feldman *et al.*, 2003; Turner *et al.*, 2007b). Furthermore, immunohistochemical staining of estrogen receptor-negative, progesterone receptor-negative breast tumors demonstrate that PDEF protein loss is associated with a more aggressive subset of these tumors (Doane *et al.*, 2006).

In response to stimulus, migratory cells must establish directional polarity by forming a dominant leading lamellipodia in the direction of migration that adheres to the extracellular matrix (ECM) through transmembrane complexes called focal adhesions (Bailly *et al.*, 1998; Pantaloni *et al.*, 2001; Danuser, 2005; Mogilner, 2006). Using focal adhesions for traction, cells then propel themselves forward by contracting the cell body and releasing focal adhesions at the rear of the cell by proteolytic degradation (Wehrle-Haller and Imhof, 2002; Gupton and Waterman-Storer, 2006). Our previous work has demonstrated that constitutive and inducible PDEF expression in multiple invasive breast cancer cell lines reduces metastatic potential by inhibiting cell migration and invasion (Feldman *et al.*, 2003; Turner *et al.*, 2007b). Expression of PDEF impacts cell migration by causing a remodeling of the actin cytoskeleton and altered focal adhesion localization, resulting in a loss of the morphological polarization required for directed migration (Turner *et al.*, 2007b). Reciprocal in vitro and in vivo loss of function studies in noninvasive cells result in a concomitant increase in migration and tumorigenic potential (Ghadersohi *et al.*, 2006; Turner *et al.*, 2007b).

This study uses whole genome expression analysis, in three invasive breast cancer cell lines (MDA MB 231, MDA MB 157, and BT549) to identify putative PDEF-regulated genes that may mediate its inhibitory effects on metastasis. Literature database searches and gene ontology associations identified biological pathways and candidate target genes that were known effectors of cell migration via cytoskeleton reorganization. Focal adhesion, adherens junctions, cell adhesion molecules and regulation of the actin cytoskeleton were all impacted by PDEF expression to a greater extent than that expected by chance. Differential expression of four mRNAs, urokinase plasminogen activator (uPA [PLAU]) and its receptor urokinase plasminogen activator receptor (uPAR [PLAUR]), LIM and SH3 protein (LASP1), and vasodilator-stimulated protein (VASP) were validated at the RNA, and protein levels and their roles in PDEF-mediated inhibition of cell migration were investigated. Significantly, chromatin immunoprecipitation (ChIP) experiments demonstrate that uPA is a direct transcriptional target for PDEF regulation, and phenotypic rescue experiments identified uPA repression by PDEF as a critical interaction in the migratory phenotype of invasive breast cancer cells.

MATERIALS AND METHODS

Cell Culture

Human breast epithelial cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained at 37°C with 5% CO₂ in medium

supplemented with 10% fetal bovine serum and 100 U of penicillin/streptomycin. MDA MB 231, MDA MB 157, and BT549 were grown in DMEM. MCF7 was also grown in DMEM supplemented with 1 mM sodium pyruvate, 1 mM sodium bicarbonate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 0.01 mg/ml insulin.

MDA MB 157 stable clones expressing doxycycline-inducible human PDEF were grown in DMEM containing 150 µg/ml hygromycin and 200 µg/ml G418 (Invitrogen, Carlsbad, CA). Stable cell lines were grown in growth media supplemented with 200 µg/ml G418. All tissue culture reagents were purchased from Invitrogen.

Adenoviral Infection

The construction of a PDEF-expressing adenovirus has been described previously (Feldman *et al.*, 2003). Cells were infected in normal growth medium at 5–10 multiplicity of infection with either control virus expressing green fluorescent protein (GFP), or virus expressing PDEF/GFP from a bicistronic promoter. Infected cells were then grown for 16 h. Under these conditions, >95% of the cells were infected as assessed by GFP expression (Turner *et al.*, 2007b).

Doxycycline-induced Expression

The construction of the PDEF-inducible expression system has been described previously (Turner *et al.*, 2007b). Unless otherwise stated, PDEF expression was induced in cells at 70–80% confluence by using 1 µg/ml doxycycline (Thermo Fisher Scientific, Rockville, MD) and incubated for 24 h before performing experiments.

PDEF Gene Knockdown

MCF7 cells at 70% confluence were transfected with a short hairpin RNA (shRNA) vector construct in pSuppressor (Imgenex, San Diego, C.A.), by using Lipofectamine 2000 (Invitrogen), as per the manufacturer's instructions. A 19-nucleotide loop (GCTATGGCCGCTTCATTAG) located at the position 1206–1224 of the open reading frame (ORF) region of the PDEF gene (GenBank accession no. NP 036523) was used to target PDEF mRNA. Cells were cultured for 48 h before RNA and protein collection. Controls consisted of cells transfected with 1.5 µg of vector only. Each transfection contained 1.5 µg of total DNA, consisting of the indicated amounts of PDEF shRNA vector adjusted to total amount with the vector DNA. GFP expression from a bicistronic promoter was used to assess transfection efficiency.

Expression Constructs

uPA cDNA was obtained from American Type Culture Collection. PDEF and LASP1 cDNA coding regions were amplified by polymerase chain reaction (PCR) using the primers detailed in Supplemental Table 1 and ligated into pcDNA 3.1, by using appropriate restriction sites. Plasmid DNA from positive clones was isolated using the ENDO-free plasmid purification kit (QIAGEN, Valencia, CA) and the sequence verified. The VASP expression construct (VSV-VASP) was a kind gift from Dr. Alan Howe (UVM College of Medicine, Burlington, VT). Plasmid DNA (1 µg) was transfected into cells using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions and then incubated for 48 h before RNA and protein collection, and/or assessment of migration and localization.

Microarray Analysis

The cDNA microarray slides containing ~42,000 gene elements were obtained from Stanford University (Stanford, CA) and were processed and hybridized as described previously (Yordy *et al.*, 2005). Competitive hybridizations were performed using RNA collected from three invasive breast cancer cell lines, MDA MB 231, MDA MB 157 and BT 549 that were transduced with either PDEF/GFP- or GFP-expressing adenovirus, respectively. Probes were labeled as described previously (Yordy *et al.*, 2005). Briefly, the purified cDNA with the incorporated amino-modified bases was covalently labeled with a succinimidyl ester Alexa Fluor dye, either Alexa Fluor 555 or Alexa Fluor 647 from the Alexa Fluor Reactive Dye decapacks (Invitrogen) following the manufacturer's instructions. Data were extracted using a GenePix Pro4.1 Axon scanner (Molecular Devices, Toronto, ON, Canada), and normalization and data visualization were performed using MIDAS and MEV, as part of the TIGR TM4 analysis package (Dudoit *et al.*, 2003). The data normalization cut-off for acceptable feature extraction by using MIDAS was a background checking signal-to-noise threshold of 2.0 (mean value at least 2 times the median of the background in both channels) (Yordy *et al.*, 2005). Block Locfit (LOWLESS) normalization was performed for each slide with a smooth parameter of 0.33, followed by block SD regularization to appropriately scale each print tip block. SD regularization with an Alexa Fluor 555 threshold of 10,000 was used to scale normalize for cross slide analysis (Geller *et al.*, 2003). A differential expression cut-off of 1.5-fold was used to filter differentially expressed genes resulting from PDEF expression in the invasive cell lines. Gene ontology classification was performed using Pathway-Express by using the default parameters (Khatri *et al.*, 2005; Draghici *et al.*, 2007; Khatri *et al.*, 2007).

