

PDEF Promotes Luminal Differentiation and Acts as a Survival Factor for ER-Positive Breast Cancer Cells

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SUMMARY

Breast cancer is a heterogeneous disease and can be classified based on gene expression profiles that reflect distinct epithelial subtypes. We identify prostate-derived ETS factor (PDEF) as a mediator of mammary luminal epithelial lineage-specific gene expression and as a factor required for tumorigenesis in a subset of breast cancers. PDEF levels strongly correlate with estrogen receptor (ER)-positive luminal breast cancer, and *PDEF* transcription is inversely regulated by ER and GATA3. Furthermore, PDEF is essential for luminal breast cancer cell survival and is required in models of endocrine resistance. These results offer insights into the function of this ETS factor that are clinically relevant and may be of therapeutic value for patients with breast cancer treated with endocrine therapy.

INTRODUCTION

Human breast cancer can be clustered into subtypes including basal, luminal, and ERBB2⁺ tumors (among others) based on gene expression profiling (Sørlie et al., 2001). Basal-like breast cancer is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and ERBB2/HER2 and is commonly more aggressive and invasive, whereas luminal breast cancer is typically ER⁺ and is generally associated with a better prognosis. ER regulates gene expression in cooperation with coactivator or corepressor proteins and drives breast cancer cell survival and growth. This dependence is the basis for the treatment of ER⁺ luminal tumors with endocrine therapies including aromatase inhibitors that reduce estrogen levels and direct ER antagonists such as tamoxifen and fulvestrant (Osborne and Schiff, 2011). However, the long-term efficacy of

these treatments is diminished by recurrence of resistant tumors that have lost dependence on estrogen for growth. Although some of the mechanisms underlying this acquired resistance have been identified, including altered ER regulation and increased HER2 activity, identification of other pathways is essential to inform the design of additional therapies for these patients.

The gene expression differences between basal and luminal breast cancers represent distinctions in the expression of lineage markers identified in the two types of normal mammary epithelial cells: basal myoepithelial cells surrounding the branching ductal structures, and ductal luminal epithelial cells that generate milk-producing alveolar cells during pregnancy. These cells arise from a common multipotent stem/progenitor cell through a process of lineage commitment (Stingl et al., 2005). Altered regulation of developmental pathways has been

Significance

ER is the defining transcription factor of luminal breast tumors, and endocrine agents that target ER are well-established standards of care in breast cancer. However, intrinsic and acquired resistance limit the success of this therapeutic strategy, highlighting the need to identify additional pathways critical for luminal tumor growth and recurrence. Our findings provide evidence that prostate-derived ETS factor (PDEF) can drive luminal differentiation of basal mammary epithelial cells, regulate the survival of luminal tumor cells, and contribute to endocrine resistance. These findings suggest that increased PDEF expression may play a role in tumor recurrence following endocrine therapy and may be a clinically useful target for the treatment of patients with luminal breast cancer.