Western Blot

Cells were lysed in radioimmunoprecipitation assay buffer, containing protease inhibitors (Complete protease inhibitors; F. Hoffman-La Roche, Nutley, NJ) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Equal amounts of total protein (as determined using bicinchoninic acid protein assay kit; Pierce Chemical, Rockford, IL) were resolved by SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis by using enhanced chemiluminescence (Pierce Chemical). Blots were probed using commercially available antibodies as detailed below. Secreted uPA protein levels were assessed in conditioned media. Treated cells were washed twice in PBS and incubated for 16 h in serum-free growth media. The conditioned media were collected and concentrated ~50-fold by using a 10-kDa cut-off Centricon filter (Millipore, Billerica, MA). Human PDEF antibody was generated as described previously (Feldman *et al.*, 2003) and was purified against truncated N-terminal PDEF protein (amino acids 1-141) lacking its pointed domain and ETS domain. Commercial antibodies used were uPA mouse monoclonal Ab-2 (Neomarkers, Fremont CA), uPAR mouse monoclonal (American Diagnostica, Stamford, CT), LASP1 rabbit polyclonal (Millipore), VASP rabbit polyclonal (Calbiochem, San Diego, CA), zyxin N-19 goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), p^{Y397}FAK (BD Biosciences, San Jose, CA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit polyclonal (Abcam, Cambridge, MA). Anti-rabbit, anti-mouse, and anti-goat horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare (Piscataway, NJ).

Transwell Migration Assay

Treated or untreated control cells were seeded in triplicate into the upper chamber of a transwell insert (BD Biosciences), precoated with 5 µg/ml fibronectin (Thermo Fisher Scientific), in serum-free media at a density of 50,000 cells per well. Media containing 10% serum was placed in the lower chamber to act as a chemoattractant, and cells were further incubated for 6 h. Nonmigratory cells were removed from the upper chamber by scraping and the cells remaining on the lower surface of the insert were stained using Diff-quick (Dade Behring Newark, DE). Cells were quantified as the number of cells found in 10 random microscope fields. Error bars represent the SD from three separate experiments.

Immunofluorescence

Cells were seeded onto sterile coverslips (18 mm in diameter) coated with 5 µg/ml fibronectin and allowed to attach overnight. After doxycycline-induced PDEF expression, cells were incubated for a further 24 h before being fixed with 2% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked in 2% bovine serum albumin for 1 h at room temperature. LASP1, VASP, uPAR, zyxin, and p^{Y397}FAK localization was examined using the antibodies detailed above and visualized using appropriate (480 and 540 nm) Alexa Fluor secondary antibodies (Invitrogen). Immunofluorescence was examined using an Olympus IX70 confocal microscope.

Real-Time PCR

Total RNA was purified from cells using QIAshredder and RNeasy Plus isolation kits (QIAGEN). cDNA was reverse transcribed from 1 µg of RNA by using the iScript cDNA synthesis kit as per the manufacturer's instructions (Bio-Rad, Hercules, CA). One microliter of a 1 in 5 dilution of cDNA was used in each real-time reaction, which was conducted using a LightCycler (Roche Diagnostics, Basel, Switzerland) with the Platinum SYBER Green qPCR SuperMix (Invitrogen), as per the manufacturers' instructions. Primers were used at a concentration of 250 nM, and the cycling conditions were as follows: preincubation, 95°C for 10 min, 95°C for 2 min, followed by 40–50 cycles of denaturation at 95°C; annealing at 58°C and extension at 72°C, all for 20 s, with a single data acquisition at the end of each extension. Melting curve analysis was carried out as per the manufacturer's recommendations, and relative expression analysis was carried out using LinRegPCR, as per the suggested specifications (Ramakers *et al.*, 2003). All primer sequences are listed in Supplemental Table 1.

ChIP

Chromatin was prepared and immunoprecipitation performed using MCF7 cells (express endogenous PDEF) or MDA MB 231 cells infected with FLAG-tagged PDEF/GFP or control GFP-expressing virus, in a two-step cross-linking protocol, as described previously (Nowak *et al.*, 2005). Chromatin was fragmented into 500- to 1000-bp fragments by sonicating the cells eight times for 10 s at level three in an ethanol ice bath by using a Virsonic 475 sonicator (Virtis, Gardiner, NY). Soluble chromatin was quantified (absorbance at 260 nM) and 10 absorbance units were incubated with 2 µg of PDEF rabbit polyclonal antibody (MCF7) or FLAG M5 (Sigma-Aldrich) monoclonal antibody (MDA MB 231) or immunoglobulin G (IgG) alone for 4 h. Collection, washing, and reverse cross-linking of immune complexes was as described previously (Nowak *et al.*, 2005). Primers (Supplemental Table 1) spanning a known ETS binding site (EBS) situated 2.4 kb upstream of the transcriptional start site of uPA (D'Orazio *et al.*, 1997) were used to examine PDEF occupancy. To normalize for PDEF enrichment levels, chromatin PCR analysis was

repeated using primers encompassing the uPA ORF to establish the background levels in each sample. Relative enrichment was expressed as percentage of total input.

Phenotypic Rescue

Invasive breast cancer cells were transfected with 1) pcDNA vector, 2) pcDNA expressing PDEF, 3) pcDNA expressing uPA, and 4) two pcDNA constructs expressing PDEF and uPA, respectively. After transfection cells were incubated for 48 h in normal growth media before harvesting by trypsinization. Rescue of the migratory phenotype was examined using transwell migration assays as described above.

Statistical Analysis

Statistical analyses of all assays were performed using Student's *t* test for paired data. *p* < 0.05 was considered significant.

RESULTS

Microarray Analysis Identifies Potential PDEF Transcriptional Targets

Re-expression of PDEF in invasive cells reduces metastatic potential by inhibiting cell migration and invasion in breast and prostate cancer cells (Feldman *et al.*, 2003; Gu *et al.*, 2007; Turner *et al.*, 2007b). The current study used microarray gene expression analysis to identify putative PDEF-regulated target genes that may mediate the observed reduction in metastatic potential. Competitive hybridizations were performed using RNA collected from three invasive breast cancer cell lines (MDA MB 231, MDA MB 157, and BT 549) infected with GFP-expressing adenovirus, and RNA collected from the same cells expressing constitutive PDEF protein (Figure 1A). Using a differential expression cut-off of 1.5-fold, the presence of PDEF differentially altered the expression of 1723 genes (1004 reduced and 719 increased) in MDA MB 231, 1980 genes (959 reduced and 1021 increased) in MDA MB 157, and 2210 genes (1018 reduced and 1192 increased) in BT 549 (Supplemental Table 2). A combined analysis of all the data identified 223 (101 down-regulated, 122 up-regulated) putative regulatory genes that display common differential expression patterns upon the introduction of PDEF into each of the three invasive breast cancer cell lines (Supplemental Table 3).

Increased PDEF Expression Impacts Biological Pathways Involved in Cell Migration

To isolate putative PDEF regulatory target genes that are associated with cell migration, gene ontology associations were examined using Pathway Express (PE) (Khatri *et al.*, 2005; Draghici *et al.*, 2007; Khatri *et al.*, 2007), a freely available web based tool (<http://vortex.cs.wayne.edu>). PE associates lists of genes with biological interaction pathways and provides an assessment of the impact that those genes have on the pathway of interest. Impact factors (IF) are calculated by taking into account the magnitude of normalized differential expression, the statistical significance of those genes, their location in the pathway, and their associated interactions (Draghici *et al.*, 2007). Using the normalized gene lists for each cell line displaying a greater than 1.5-fold differential expression, four cytoskeleton/migration associated pathways were impacted with highly significant *p* values in each cell line: focal adhesions; cell adhesion molecules; adherens junctions and regulation of the actin cytoskeleton (Figure 1B). Gene ontology analysis and a search of the literature database identified three classifications of differentially expressed genes for each of these pathways (Figure 1C): class 1, differentially expressed genes in all three cell lines; class 2, differentially expressed genes in at least two cell lines; and class 3, differentially expressed genes in a single cell line. The number of genes identified for each

Figure 1. Microarray and gene ontology analysis identify interaction pathways impacted by PDEF expression. (A) Flow chart of microarray analysis protocol. Competitive hybridizations were performed in three invasive breast cancer cell lines expressing adenoviral mediated PDEF/GFP or GFP alone. Subsequent normalization and gene analysis identified differentially expressed genes for each cell line. A combined analysis of the data identified genes that displayed a common differential expression pattern in all three cell lines. (B) Gene ontology associations identify that PDEF expression in invasive breast cancer cells significantly impacts interaction pathways involved in cell migration. Calculated impact factors are displayed under individual bars on the graph and significance *p* values are shown above. (C) Tabulation of the number of genes displaying differential expression in each of the pathways identified as being impacted by PDEF expression. Classification of genes is defined in Table 1.

classification is shown in Figure 1C and full gene lists for each ontology pathway are provided in Supplemental Tables 4–7.