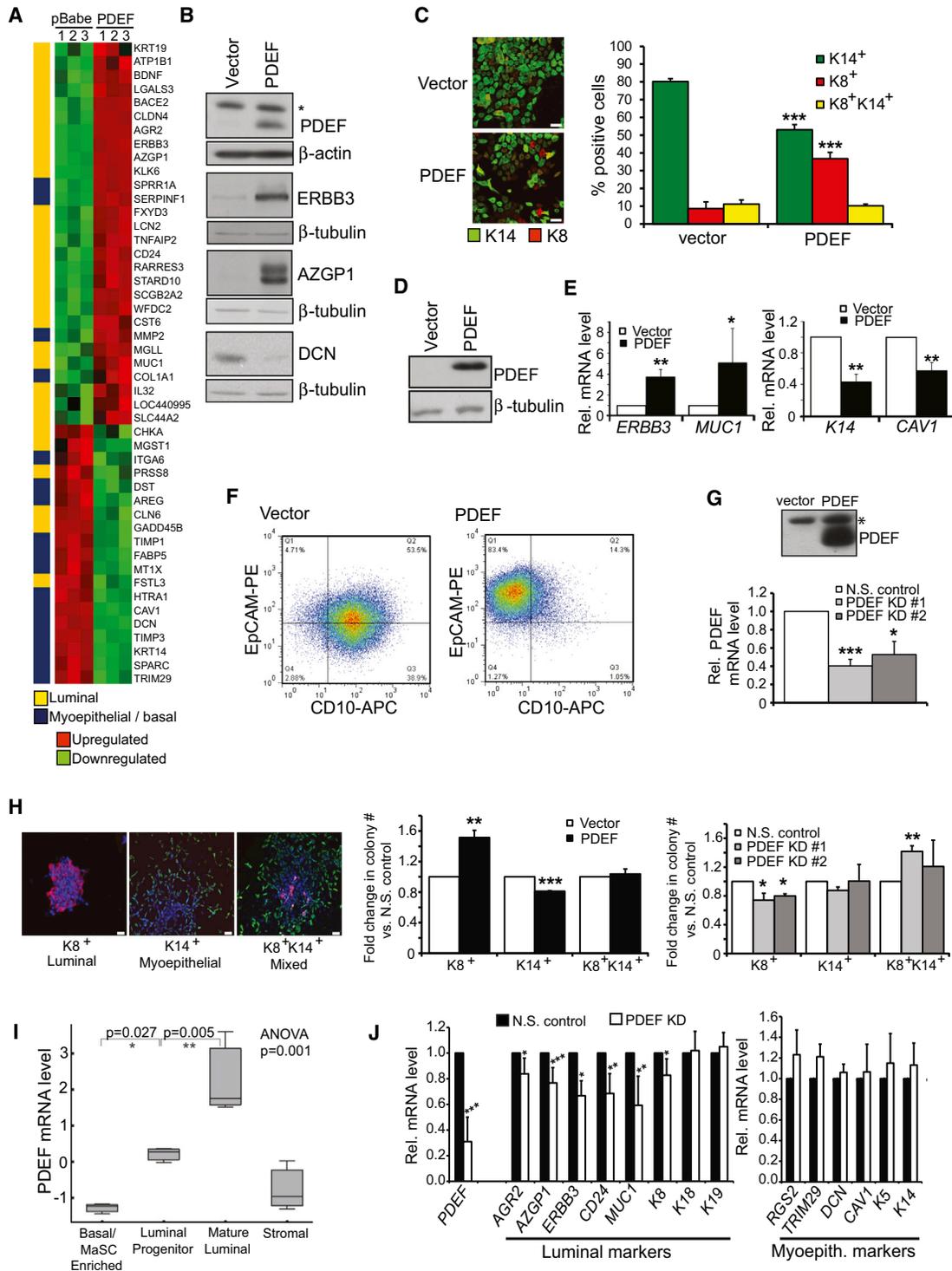


Figure 1. PDEF Induces a Luminal Lineage-Specific Gene Expression Profile in Non-Transformed Mammary Epithelial Cells

(A) Heatmap generated from microarray analysis of triplicate samples of MCF-10A cells expressing either vector control (pBabe) or PDEF. Differentially expressed mammary epithelial marker genes are shown. These expression changes were statistically significant ($p = 4.14 \times 10^{-14}$ by Fisher's exact test).

(B) Western blot analysis of vector control or PDEF-expressing MCF-10A cells (*NS band).

(C) Immunofluorescent staining for keratins 8 and 14 in MCF-10A cells. Representative images of vector control and PDEF-expressing cells are shown. Scale bars represent 100 μ M. Quantitation is shown in graph. Data represent mean \pm SEM (** $p < 0.0007$).

(D) Western blot confirming PDEF overexpression in HMECs.

(E) Real-time PCR analysis of luminal (left) and basal (right) marker gene expression in HMECs overexpressing PDEF. Results were normalized to the housekeeping gene *RPLP0* and are presented as mean fold change \pm SD (* $p < 0.05$, ** $p < 0.008$).

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proposed to play an important role in tumorigenesis and to contribute to the observed heterogeneity in breast cancer (Dontu et al., 2003). Identification of other factors that regulate mammary gland differentiation is important for understanding the mechanisms of breast cancer initiation and progression and for developing targeted treatments for each tumor subtype. Several transcription factors involved in both mammary development and breast cancer progression have been identified, including GATA3, which promotes luminal epithelial differentiation and exerts a tumor-suppressive function by sustaining differentiation and inhibiting metastasis (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2008).

Members of the ETS family, such as ELF3, ELF5, and ETV4, have also been implicated in mammary development (Shepherd and Hassell, 2001) and are overexpressed in breast cancer, supporting a role in epithelial tumorigenesis (Galang et al., 2004; Kalyuga et al., 2012). Unlike most ETS proteins, the expression of prostate-derived ETS factor (PDEF/SPDEF), which was first identified as an activator of prostate specific antigen, (Oettgen et al., 2000) is largely restricted to epithelial tissues including the lung, stomach, colon, and hormone-regulated epithelia such as the prostate, breast, and ovary. In these tissues, PDEF mediates epithelial cell fate decisions and secretory cell differentiation (Gregorieff et al., 2009; Park et al., 2007); however, it is unclear whether PDEF functions similarly in the mammary gland. There is also little known about the regulation of PDEF expression apart from miRNA-mediated suppression in basal breast cancer cell lines (Findlay et al., 2008).

PDEF also appears to play a role in regulating tumor growth and loss of PDEF is associated with a more aggressive phenotype in prostate and colon cancer (Gu et al., 2007; Moussa et al., 2009). However, the role of PDEF in breast cancer is controversial, as several studies have demonstrated that PDEF expression is lost in invasive basal breast cancer cell lines, and that its re-expression inhibits the growth and migration of these cells, supporting a tumor-suppressive function (Feldman et al., 2003; Turner et al., 2007). In contrast, PDEF cooperates with known oncogenes to stimulate MCF-10A cell transformation (Gunawardane et al., 2005) and is one of the most highly overexpressed genes in human and mouse mammary tumors and in lymph node metastases (Galang et al., 2004; Ghadersohi and Sood, 2001). Furthermore, PDEF expression is enriched in luminal tumors and correlates with poor overall survival in ER⁺ breast cancer patients, suggesting instead a

possible oncogenic role (Sood et al., 2007, 2009; Gunawardane et al., 2005).

In this study we sought to investigate and clarify the role of PDEF in both normal mammary gland development and breast cancer. We hypothesize that PDEF may be important for the differentiation of normal mammary luminal cells and that this transcription factor may function differentially in luminal versus basal breast tumors.

RESULTS

PDEF Induces a Luminal Epithelial Gene Expression Program in Mammary Epithelial Cells

To investigate PDEF function in the mammary gland, we examined the effect of this transcription factor on gene expression using microarray-based profiling of MCF-10A cells. These cells are nontransformed mammary epithelial cells that express protein and gene expression programs of basal epithelial cells (Neve et al., 2006) and undetectable levels of endogenous PDEF (Gunawardane et al., 2005). Bioinformatics analysis of the genes induced or repressed by PDEF overexpression in MCF-10A cells revealed a striking effect on expression of luminal and myoepithelial cell markers. The heat map in Figure 1A shows the effects of PDEF overexpression on genes previously identified to be most specifically expressed in purified populations of primary luminal (left column, yellow) or myoepithelial cells (left column, blue) (Allinen et al., 2004; Jones et al., 2004). PDEF overexpression induced a switch in gene expression, resulting in the induction of luminal markers such as *ERBB3*, *MUC1*, and *AZGP1* and the concomitant suppression of myoepithelial marker genes, including *KRT14*, *CAV1*, and *DCN*. A complete list of the significantly regulated genes from the microarray is shown in Table S1 (available online).