Key Mediators of the Migratory Phenotype Are Differentially Regulated upon PDEF Expression

More than 100 protein components have been associated with the formation of focal adhesion complexes. Focal adhesions function not only as cell anchorage complexes but also as environmental sensors and signaling focal points between the actin cytoskeleton and the ECM via clustered integrin complexes (McLean *et al.*, 2005). Combined ontology and literature analysis identified eight potential class 1 focal adhesion-associated PDEF regulatory genes: *baculoviral IAP repeat-containing 3*; *epidermal growth factor receptor (EGFR)*; *integrin, alpha 5 (ITGA5)*; *integrin, alpha 6 (ITGA6)*; and *LASP1*, *VASP*, *uPA*, and *uPAR* (expression changes are provided in Table 1). Given our previous data demonstrating PDEF-mediated relocalization of focal adhesion complexes and the high *p* values assigned to the ontology derived impact factor for this pathway in all three cell lines (Figure 1B), we examined the associated genes in more detail

(Figure 2). Significantly, PDEF expression in invasive breast cancer cell lines initially impacts the focal adhesion regulatory pathway at the level of cell membrane receptor. ECM/integrin, receptor tyrosine kinase, and uPA/uPAR interactions were all impacted by PDEF expression (Figure 2). Further downstream, PDEF impacted the expression of genes involved in F-actin and focal adhesion turnover, including the actin-binding proteins *LASP1* and *VASP*. Crucially, *LASP1*, *VASP*, *uPA*, and *uPAR*, also have functional associations with adherens junctions, cell adhesion, and actin cytoskeleton regulation. *VASP* (2.1- to 3.3-fold up-regulated; Table 1) is an actin-binding protein that localizes to focal adhesions, stress fibers, and the tips of lamellipodia and filopodia, where it mediates actin filament elongation (Holt *et al.*, 1998; Krause *et al.*, 2002; Krause *et al.*, 2003). *LASP1* (1.6–2.4-fold down-regulated; Table 1) also localizes to sites of dynamic actin assembly and has known associations with cytoskeleton reorganization as well as focal adhesion complexes, although its exact function is still unknown (Schreiber *et al.*, 1998; Li *et al.*, 2004; Lin *et al.*, 2004). Interestingly, *LASP1* has recently been reported to be over-expressed in >50% of invasive breast cancer tissue samples

Table 1. PDEF responsive genes associated with focal adhesion, adherens junction, cell adhesion, and actin cytoskeleton regulation

	MDA MB 157	MDA MB 231	BT 549		MDA MB 157	MDA MB 231	BT 549
Focal adhesion	Fold expression change			Adherens junctions	Fold expression change		
BIRC3	-2.5	-3.7	-2.1	EGFR	-1.6	-1.6	-2
EGFR	-1.6	-1.6	-2	FGFR1	1.9	2.2	1.5
ITGA5	2.2	1.8	2.6	PTPRF	1.5	1.6	1.5
ITGA6	3.7	2.2	4.4	SNAI2	-2.1	-2	-1.6
LASP1	-2.1	-2.4	-1.6	PLAU	-7.8	-6.5	-2.5
PLAU	-7.8	-6.5	-2.5	PLAUR	6.5	5	4.6
PLAUR	6.5	5	4.6	VASP	2.8	3.3	2.1
VASP	2.8	3.3	2.1				
Cell adhesion molecules				Regulators of actin cytoskeleton			
CD40	-2.5	-3.7	-2.1	APC	2.1	1.9	2
CLDN7	-1.6	-1.6	-2	CYFIP1	1.8	1.5	3
ITGA6	3.7	2.2	4.4	EGFR	-1.6	-1.6	-2
PTPRF	1.5	1.6	1.5	FGFR1	1.9	2.2	1.5
PLAU	-7.8	-6.5	-2.5	ITGA5	2.2	1.8	2.6
PLAUR	6.5	5	4.6	ITGA6	3.7	2.2	4.4
VASP	2.8	3.3	2.1	PLAU	-7.8	-6.5	-2.5
				PLAUR	6.5	5	4.6
				VASP	2.8	3.3	2.1

and correlates with increased tumor size and rate of nodal positivity (Grunewald *et al.*, 2007a). Previous work from this laboratory (Feldman *et al.*, 2003) demonstrated that increased PDEF expression negatively modulates uPA mRNA expression. The serine protease uPA (2.5- to 7.8-fold down-regulated; Table 1) is marker of poor prognosis and together with its receptor uPAR (4.6- to 6.5-fold up-regulated; Table 1) is best studied for its role in extracellular proteolysis activation (Aguirre Ghiso *et al.*, 1999; Choong and Nadesapillai, 2003; Han *et al.*, 2005). In addition, the uPA/uPAR system is also involved in signal transduction during cell migration, which is mediated by conferred changes in cytoskeleton and focal adhesion organization (Aguirre Ghiso *et al.*, 1999; Kjoller, 2002; Han *et al.*, 2005). Combined ontology and literature analysis also identified seven potential class 1 genes in cell adhesion, seven in adherens junctions, and 10 in regulation of actin cytoskeleton. Interestingly, VASP, uPAR (PLAUR), and uPA (PLAU) were found to impact each category (Figure 3C and Table 1).

Validation of uPA, uPAR, LASP1, and VASP Expression Levels

Differential expression validation was performed using two PDEF expression systems. The first approach used the same adenoviral expression system used to generate the microarray data. Infection with PDEF-expressing adenovirus produced high levels of PDEF protein in each of the three invasive breast cancer cell lines examined (MDA MB 231, MDA MB 157, and BT549) and control cells infected with GFP-expressing virus had no detectable endogenous PDEF expression (Figure 3B), consistent with previous reports (Feldman *et al.*, 2003; Turner *et al.*, 2007b). Real-time PCR analysis of mRNA levels confirmed the PDEF-mediated down-regulation of LASP1 and uPA, and the up-regulation of VASP and uPAR expression in all three cell lines (Figure 3A). Although mRNA levels were significantly reduced in all cell lines examined, the differential levels of uPA protein observed upon PDEF re-expression in BT549 cells were lower than that observed for MDA MB 231 and MDA MB 157. The reason for this observation is not clear, but it suggests that the uPA protein itself is more stable in the BT

549 cell line. The differential mRNA expression was accompanied by a concomitant reduction (LASP1 and uPA) and increase (VASP and uPAR) at the protein level as visualized by Western blot analysis (Figure 3B). Three major VASP phosphorylation sites have been identified, Ser157, Ser239, and Thr238 (Reinhard *et al.*, 2001). Using antibody against total VASP protein, PDEF expression was found to also increase both unphosphorylated and phosphorylated VASP (Figure 3B).

Adenoviral-mediated expression of PDEF produces high levels of PDEF protein compared with that endogenously expressed in the noninvasive breast cancer cell line MCF7 (Turner *et al.*, 2007b). To look at the effect of altered PDEF expression at a more physiological level, a stably transfected doxycycline-inducible expression system was used to modulate PDEF protein levels in a time- and dose-dependent manner (Figure 3, C and D). Inducible expression of PDEF over an 8-h period confirmed the results obtained using the adenoviral system and produced a steady decrease in uPA and LASP1 and increase in uPAR mRNA levels (Figure 3C). After an initial high level of expression VASP mRNA levels fall thereafter, but always remain above that observed in wild-type cells; thus, there is always an overall increase in expression over the 8-h period (Figure 3C). Furthermore, dose-dependent PDEF expression over a 24-h period again produced results consistent with those obtained after adenoviral expression, with an observed decrease in uPA and LASP1, and increase in uPAR and VASP protein expression (Figure 3D). These results further validate the data obtained from the microarray analysis and demonstrate PDEF-dependent modulation of these genes under physiological conditions.

Increased PDEF Mediates the Subcellular Relocalization of LASP1, VASP, and uPAR Proteins

The correct spatial and temporal localization of proteins is crucial to their function and is critical to ensuring cells adopt the correct morphological polarity required for efficient migration (Gupton and Waterman-Storer, 2006). In the absence of PDEF expression, ~15–20% of the morphologically heterogeneous population of cells displayed the classic morphological polarity of a migrating cell at any

Figure 2. Focal adhesion pathway interaction map. Pathway express and literature analysis identified critical interactions within the focal adhesion pathway that were impacted by PDEF expression. Classification of genes is defined in Table 1.

one time. This is compared with ~1–2% of total cells expressing PDEF.

Stimulation of nonmotile cells with growth factor or ECM protein mediates the relocation of LASP1 to nascent focal adhesions and sites of actin polymerization at the periphery of the leading lamellipodia of migrating cells (Lin *et al.*, 2004). In a morphologically heterogeneous population of cells that do not express PDEF, invasive breast cancer cells display a similar defined localization of LASP1 at the leading lamellipodia and at the trailing edge (Figure 4A, top) and in the cytoplasmic region. Furthermore, LASP1 is colocalized with the focal adhesion protein zyxin (Figure 4A, top), as reported previously (Li *et al.*, 2004; Grunewald *et al.*, 2006, 2007b). However, upon inducible expression of PDEF, LASP1 immunofluorescence is observed at multiple sites around the cell periphery and not defined to a single lamellipodia, although cytoplasmic LASP1 remains unchanged

and LASP1-zyxin colocalization is still observed (Figure 4A, bottom).

VASP facilitates filamentous actin formation and evidence suggests a widespread role in cell adhesion and motility (Reinhard *et al.*, 2001). Similarly to LASP1, in PDEF minus (migratory) cells, VASP localization is limited to a few complexes at the leading and trailing edge of migrating cells, but it is also observed at high levels within the cytoplasm. VASP also colocalizes with zyxin (Figure 4B, top). In cells expressing PDEF, VASP complexes are more numerous and like LASP1, they are localized all around the cell periphery and throughout the cell body (Figure 4B, bottom). Although almost all VASP protein is colocalized with zyxin at the cell periphery and within the cytoplasm in PDEF minus (migratory) cells, in PDEF-expressing cells there are distinct complexes of VASP protein just within the cell periphery that are not associated with zyxin (Figure 4B, inset). To examine the

Figure 3. Validation of differential expression of uPA, uPAR, LASP1, and VASP at the mRNA and protein level. (A) Real time PCR analysis of PDEF mRNA expression in invasive breast cancer cell lines infected with GFP- or PDEF/GFP-expressing adenovirus. Expression was normalized to S26 mRNA levels. Error bars represent the SD obtained from three biological replicates. (B) Western blot analysis of PDEF, uPA, uPAR, LASP1, and VASP expression in total cell lysates obtained from invasive breast cancer cell lines infected with GFP- (G) or PDEF/GFP (P)-expressing adenovirus. GAPDH was used as a loading control. (C) Real-time PCR analysis over time of PDEF mRNA expression in stably transfected inducible MDA MB 157 cells expressing and not expressing PDEF. PDEF expression was induced with 1 μ g/ml doxycycline. Expression was normalized to S26 mRNA levels. Error bars represent the SD obtained from three biological replicates. (D) Western blot analysis of PDEF, uPA, uPAR, LASP1, and VASP expression in total cell lysates obtained from stably transfected inducible MDA MB 157 cells expressing dose-dependent doxycycline induced PDEF. GAPDH was used as a loading control.

biological impact of these altered localizations, either LASP1 or VASP cDNA was expressed in MDA MB 231, MDA MB 157, and BT549 cells. Transwell assays demonstrated that either gene inhibited cell migration across fibronectin-coated membranes (Figure 5, A and B).

Most characterized uPA-mediated effects are conferred through interactions with its receptor uPAR. In invasive breast cancer cells, PDEF expression mediated the down-regulation of uPA, but it increased uPAR mRNA and protein levels. In a similar manner to that observed for LASP1 and VASP, PDEF expression also altered the cellular localization of uPAR. In PDEF minus control cells, uPAR is colocalized with phosphorylated focal adhesion kinase (pFAK) to the leading lamellipodia, at the perinuclear membrane, and within the cytoplasm (Figure 4C, top). On PDEF expression, uPAR is localized all around the cell periphery, with little or no colocalization with pFAK complexes, although the perinuclear membrane uPAR and pFAK are still colocalized (Figure 4C, bottom).

RNA Interference (RNAi)-mediated PDEF Gene Knockdown Produces Reciprocal Effects on uPA, LASP1, and VASP Expression

PDEF protein is endogenously expressed in the noninvasive breast cancer cell line MCF7 (Feldman *et al.*, 2003; Turner *et al.*, 2007b). Using vector-expressed shRNA directed against PDEF, protein levels were significantly reduced in a dose-dependent manner (Figure 6A). Based upon the expression of GFP, ~60% transfection efficiency was observed. Real-time PCR analysis demonstrates that the knockdown of PDEF protein results in the increased expression of both uPA and LASP1 transcripts and a reduction in the expression of VASP (Figure 6B). However, uPAR transcript levels in noninvasive cells were increased upon PDEF knockdown (Figure 6B), giving a similar result as to that observed when PDEF is overexpressed in invasive cells (Figure 2B).

Exogenous uPA Expression Restores the Migratory Phenotype Inhibited by PDEF Regulation of the uPA Promoter

Elevated expression of uPA in breast cancer cells increases their migratory ability (Han *et al.*, 2005), similar to that observed upon loss of PDEF protein expression (Feldman *et al.*, 2003; Turner *et al.*, 2007b). Furthermore, re-expression of PDEF in invasive breast cancer cells inhibits migration and invasion (Feldman *et al.*, 2003; Turner *et al.*, 2007b) and reduces uPA mRNA and protein expression (Figure 2). In vivo ChIP analysis demonstrated the direct binding of exogenous (MDA MB 231) and endogenous (MCF7) PDEF protein at an EBS situated 2.4 kb upstream from the transcriptional start site of *uPA*. A significantly high level of enrichment was observed in MDA MB 231 cells infected with PDEF-expressing virus, and a lower but still significant enrichment was also observed in MCF7 cells endogenously expressing PDEF (Figure 7A). No such PDEF enrichment was observed in similar reactions by using primers encompassing a different region of the *uPA* gene (i.e., the coding region of *uPA*) (data not shown). Because PDEF was identified as a direct negative regulator of *uPA* transcriptional expression, phenotypic rescue experiments were used to examine the ability of exogenous uPA to restore the migratory phenotype in cells expressing PDEF. The three invasive breast cancer cell models used in the microarray analysis were transfected with pcDNA3 vector (control), pcDNA3-PDEF, or pcDNA-uPA, respectively, and also dual transfected with both pcDNA-PDEF and pcDNA-uPA. Protein expression levels of PDEF and uPA were confirmed by Western blot analysis (Figure 7B). As demonstrated previously (Figure 3), the re-expression of PDEF in cells expressing only endogenous uPA resulted in a reduction of uPA mRNA and protein (Figure 7, B and C). In the presence of both endogenous and exogenous uPA, the re-expression of PDEF had little or no significant effect on total uPA levels (Figure 7, B and C). Phenotypic analysis using transwell migration assays confirmed that PDEF expression inhibits, and exogenous uPA expression increases, the migratory ability of invasive breast cancer cells (Figure 7D). Signifi-