These results were validated in MCF-10A cells at the mRNA level using quantitative real-time PCR (Figures S1A and S1B) and at the protein level by western blot (Figure 1B). Expression of PDEF also resulted in a shift to a luminal, EpCAM⁺CD10⁻ phenotype (76.5% versus 45.9% in vector control cells) as determined by flow cytometry surface marker analysis (Figure S1C), as well as a significant increase in luminal keratin-8⁺ (K8) cells with a simultaneous decrease in the percentage of myoepithelial keratin-14⁺ (K14) cells (Figure 1C). In addition, PDEF expression in a second, independent cell line, non-transformed human mammary epithelial cells (HMECs) (Figure 1D), also resulted in

(F) Flow cytometry surface marker analysis of HMECs using antibodies for the luminal gene EpCAM and the myoepithelial gene CD10. A representative experiment is shown; similar results were obtained in two independent experiments.

(G) PDEF overexpression in COMMA-1D murine mammary epithelial cells was detected by western blot (top). PDEF downregulation was detected by real-time PCR (bottom); two independent shRNAs (PDEF KD: KnockDown #1 and #2) were used and compared to a NS shRNA control. Results were normalized to the housekeeping gene *RPLP0* and are presented as mean fold change \pm SEM (* $p < 0.02$, *** $p < 0.0005$).

(H) COMMA-1D cells infected with lentivirus to modulate PDEF expression were plated on a layer of irradiated fibroblast feeder cells for colony formation assays. Colonies were scored based on K8 (red) and K14 (green) staining and categorized into three types of colonies: K8⁺ luminal, K14⁺ myoepithelial, or K8⁺K14⁺ mixed colonies. Representative images of each type of colony are shown (left), scale bars represent 100 μ M. Quantitation of PDEF-overexpressing cells (middle, \pm SEM) and PDEF-downregulated cells (right, \pm SD) are shown as the average fold change in colony number across three experiments compared to vector or NS control cells (* $p < 0.05$, ** $p < 0.006$, *** $p < 0.0001$).

(I) Box plot displaying PDEF mRNA levels in MaSC, luminal progenitors, mature luminal cells, and stromal cells derived from gene expression analysis of purified human mammary epithelial subpopulations (Lim et al., 2009).

(J) Relative mRNA expression levels of luminal (left) and myoepithelial (right) markers in luminal progenitor cells transduced with PDEF-targeting shRNA (KD) or NS shRNA. Cells were harvested 5 days after infection. Bars represent fold change \pm SD of two independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

See also Figure S1 and Table S1.

induction of luminal gene expression and inhibition of myoepithelial marker genes (Figure 1E) and a shift to a luminal, EpCAM⁺ CD10⁻ phenotype (83.4% versus 4.71% in vector control cells) (Figure 1F).

To further validate the possibility that PDEF regulates luminal differentiation, we examined the effect of modulating PDEF expression in a murine mammary epithelial cell line, COMMA-1D cells. These cells are derived from a mid-pregnant mouse and contain a population of stem/progenitor cells that are capable of both in vitro differentiation and mammary gland repopulation in transplantation assays (Danielson et al., 1984). Endogenous PDEF levels in these cells are low but can be detected using real-time PCR (Figure 1G). COMMA-1D cells were infected with lentiviral vectors expressing either PDEF cDNA or shRNAs targeting PDEF and then assayed for colony formation and scored for K8 and K14 staining (Figure 1H, left). PDEF mRNA was efficiently downregulated with each of two independent shRNAs (PDEF KnockDown #1 and #2) compared to a nonspecific (NS) shRNA control. Similar to the effects of PDEF on keratin expression in MCF-10A cells, PDEF overexpression in COMMA-1D cells led to enhanced formation of K8⁺ luminal colonies and a reduction in K14⁺ colonies (Figure 1H, middle). In contrast, downregulation of PDEF expression decreased the formation of K8⁺ luminal colonies and resulted in an increased proportion of K8⁺K14⁺ mixed colonies (Figure 1H, right), suggesting a block in luminal differentiation and the accumulation of bipotent double-positive colonies in the absence of PDEF.

In addition, gene expression analysis of FACS-sorted primary human breast epithelial subpopulations (Lim et al., 2009) revealed that PDEF mRNA levels are enriched in the CD49f⁺ EpCAM⁺ luminal progenitor cell population and are further enriched in CD49f⁺ EpCAM⁺ mature luminal cells, as compared to the basal/mammary stem cell-enriched (Basal/MaSC) and stromal fibroblast populations (Figure 1I). To determine whether PDEF regulates the expression of luminal and myoepithelial differentiation markers, we used this same FACS-sorting approach to purify mammary epithelial subpopulations from a reduction mastectomy specimen (Figures S1D and S1E). Although sorted mature luminal cells could not be cultured, we were able to successfully grow luminal progenitor cells in vitro and evaluated the expression of several luminal and myoepithelial markers after PDEF silencing in these cells (Figure 1J). Interestingly, PDEF knockdown significantly decreased the expression of multiple luminal markers, whereas myoepithelial marker expression was not significantly affected. These results further support a role for PDEF in driving the expression of luminal-specific genes and promoting the differentiation of this subset of mammary epithelial cells.

PDEF Is Co-Expressed with ER and Is Regulated by ER-Cooperating Factors GATA3 and FOXA1

PDEF mRNA is significantly overexpressed in ERBB2⁺ and luminal A and B tumors in two independent data sets, as compared to basal-like tumors (Figure 2A). This correlation with the luminal subtype was also confirmed in breast cancer cell lines (Figure 2B). Because ER expression is the major molecular determinant of luminal tumors (Perou et al., 2000), PDEF is significantly overexpressed in ER⁺ tumors (Figure 2C). Interestingly, PDEF is the only ETS transcription factor displaying a

positive correlation with ER expression among the 28 members of the family (Figure 2D). This correlation suggests that ER may regulate PDEF transcription.

To determine if ER regulates PDEF expression, we analyzed a previously established ER cistrome data set (Carroll et al., 2006) and found three ER-binding regions located in the PDEF gene locus (Figure 3A). Two enhancers (1 and 2) are located within the first intron of the gene and the third one (3) is located after the 3'-end of the gene. Direct ER chromatin immunoprecipitation (ChIP) in MCF7 cells showed an estradiol (E2)-induced recruitment of ER at these three enhancers, thus confirming PDEF as a direct ER target.

However, a time course analysis of MCF7 cells treated with increasing E2 doses did not reveal PDEF mRNA induction, in contrast with the strong increase of PR mRNA, a known direct ER target (Figure 3B). Direct RNA polymerase II (Pol2) ChIP corroborated the absence of PDEF induction by E2. Pol2 recruitment at the PDEF promoter was not increased after E2 treatment, whereas its recruitment at the PR promoter was strongly induced (Figure 3C). We investigated whether the three enhancers could be responsible for the activation of the genes adjacent to PDEF (PACSL1 and c6orf106, Figure S2A). However, neither of these neighboring genes demonstrated a correlation with ER expression in breast carcinomas (Figure S2B), and E2 stimulation did not induce their expression (Figure S2C). The absence of PDEF mRNA induction by E2 was further validated in another ER⁺ breast cancer cell line (T47D, Figure S2D). In addition, ER downregulation in MCF7 cells did not affect PDEF mRNA levels (Figure 3D), further confirming the absence of regulatory activity, despite direct ER recruitment at the PDEF gene locus in an E2-dependent manner.

These results suggested that a potential repressor prevents transactivation of the PDEF gene by ER. Therefore, we examined the roles of the known ER-cooperating factors FOXA1 (Lupien et al., 2008) and GATA3 (Theodorou et al., 2013) as potential regulators of PDEF expression. FOXA1 downregulation in MCF7 cells led to a decrease in PDEF mRNA and protein levels, indicating a positive role of FOXA1 in the regulation of PDEF expression (Figure 3D). FOXA1 downregulation in another luminal cell line (SKBR3) also decreased PDEF mRNA levels (Figure S2E). In contrast, GATA3 downregulation in MCF7 cells triggered an increase of PDEF mRNA and protein levels (Figure 3D), demonstrating a negative role of GATA3 in the regulation of PDEF expression. Additionally, GATA3 overexpression in two luminal cell lines expressing low endogenous GATA3 levels resulted in decreased PDEF expression (Figure S2F). These regulatory effects occur through direct recruitment of FOXA1 and GATA3 at the PDEF locus in MCF7 cells because direct ChIP-qPCR revealed FOXA1 recruitment at the PDEF promoter and enhancer 3, whereas GATA3 was recruited at the most proximal enhancer (Figure 3E). These data suggest that PDEF is a downstream target of ER, FOXA1, and GATA3. As a control, we verified that PDEF downregulation did not affect the expression of ER, FOXA1, or GATA3, indicating that there is no reciprocal regulatory loop between these genes (Figure S2G).

GATA3 Prevents ER-Mediated Induction of PDEF

As a direct PDEF repressor, GATA3 could be responsible for the absence of E2-mediated induction of PDEF mRNA. To test this