Figure 4. PDEF expression alters LASP1, VASP, and uPAR subcellular localization. (A) Immunofluorescence staining of LASP1 localization in MDA MB 157 stable PDEF-inducible cell lines, after treatment with (plus PDEF, bottom) or without (minus PDEF, top) doxycycline. LASP1 localization was visualized at 480 nm, and adhesion complex localization was visualized using immunofluorescent staining of zyxin at 540 nm. Colocalization is shown as a merged image at 480 nm/540 nm. (B) Immunofluorescence staining of VASP localization in MDA MB 157 stable PDEF-inducible cell lines after treatment with (plus PDEF, bottom) or without (minus PDEF, top) doxycycline. VASP localization was visualized at 480 nm, and adhesion complex localization was visualized using immunofluorescent staining of zyxin at 540 nm. Colocalization is shown as a merged image at 480 nm/540 nm. A magnified view of VASP and zyxin nonlocalization is shown in the inset. (C) immunofluorescence staining of uPAR localization in MDA MB 157 stable PDEF-inducible cell lines after treatment with (plus PDEF, bottom) or without (minus PDEF, top) doxycycline. uPAR localization was visualized at 480 nm, and adhesion complex localization was visualized using immunofluorescent staining of phosphorylated focal adhesion kinase pFAK at 540 nm. Colocalization is shown as a merged image at 480 nm/540 nm.

cantly, when exogenous PDEF and uPA are simultaneously expressed, the exogenous uPA expression rescues the migratory phenotype to levels observed in wild-type cells expressing only endogenous uPA (Figure 7D). In additional experiments performed in a pool MDA MB 231 cells stably transfected with the uPA vector, phenotypic rescue by ex-

ogenous uPA expression was retained even at the high levels of PDEF protein produced by adenoviral mediated expression (Figure 7, E and F). Furthermore the coexpression of uPA/PDEF was able to restore the colocalization of LASP1, VASP, and uPAR to the leading and trailing edge of migrating cells (Supplemental Figure 1).

Figure 5. LASP1 and VASP expression inhibit cell migration in invasive breast cancer cells. The migratory ability of invasive breast cancer cells expressing exogenous LASP1 and VASP, respectively, was analyzed using transwell migration assays. The columns represent the average number of cells migrating per 10 microscope fields. Error bars represent the SD obtained from three biological replicates. **p value <0.05.

DISCUSSION

The progression to metastasis is the most deadly phase of cancer and central to that process is the acquisition of motile

function. Expression of PDEF in invasive breast and prostate cancer inhibits cell migration and invasion (Feldman *et al.*, 2003; Gu *et al.*, 2007; Turner *et al.*, 2007b; Turner and Watson, 2008). To date, few PDEF target genes have been identified, and the precise mechanism by which PDEF leads to an inhibition of migration and invasion is not clear. Using microarray, multiple potential PDEF response genes were identified based upon their differential regulation upon PDEF reprogramming of gene expression. Gene ontology analysis coupled with literature searches identified *uPA*, *uPAR*, *LASP1*, and *VASP* as putative PDEF target genes with biological associations with multiple critical pathways involved in cell migration during metastasis. Subsequent validation studies confirmed the differential expression of all of these genes in three invasive breast cancer cell lines, by using both adenoviral and inducible PDEF expression, and served to indirectly support the robustness of the overall data set. In noninvasive MCF7 breast cancer cells, concomitant up-regulation of *uPA* and *LASP1* and down-regulation of *VASP* mRNA levels were observed upon reduction of PDEF protein expression. Knockdown of PDEF expression in MCF7 has been shown previously to increase cell migration in vitro (Turner *et al.*, 2007b) and tumor formation in vivo (Ghadersohi *et al.*, 2006), providing biological significance for the observed molecular changes. Overall, these data demonstrate that PDEF has reciprocal effects on *uPA*, *LASP1*, and *VASP* gene expression in noninvasive and invasive breast cancer cells.

Significantly, the ChIP experiment presented in this study provides the first evidence that the metastasis associated gene *uPA* is a direct transcriptional target of PDEF-negative regulation. Furthermore, by rescuing the antimigratory phenotype and subcellular localization of *LASP1*, *VASP* and *uPAR* through the exogenous expression of *uPA*, this study assigns one of the functional consequences of this single PDEF target gene to the migratory ability of invasive breast cancer cells. High levels of *uPA* in primary breast cancer are independently associated with adverse outcome and have recently been recommended as a tumor marker for breast cancer patient prognosis by the American Society of Clinical Oncology (Harris *et al.*, 2007). The negative regulation of *uPA* by PDEF may alter the migratory ability of cancer cells through several possible mechanisms. The *uPA* ligand and its receptor mediate numerous processes during cancer progression, including cell proliferation, angiogenesis, cell signaling, growth factor secretion, remodeling of the extracellular matrix, cell migration, and cell adhesion (Aguirre Ghiso *et al.*, 1999; Choong and Nadesapillai, 2003; Han *et al.*, 2005). The *uPA/uPAR* system is most extensively studied for its role in the activation of proteolytic enzymes, such as plasminogen and matrix metalloproteases (MMPs). The activation of these enzymes is crucial for the degradation of the ECM and for the efficient turnover of nascent focal adhesions, and it has possible implications in both cell adhesion and adherens junction turnover (Abu-Ali *et al.*, 2005). Although minimal effects on the mRNA expression level of MMPs were observed, it remains to be determined whether PDEF expression affects these or additional proteases at either the total protein level or their activation state. Interestingly, members of the serine protease inhibitor (serpin) family (*SERPINB8*, *SERPINI2*) are up-regulated after PDEF expression (Supplemental Table 3). The *uPA/uPAR* system also has a critical role in intracellular cell signaling, which is independent of its proteolytic function and includes interactions with the tyrosine kinase (Blasi and Carmeliet, 2002) and EGFR (Festuccia *et al.*, 2005) signaling pathways, as well as signaling associated integrin family members (Ossowski

Figure 6. RNAi-mediated down-regulation of PDEF modulates *uPA*, *uPAR*, *LASP1*, and *VASP* expression in noninvasive cells. (A) Western blot analysis of PDEF protein levels in total cell lysates obtained from MCF7 cells transfected with increasing concentrations of PDEF-targeting shRNA. GAPDH was used as a loading control. (B) Real time PCR analysis of *uPA*, *uPAR*, *LASP1*, and *VASP* mRNA expression in noninvasive MCF7 breast cancer cell lines transfected with shRNA targeting PDEF expression. Expression was normalized to *S26* mRNA levels. Error bars represent the SD obtained from three biological replicates. *p value <0.01, **p value <0.05.