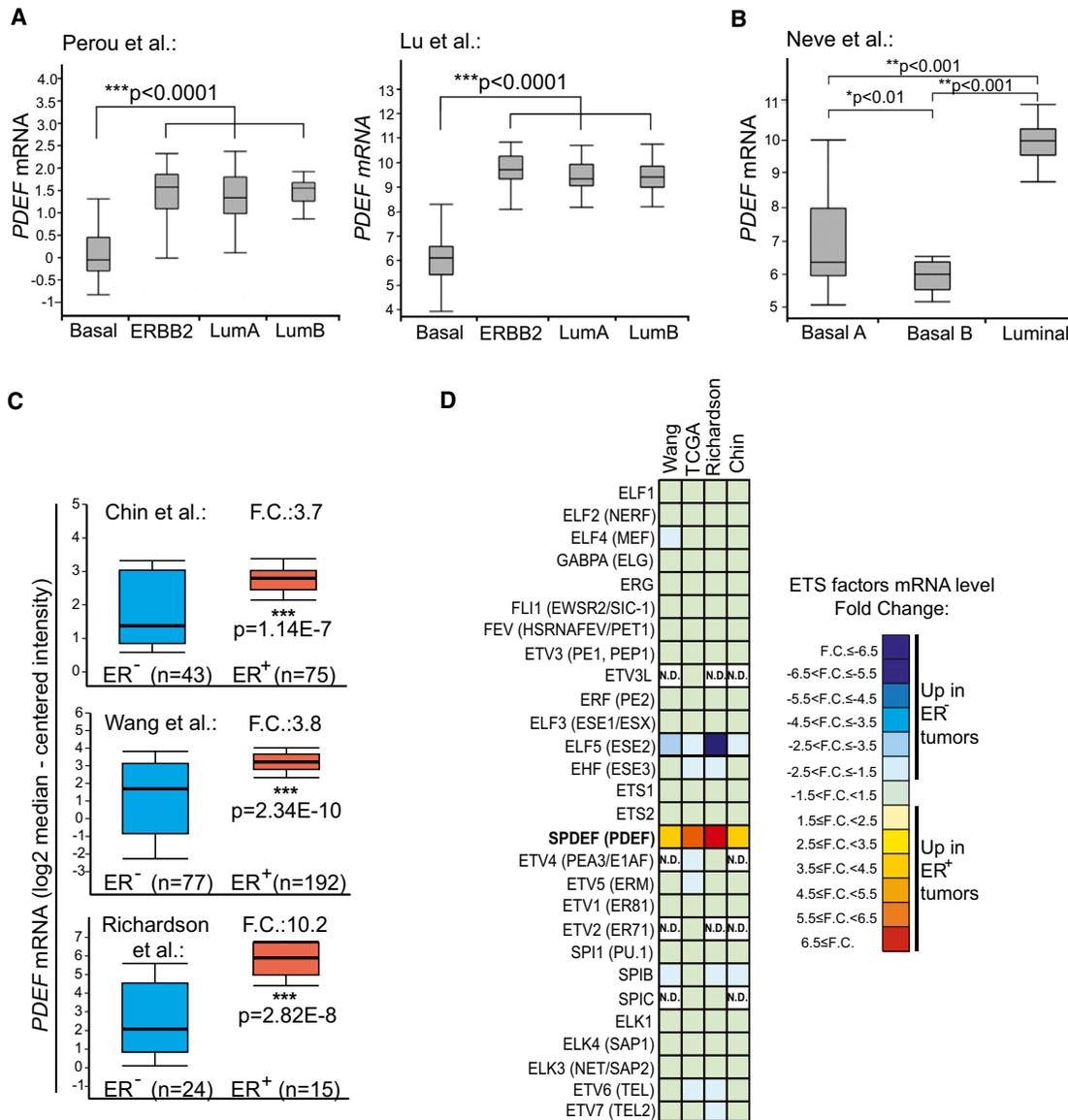


Figure 2. PDEF Is Enriched in Luminal Tumors and Strongly Correlates with ER Expression

(A and B) Box plots displaying *PDEF* mRNA levels between Basal-like, ERBB2⁺, and Luminal (Lum) subtypes of breast cancer (A) and breast cancer cell lines (B) using published microarray data sets (Lu et al., 2008; Perou et al., 2000; Neve et al., 2006).

(C) Box plots displaying *PDEF* mRNA levels between ER⁻ and ER⁺ breast carcinoma (F.C., fold change) in published microarray data sets (Chin et al., 2006; Richardson et al., 2006; Wang et al., 2005).

(D) Heatmap displaying the relative expression levels of the 28 ETS transcription factors in ER⁻ and ER⁺ breast cancer subtypes (Wang et al., 2005; Richardson et al., 2006; Chin et al., 2006) (N.D., not determined).

hypothesis, we compared the effect of E2 treatment in the presence or absence of GATA3 expression (Figure 4A). As expected, *PDEF* mRNA levels were unchanged in MCF7 cells transfected with a non-specific siRNA (NS). In contrast, GATA3 knockdown enabled the induction of *PDEF* mRNA (Figure 4A) and protein (Figure 4B) by E2 treatment. We then examined whether modulation of GATA3 expression affected the recruitment of various transcriptional regulators to the *PDEF* promoter and enhancer regions using CHIP in E2-treated MCF7 cells (Figure 4C). Indeed, Pol2 displayed a higher recruitment level at the *PDEF* promoter in GATA3-knockdown conditions, whereas the promoter of the ER

target gene *MYC* did not show differential Pol2 recruitment. ER recruitment to the three enhancers was also increased upon GATA3 depletion, with a more pronounced increase at enhancer 1, in contrast to the ER-recruiting enhancer located upstream of the *MYC* gene, which did not show differential ER recruitment. In the absence of estrogen stimulation (vehicle condition), ER recruitment at the *PDEF* enhancers was close to background level, and modulation of GATA3 expression did not affect ER recruitment (Figure S3). Furthermore, the transcriptional coactivator p300, known to associate with ligand-bound ER and to augment ligand-dependent activation by ER (Hanstein et al.,

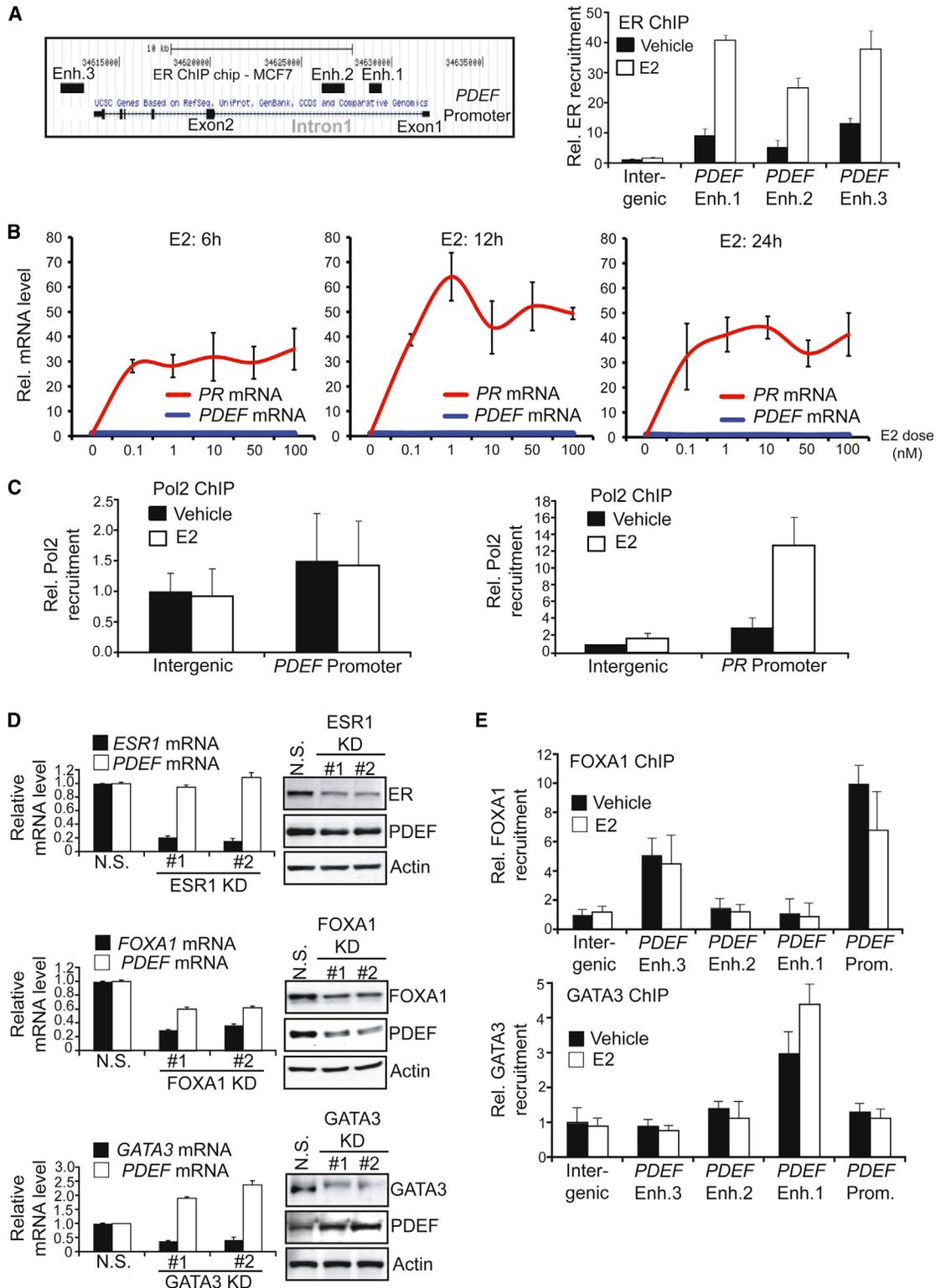


Figure 3. PDEF Is Not Directly Regulated by ER, but Is Activated by FOXA1 and Repressed by GATA3

(A) Left: Schematic diagram of the ER-binding regions within the *PDEF* gene locus as defined by ER ChIP on chip in MCF7 cells (Carroll et al., 2006). Enh., enhancer. Right: direct ER chromatin immunoprecipitation (ChIP) followed by qPCR after treatment of MCF7 cells with vehicle (black bars) or 10 nM estradiol (E2, white bars). Data represent means \pm SD.

(B) *PDEF* and *PR* mRNA levels were determined by real-time PCR after MCF7 cells were treated with increasing E2 doses for 6 hr (left), 12 hr (middle), and 24 hr (right). mRNA levels are presented as means \pm SD and normalized to the housekeeping gene *GAPDH*.

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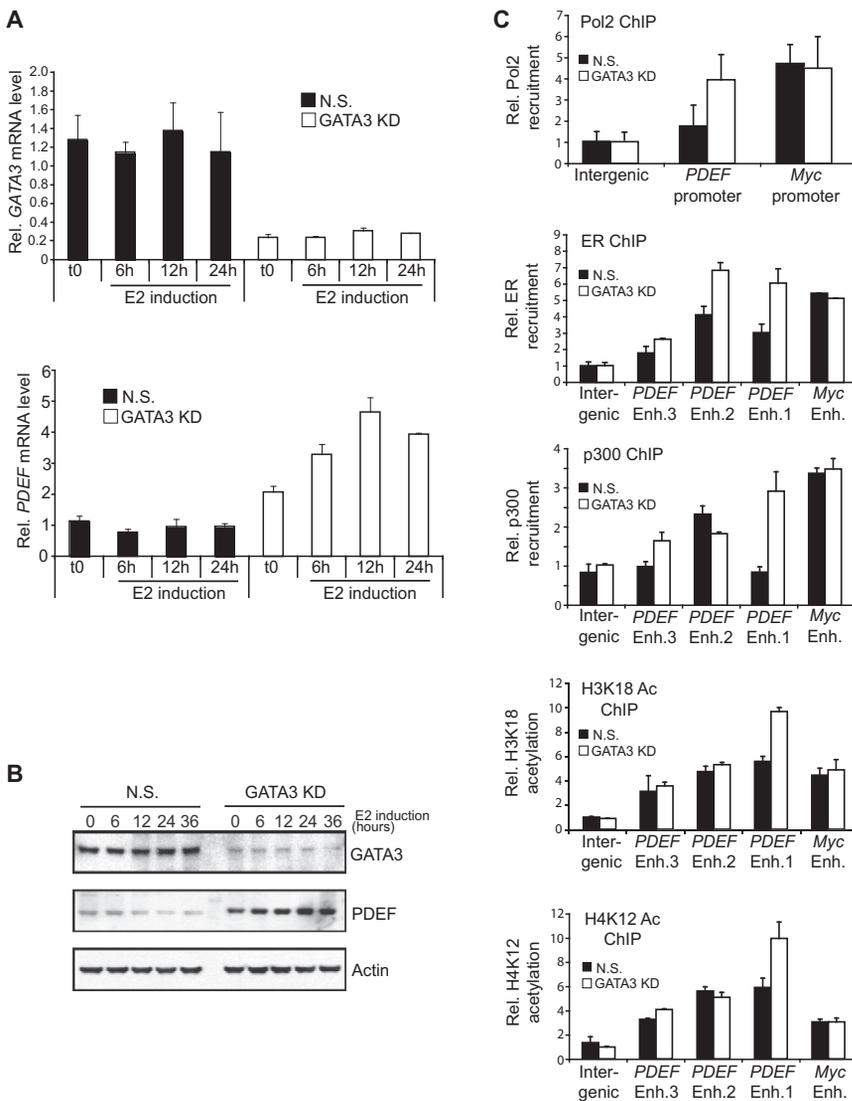


Figure 4. GATA3 Prevents PDEF Induction by ER

(A) *GATA3* (upper) and *PDEF* (lower) mRNA levels were determined by RT-qPCR after MCF7 cells were transfected with a NS or *GATA3*-targeting (KD, KnockDown) siRNAs and challenged with 10 nM estradiol (E2). Data represent means \pm SD. (B) Western blot lysates from MCF7 cells transfected with NS or *GATA3*-targeting (KD) siRNAs, and challenged with 10 nM estradiol.