Figure 7. Exogenous expression of uPA restores the anti-migratory phenotype of invasive cells expressing PDEF. (A) In vivo ChIP analysis identifies PDEF as a direct negative regulator of *uPA* gene expression. PDEF promoter occupancy was demonstrated with endogenous (MCF7 probed with PDEF antibody) and exogenous PDEF expression (MDA MB 231 cells expressing adenoviral-mediated PDEF probed with FLAG antibody). Control immunoprecipitations were performed in the presence of preimmune IgG alone. PCR was performed on the enriched chromatin fragments using the *uPA* promoter-specific primers detailed in Supplemental Table 1. Numbers represent the percentage of total input. (B) Western blot analysis of PDEF and uPA expression in total cell lysates obtained from cells singularly and dual transfected with pcDNA3 vector expressing PDEF or uPA protein in MDA MB 231. Cells transfected with pcDNA vector alone served as controls. GAPDH was used as a loading control. (C) Real-time PCR analysis of uPA mRNA expression in invasive breast cancer cell lines transfected with pcDNA3 vector expressing PDEF and/or uPA. Cells transfected with pcDNA vector alone served as controls. Expression was normalized to S26 mRNA levels. Error bars represent the SD obtained from three biological replicates. (D) Phenotypic rescue of the migratory phenotype upon exogenous uPA expression. The migratory ability of invasive breast cancer cells treated as described in C was analyzed using transwell migration assays. The columns represent the average number of cells migrating per ten microscope fields. Error bars represent the SD obtained from three biological replicates. (E) Western blot analysis of PDEF and uPA expression in total cell lysates obtained from MDA MB 231 cells stably expressing uPA. PDEF expression was mediated by adenoviral infection. Cells stably expressing uPA but infected with GFP-expressing adenovirus served as controls. GAPDH was used as a loading control. (F) Phenotypic rescue of the migratory phenotype upon exogenous uPA expression. The migratory ability of invasive breast cancer cells treated as described in D was analyzed using transwell migration assays. The columns represent the average number of cells migrating per ten microscope fields. Error bars represent the SD obtained from three biological replicates.

and Aguirre-Ghiso, 2000; Chapman and Wei, 2001). Although not directly validated, EGFR and integrin family members ITGA6 and ITGA5 are differentially expressed upon PDEF expression in all three cell lines examined by microarray analysis (Table 1). It is believed that uPA-induced conformational changes in uPAR structure allow the formation of signal transduction complexes, which mediate signal transduction (Blasi and Carmeliet, 2002). The direct negative regulation of *uPA* by PDEF therefore would be expected to reduce uPA receptor binding and significantly alter cell signaling patterns within the cell and inhibit cell migration. The reason for the elevated uPAR mRNA levels observed upon both increased and decreased of PDEF protein expression is not clear, but it may reflect cell-specific

phenotypes. In noninvasive MCF7 cells, PDEF knockdown increases uPA expression, which in turn may stimulate the expression of its binding partner uPAR. In invasive cells (MDA MB 231, MDA MB 157, and BT549), the observed increase in uPAR mRNA levels after PDEF expression may be a compensatory mechanism for the loss of uPA. Interestingly, studies have shown that elevated soluble uPAR (suPAR) can impair many of the functions of the urokinase system, including proteolysis and tumor growth to reduce metastatic potential in breast cancer cells (Kruger *et al.*, 2000). PDEF expression may stimulate the expression of suPAR further increasing its inhibitory effects on cancer progression. Further studies are required to investigate this possibility.

Central to the activation of motile function is the acquisition of cell polarity. Migratory polarization results from the spatial and temporal organization of a single lamellipodium that must adhere to the ECM through nascent focal adhesions to sustain its development into the polarized dominant lamellipodium required for directed cell migration. Failure to adhere to the ECM results in the retraction of the lamellipodium, and migration is subsequently inhibited (Bailly *et al.*, 1998; Pantaloni *et al.*, 2001; Wehrle-Haller and Imhof, 2002; Danuser, 2005). In fluorescent migration track assays, cells expressing PDEF produce rounded areas of cleared fluorescence rather than the long migration tracks associated with a migratory cell (Turner *et al.*, 2007b). Time-lapse microscopy demonstrated that the rounded migration tracks were due to the extension and retraction of lamellipodia, indicating a failure to adhere to the ECM through nascent focal adhesion formation. This previous study concluded that cells expressing PDEF could no longer form the morphological polarity required for efficient directional cell migration. As outlined above, uPA may have multiple roles in mediating this effect; moreover, both VASP and LASP1 also have functional roles in defining cell polarity during cell migration. VASP along with MENA and EVL are members of the ENA/VASP family of actin-binding proteins. Elevated levels of ENA/VASP protein localizes to the cell membrane and produces multiple lamellipodia, that rapidly protrude and retract resulting in an inhibition of migration in a similar manner to that observed upon PDEF expression (Bear *et al.*, 2002; Krause *et al.*, 2002). Additionally, during cell migration, LASP1 is relocalized from the cell periphery to the leading edge of a single lamellipodium and to the nascent focal adhesions it contains (Lin *et al.*, 2004), where it is associated with the correct localization of the focal adhesion regulator zyxin (Grunewald *et al.*, 2006, 2007b). If LASP1 is not recruited to the leading edge, then cell migration is inhibited (Lin *et al.*, 2004). This study identifies that the PDEF-mediated expression of PDEF decreases LASP1 levels and increases VASP levels to inhibit migration, but also demonstrates that the overexpression of LASP1 can inhibit migration. Previous studies have also shown that both reduced and increased LASP1 (Lin *et al.*, 2004; Grunewald *et al.*, 2007b) and VASP (Reinhard *et al.*, 2001; Krause *et al.*, 2002) intracellular levels can inhibit the migratory ability in alternative cell lines. This indicates that their precise cellular protein concentration, as well as their subcellular localization, is crucial to their function as cytoskeleton regulators during cell migration. It is unclear at this stage whether the disruption of the uPA/uPAR system by PDEF is directly responsible for the altered expression and localization of LASP1 and VASP, but it remains a possibility requiring further analysis.

Although overall VASP mRNA levels remain above wild-type upon PDEF expression, they do decline thereafter (Figure 3C). The reason for this is not clear; however, the induction kinetics and sustainability of activation are specific for each gene. Transcriptional complexes on defined promoters are also gene specific. The concentration of protein and other kinetic parameters, such as protein cofactors, basal rates, decay rates, and sensitivities, can all affect transcriptional expression patterns of an individual target gene (Nasiadka and Krause, 1999; Sandmann *et al.*, 2006). The transcriptional effect of PDEF on VASP expression may be temporal due to a disruption of any one or more of these factors.

ETS transcriptional regulation of uPA has been well established. ETS1 and ETS2 have been implicated in the increased expression of uPA and a subsequent increase in the migratory ability of cancer cells (Watabe *et al.*, 1998; Nakada *et al.*,

1999; Behrens *et al.*, 2001; Buggy *et al.*, 2004; Buggy *et al.*, 2006). Significantly, the specific EBS occupied by PDEF was originally identified as a regulatory element for the ETS transcription factor PEA3, and its expression has been shown to increase uPA promoter activity (D'Orazio *et al.*, 1997). Together, these data indicate the presence of an ETS mediated pro- (PEA3, ETS1, and ETS2) and antimetastatic (PDEF) regulatory network in the regulation of a metastasis-associated gene. It is becoming increasingly apparent that to understand the transcriptional regulation of normal and cancer associated genes, the function of transcription factors must be analyzed in the context of a transcriptional network rather than as individual entities (Turner *et al.*, 2007a). Global analyses that examine gene expression patterns and promoter occupancy (ChIP on chip microarray) will allow the identification of these transcriptional networks, a prerequisite for assessing their impact on cancer progression. Such an understanding of transcriptional regulation may allow the development of novel therapeutics that may molecularly prevent or reverse the metastatic phenotype and has the potential to significantly lower morbidity and improve the quality of life of cancer patients.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grant P01-CA78582 (to D.K.W.).