(C) Direct RNA polymerase II (Pol2), ER, p300, acetylated histone H3K18, and H4K12 ChIPs followed by real-time PCR after MCF7 cells were transfected with NS or *GATA3*-targeting (KD) siRNAs, and challenged with 10 nM E2. Data represent means \pm SD. Enh., enhancer. See also Figure S3.

analysis showed a statistically significant inverse correlation between *PDEF* and *GATA3*. Conversely, *PDEF* and *FOXA1* levels were positively correlated, further confirming the positive regulation of *PDEF* expression by *FOXA1*. Validation of the negative correlation between *PDEF* and *GATA3* expression was also performed in four ER⁺ breast cancer cell lines (Figure 5B).

PDEF Is Required for the Growth of ER⁺ Breast Cancers

To investigate the role of *PDEF* in breast cancer progression, we first analyzed gene expression profiling and clinical outcome data from published studies for correlations between *PDEF*/*GATA3* expression levels and overall survival (Figure 5C). Due to the strong association between *PDEF* and ER expression, only

ER⁺ breast tumors were included in this analysis. Patients were divided into two equal groups, high and low, based on the median *PDEF* expression level. Kaplan-Meier survival analyses in three independent data sets demonstrated that high *PDEF* expression is associated with worse overall survival for patients with ER⁺ breast cancer. We found that *PDEF* is a significant predictor of survival when treated as a continuous variable in Cox regression models (Chin $p = 0.0102$, Ivshina $p = 3.74 \times 10^{-3}$, Desmedt $p = 0.0476$), consistent with a previous report (Sood et al., 2009). In contrast, *GATA3* is not a significant predictor of survival in the ER⁺ breast samples in these three studies when tumors are classified based on the median *GATA3* expression or when *GATA3* expression is treated as a continuous variable

1996), also displayed a stronger recruitment in *GATA3*-knock-down conditions, specifically at enhancer 1. Consistent with the histone acetyltransferase activity of p300 (McManus and Hendzel, 2003), we found higher levels of the active histone marks acetylated-H3K18 and -H4K12 at *PDEF* enhancer 1. Collectively, these data demonstrate that the loss of *GATA3* allows for ER-mediated *PDEF* induction.

To evaluate the repressive effect of *GATA3* on *PDEF* expression in other cell lines, we examined the mRNA levels of these transcription factors in a subset of ER⁺ breast cancer cell lines (Neve et al., 2006). Clustering analysis revealed a clear distinction between [*GATA3*]^{high} [*PDEF*, *FOXA1*]^{low} and [*GATA3*]^{low} [*PDEF*, *FOXA1*]^{high} cell lines (Figure 5A). Pairwise correlation

(C) Direct RNA polymerase II (Pol2) ChIP followed by qPCR after treatment of MCF7 cells with vehicle or 10 nM E2 using primers flanking the *PDEF* promoter (left) and *PR* promoter (right). Data represent means \pm SD.

(D) *PDEF*, *ESR1*, *FOXA1*, and *GATA3* mRNA and protein levels were determined by RT-qPCR (left panels) or western blot (right panels) after MCF7 cells were transfected with a NS or two independent siRNAs targeting *ESR1*/*FOXA1*/*GATA3* (KD, KnockDown). Data represent means \pm SD.

(E) Direct *FOXA1* (upper) and *GATA3* (lower) ChIPs followed by qPCR after treatment of MCF7 cells with vehicle or 10 nM E2. Enh., enhancer; Prom., promoter. Data represent means \pm SD.

See also Figure S2.