REFERENCES

- Abu-Ali, S., Sugiura, T., Takahashi, M., Shiratsuchi, T., Ikari, T., Seki, K., Hiraki, A., Matsuki, R., and Shirasuna, K. (2005). Expression of the urokinase receptor regulates focal adhesion assembly and cell migration in adenoid cystic carcinoma cells. *J. Cell Physiol.* 203, 410–419.
- Aguirre Ghiso, J. A., Alonso, D. F., Farias, E. F., Gomez, D. E., and de Kier Joffe, E. B. (1999). Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. *Eur. J. Biochem.* 263, 295–304.
- Bailly, M., Yan, L., Whitesides, G. M., Condeelis, J. S., and Segall, J. E. (1998). Regulation of protrusion shape and adhesion to the substratum during chemotactic responses of mammalian carcinoma cells. *Exp. Cell Res.* 241, 285–299.
- Bear, J. E. *et al.* (2002). Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell* 109, 509–521.
- Behrens, P., Rothe, M., Wellmann, A., Krischler, J., and Wernert, N. (2001). The Ets-1 transcription factor is up-regulated together with MMP 1 and MMP 9 in the stroma of pre-invasive breast cancer. *J. Pathol.* 194, 43–50.
- Benz, C. C. *et al.* (1997). HER2/Neu and the Ets transcription activator PEA3 are coordinately upregulated in human breast cancer. *Oncogene* 15, 1513–1525.
- Bieche, I., Tozlu, S., Girault, I., Onody, P., Driouch, K., Vidaud, M., and Lidereau, R. (2004). Expression of PEA3/E1AF/ETV4, an Ets-related transcription factor, in breast tumors: positive links to MMP2, NRG1 and CGB expression. *Carcinogenesis* 25, 405–411.
- Blasi, F., and Carmeliet, P. (2002). uPAR: a versatile signalling orchestrator. *Nat. Rev. Mol. Cell Biol.* 3, 932–943.
- Bosc, D. G., Goueli, B. S., and Janknecht, R. (2001). HER2/Neu-mediated activation of the ETS transcription factor ER81 and its target gene MMP-1. *Oncogene* 20, 6215–6224.
- Buggy, Y., Maguire, T. M., McDermott, E., Hill, A. D., O'Higgins, N., and Duffy, M. J. (2006). Ets2 transcription factor in normal and neoplastic human breast tissue. *Eur. J. Cancer* 42, 485–491.
- Buggy, Y., Maguire, T. M., McGreal, G., McDermott, E., Hill, A. D., O'Higgins, N., and Duffy, M. J. (2004). Overexpression of the Ets-1 transcription factor in human breast cancer. *Br. J. Cancer* 91, 1308–1315.
- Chapman, H. A., and Wei, Y. (2001). Protease crosstalk with integrins: the urokinase receptor paradigm. *Thromb. Haemost.* 86, 124–129.
- Choong, P. F., and Nadesapillai, A. P. (2003). Urokinase plasminogen activator system: a multifunctional role in tumor progression and metastasis. *Clin. Orthop. Relat. Res.* S46–S58.

- Compagni, A., and Christofori, G. (2000). Recent advances in research on multistage tumorigenesis. *Br. J. Cancer* 83, 1–5.
- D'Orazio, D., Besser, D., Marksitzer, R., Kunz, C., Hume, D. A., Kiefer, B., and Nagamine, Y. (1997). Cooperation of two PEA3/AP1 sites in uPA gene induction by TPA and FGF-2. *Gene* 201, 179–187.
- Danuser, G. (2005). Coupling the dynamics of two actin networks—new views on the mechanics of cell protrusion. *Biochem. Soc. Trans.* 33, 1250–1253.
- Doane, A. S., Danso, M., Lal, P., Donaton, M., Zhang, L., Hudis, C., and Gerald, W. L. (2006). An estrogen receptor-negative breast cancer subset characterized by a hormonally regulated transcriptional program and response to androgen. *Oncogene* 25, 3994–4008.
- Draghici, S., Khatri, P., Tarca, A. L., Amin, K., Done, A., Voichita, C., Georgescu, C., and Romero, R. (2007). A systems biology approach for pathway level analysis. *Genome Res.* 17, 1537–1545.
- Dudoit, S., Gentleman, R. C., and Quackenbush, J. (2003). Open source software for the analysis of microarray data. *Biotechniques Suppl.* 45–51.
- Feldman, R. J., Sementchenko, V. I., Gayed, M., Fraig, M. M., and Watson, D. K. (2003). Pdef expression in human breast cancer is correlated with invasive potential and altered gene expression. *Cancer Res.* 63, 4626–4631.
- Festuccia, C., Angelucci, A., Gravina, G. L., Biordi, L., Millimaggi, D., Muzi, P., Vicentini, C., and Bologna, M. (2005). Epidermal growth factor modulates prostate cancer cell invasiveness regulating urokinase-type plasminogen activator activity. EGF-receptor inhibition may prevent tumor cell dissemination. *Thromb. Haemost.* 93, 964–975.
- Fidler, I. J. (2003). The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat. Rev. Cancer* 3, 453–458.
- Friedl, P., and Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat. Rev. Cancer* 3, 362–374.
- Geller, S. C., Gregg, J. P., Hagerman, P., and Rocke, D. M. (2003). Transformation and normalization of oligonucleotide microarray data. *Bioinformatics* 19, 1817–1823.
- Ghadersohi, A., Odunsi, K., Lele, S., Collins, Y., Greco, W. R., Winston, J., Liang, P., and Sood, A. K. (2004). Prostate derived Ets transcription factor shows better tumor-association than other cancer-associated molecules. *Oncol. Rep.* 11, 453–458.
- Ghadersohi, A., Pan, D., Fayazi, Z., Hicks, D. G., Winston, J. S., and Li, F. (2006). Prostate-derived Ets transcription factor (PDEF) downregulates survivin expression and inhibits breast cancer cell growth in vitro and xenograft tumor formation in vivo. *Breast Cancer Res. Treat.*
- Ghadersohi, A., and Sood, A. K. (2001). Prostate epithelium-derived Ets transcription factor mRNA is overexpressed in human breast tumors and is a candidate breast tumor marker and a breast tumor antigen. *Clin. Cancer Res.* 7, 2731–2738.
- Grunewald, T. G., Kammerer, U., Kapp, M., Eck, M., Dietl, J., Butt, E., and Honig, A. (2007a). Nuclear localization and cytosolic overexpression of LASP-1 correlates with tumor size and nodal-positivity of human breast carcinoma. *BMC Cancer* 7, 198.
- Grunewald, T. G., Kammerer, U., Schulze, E., Schindler, D., Honig, A., Zimmer, M., and Butt, E. (2006). Silencing of LASP-1 influences zyxin localization, inhibits proliferation and reduces migration in breast cancer cells. *Exp. Cell Res.* 312, 974–982.
- Grunewald, T. G., Kammerer, U., Winkler, C., Schindler, D., Sickmann, A., Honig, A., and Butt, E. (2007b). Overexpression of LASP-1 mediates migration and proliferation of human ovarian cancer cells and influences zyxin localisation. *Br. J. Cancer* 96, 296–305.
- Gu, X., Zerbini, L. F., Otu, H. H., Bhasin, M., Yang, Q., Joseph, M. G., Grall, F., Onatunde, T., Correa, R. G., and Libermann, T. A. (2007). Reduced PDEF expression increases invasion and expression of mesenchymal genes in prostate cancer cells. *Cancer Res.* 67, 4219–4226.
- Gupton, S. L., and Waterman-Storer, C. M. (2006). Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. *Cell* 125, 1361–1374.
- Han, B., Nakamura, M., Mori, I., Nakamura, Y., and Kakudo, K. (2005). Urokinase-type plasminogen activator system and breast cancer. *Oncol. Rep.* 14, 105–112.
- Harris, L., Fritsche, H., Mennel, R., Norton, L., Ravdin, P., Taube, S., Somerfield, M. R., Hayes, D. F., and Bast, R. C., Jr. (2007). American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J. Clin. Oncol.* 25, 5287–5312.
- Holt, M. R., Critchley, D. R., and Brindle, N. P. (1998). The focal adhesion phosphoprotein, VASP. *Int. J. Biochem. Cell Biol.* 30, 307–311.
- Kedrin, D., van Rheenen, J., Hernandez, L., Condeelis, J., and Segall, J. E. (2007). Cell motility and cytoskeletal regulation in invasion and metastasis. *J. Mammary Gland Biol. Neoplasia* 12, 143–152.
- Khatri, P., Sellamuthu, S., Malhotra, P., Amin, K., Done, A., and Draghici, S. (2005). Recent additions and improvements to the Onto-Tools. *Nucleic Acids Res.* 33, W762–W765.
- Khatri, P., Voichita, C., Kattan, K., Ansari, N., Khatri, A., Georgescu, C., Tarca, A. L., and Draghici, S. (2007). Onto-Tools: new additions and improvements in 2006. *Nucleic Acids Res.* 35, W206–W211.
- Kjoller, L. (2002). The urokinase plasminogen activator receptor in the regulation of the actin cytoskeleton and cell motility. *Biol. Chem.* 383, 5–19.
- Krause, M., Bear, J. E., Loureiro, J. J., and Gertler, F. B. (2002). The Ena/VASP enigma. *J. Cell Sci.* 115, 4721–4726.
- Krause, M., Dent, E. W., Bear, J. E., Loureiro, J. J., and Gertler, F. B. (2003). Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. *Annu. Rev. Cell Dev. Biol.* 19, 541–564.
- Kruger, A., Soeltl, R., Lutz, V., Wilhelm, O. G., Magdolen, V., Rojo, E. E., Hantzopoulos, P. A., Graeff, H., Gansbacher, B., and Schmitt, M. (2000). Reduction of breast carcinoma tumor growth and lung colonization by overexpression of the soluble urokinase-type plasminogen activator receptor (CD87). *Cancer Gene Ther.* 7, 292–299.
- Li, B., Zhuang, L., and Trueb, B. (2004). Zyxin interacts with the SH3 domains of the cytoskeletal proteins LIM-nebulette and Lasp-1. *J. Biol. Chem.* 279, 20401–20410.
- Lin, Y. H., Park, Z. Y., Lin, D., Brahmabhatt, A. A., Rio, M. C., Yates, J. R., 3rd, and Klemke, R. L. (2004). Regulation of cell migration and survival by focal adhesion targeting of Lasp-1. *J. Cell Biol.* 165, 421–432.
- Loberg, R. D., Bradley, D. A., Tomlins, S. A., Chinnaiyan, A. M., and Pienta, K. J. (2007). The lethal phenotype of cancer: the molecular basis of death due to malignancy. *CA Cancer J. Clin.* 57, 225–241.
- McLean, G. W., Carragher, N. O., Avizienyte, E., Evans, J., Brunton, V. G., and Frame, M. C. (2005). The role of focal-adhesion kinase in cancer—a new therapeutic opportunity. *Nat. Rev. Cancer* 5, 505–515.
- Mogilner, A. (2006). On the edge: modeling protrusion. *Curr. Opin. Cell Biol.* 18, 32–39.
- Nakada, M., Yamashita, J., Okada, Y., and Sato, H. (1999). Ets-1 positively regulates expression of urokinase-type plasminogen activator (uPA) and invasiveness of astrocytic tumors. *J. Neuropathol. Exp. Neurol.* 58, 329–334.
- Nasiadka, A., and Krause, H. M. (1999). Kinetic analysis of segmentation gene interactions in *Drosophila* embryos. *Development* 126, 1515–1526.
- Nowak, D. E., Tian, B., and Brasier, A. R. (2005). Two-step cross-linking method for identification of NF-kappaB gene network by chromatin immunoprecipitation. *Biotechniques* 39, 715–725.
- Nozawa, M., Yomogida, K., Kanno, N., Nonomura, N., Miki, T., Okuyama, A., Nishimune, Y., and Nozaki, M. (2000). Prostate-specific transcription factor hPSE is translated only in normal prostate epithelial cells. *Cancer Res.* 60, 1348–1352.
- Ossowski, L., and Aguirre-Ghisso, J. A. (2000). Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. *Curr. Opin. Cell Biol.* 12, 613–620.
- Pantaloni, D., Le Clairche, C., and Carlier, M. F. (2001). Mechanism of actin-based motility. *Science* 292, 1502–1506.
- Ramakers, C., Ruijter, J. M., Deprez, R. H., and Moorman, A. F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* 339, 62–66.
- Reinhard, M., Jarchau, T., and Walter, U. (2001). Actin-based motility: stop and go with Ena/VASP proteins. *Trends Biochem. Sci.* 26, 243–249.
- Sandmann, T., Jensen, L. J., Jakobsen, J. S., Karzynski, M. M., Eichenlaub, M. P., Bork, P., and Furlong, E. E. (2006). A temporal map of transcription factor activity: mef2 directly regulates target genes at all stages of muscle development. *Dev. Cell* 10, 797–807.
- Schreiber, V., Moog-Lutz, C., Regnier, C. H., Chenard, M. P., Boeuf, H., Vonesch, J. L., Tomasello, C., and Rio, M. C. (1998). Lasp-1, a novel type of actin-binding protein accumulating in cell membrane extensions. *Mol. Med.* 4, 675–687.
- Seth, A., and Watson, D. K. (2005). ETS transcription factors and their emerging roles in human cancer. *Eur. J. Cancer* 41, 2462–2478.
- Sporn, M. B. (1996). The war on cancer. *Lancet* 347, 1377–1381.
- Tsujimoto, Y., Nonomura, N., Takayama, H., Yomogida, K., Nozawa, M., Nishimura, K., Okuyama, A., Nozaki, M., and Aozasa, K. (2002). Utility of immunohistochemical detection of prostate-specific Ets for the diagnosis of benign and malignant prostatic epithelial lesions. *Int. J. Urol.* 9, 167–172.

- Turner, D. P., Findlay, V. J., Moussa, O., and Watson, D. K. (2007a). Defining ETS transcription regulatory networks and their contribution to breast cancer progression. *J. Cell Biochem.* 102, 549–559.
- Turner, D. P., Moussa, O., Sauane, M., Fisher, P. B., and Watson, D. K. (2007b). Prostate-derived ETS factor is a mediator of metastatic potential through the inhibition of migration and invasion in breast cancer. *Cancer Res.* 67, 1618–1625.
- Turner, D. P., and Watson, D. K. (2008). ETS transcription factors: oncogenes and tumor suppressor genes as therapeutic targets for prostate cancer. *Expert Rev. Anticancer Ther.* 8, 33–42.
- Watabe, T., Yoshida, K., Shindoh, M., Kaya, M., Fujikawa, K., Sato, H., Seiki, M., Ishii, S., and Fujinaga, K. (1998). The Ets-1 and Ets-2 transcription factors activate the promoters for invasion-associated urokinase and collagenase genes in response to epidermal growth factor. *Int. J. Cancer* 77, 128–137.
- Wehrle-Haller, B., and Imhof, B. (2002). The inner lives of focal adhesions. *Trends Cell Biol.* 12, 382–389.
- Yamazaki, D., Kurisu, S., and Takenawa, T. (2005). Regulation of cancer cell motility through actin reorganization. *Cancer Sci.* 96, 379–386.
- Yordy, J. S., Moussa, O., Pei, H., Chaussabel, D., Li, R., and Watson, D. K. (2005). SP100 inhibits ETS1 activity in primary endothelial cells. *Oncogene* 24, 916–931.