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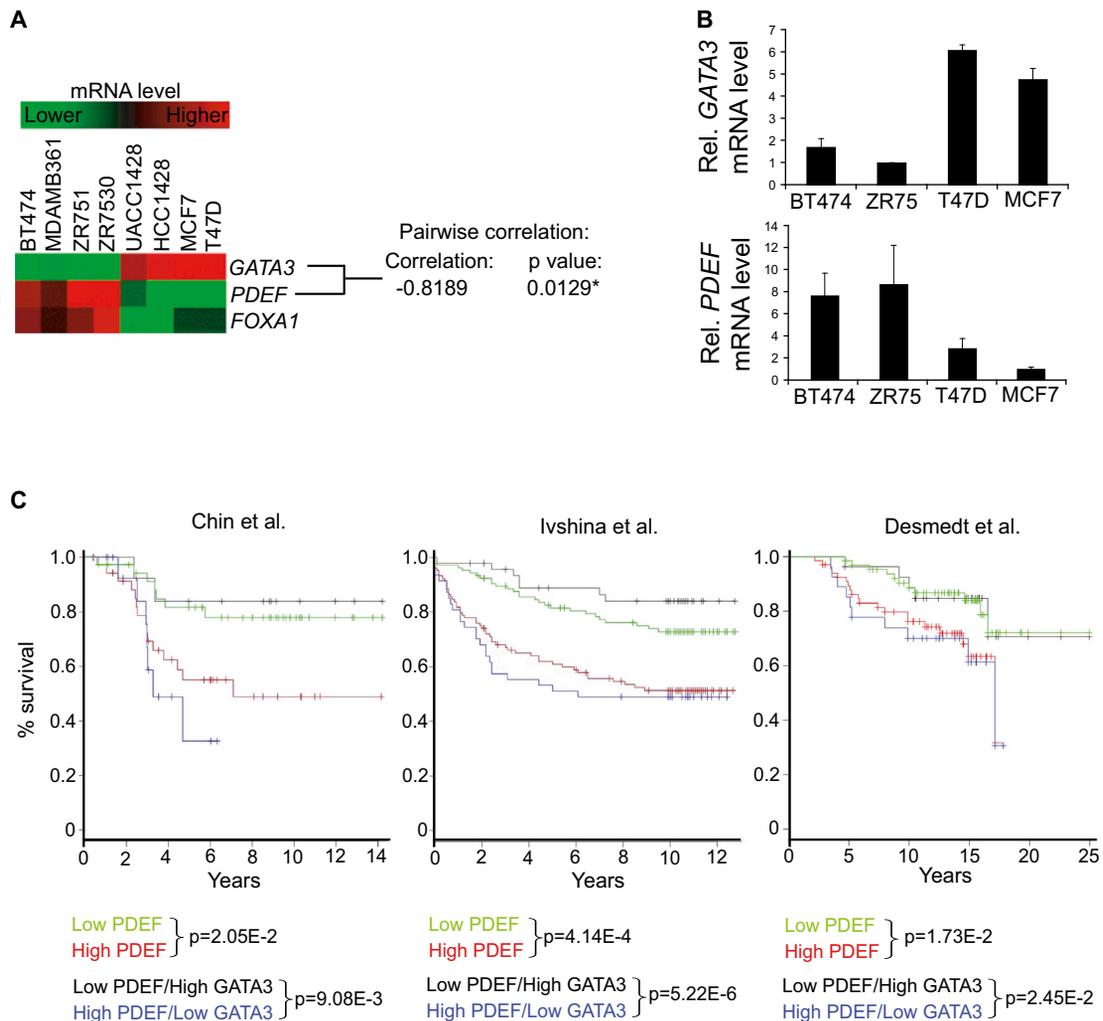


Figure 5. PDEF and GATA3 Are Inversely Correlated in ER⁺ Luminal Tumor Cell Lines, and Their Expression Levels Are of Prognostic Value for ER⁺ Breast Carcinoma Patient Survival

(A) Heatmap displaying *GATA3*, *PDEF*, and *FOXA1* mRNA levels in eight ER⁺ breast cancer cell lines (Neve et al., 2006) with pairwise correlation analysis. Scale: +1, perfect positive correlation; 0, no correlation; -1: perfect inverse correlation.

(B) *GATA3* (upper) and *PDEF* (lower) mRNA levels were determined by real-time PCR in four ER⁺ breast cancer cell lines. Data represent means \pm SD.

(C) Kaplan-Meier survival analysis of patients with ER⁺ breast carcinoma (Chin et al., 2006; Ivshina et al., 2006; Desmedt et al., 2007). Patients were stratified on the median value for each marker. The log-rank test p values are shown.

(data not shown). Interestingly, further stratification of patient groups based on inverse *GATA3/PDEF* expression improves the predictive capability of *PDEF* (Figure 5C). The significant correlation between high *PDEF* expression and poor overall survival strongly supports a potential role for *PDEF* in ER⁺ breast tumorigenesis.

To assess this hypothesis, we next investigated the biologic effects of *PDEF* knockdown on luminal breast cancer cell growth. *PDEF* downregulation by transient transfection with two independent siRNAs (Figure 6A) triggered a growth defect in MCF7 cells subjected to hormone starvation prior to E2 stimulation (Figure 6B). This growth defect was rescued by the expression of a mutant *PDEF* protein harboring a silent mutation preventing siRNA-mediated downregulation (Figures S4A and S4B). Stable *PDEF* knockdown by transduction of MCF7 cells

with lentiviral *PDEF*-targeting shRNAs also induced a growth defect in conditions of hormone depletion followed by E2 stimulation (Figures S4C and S4D).

Flow cytometry analysis did not reveal any obvious cell-cycle progression defect in *PDEF* knockdown cells subjected to hormone starvation and E2 induction. However, *PDEF* knockdown followed by treatment with nocodazole prevented a significant proportion of cells from entering mitotic arrest, suggesting that *PDEF* silencing does induce a subtle G0/G1 arrest (Figure S4E). In addition, *PDEF* knockdown cells subjected to hormone depletion displayed decreased viability with a statistically significant increase in apoptotic cells (Figure 6C). Based on this finding, we examined the effect of the ER antagonists tamoxifen and fulvestrant in cells expressing *PDEF* siRNA (Figure 6D). Increasing doses of anti-estrogens resulted in more pronounced

cytotoxicity in PDEF knockdown conditions. In addition, caspase 8 activity was also increased in MCF7 cells expressing PDEF-targeting shRNAs and subjected to hormone depletion or fulvestrant treatment (Figure S4F), further confirming a role for PDEF in mediating luminal tumor cell survival in response to hormone-related stress.

We also examined the effect of PDEF loss on luminal tumor cell survival in anchorage-independent conditions. Soft agar colony formation was markedly decreased in MCF7 cells expressing PDEF shRNA, suggesting that this ETS factor is essential for the transformation of luminal tumor cells (Figure 6E). Consistent with this notion, PDEF downregulation in three other luminal cancer cell lines, T47D, BT474, and SKBR3 (Figure S4G), resulted in similar decreases in cell growth (Figures S4H–S4J) and significantly impaired colony formation in soft agar assays (Figures S4K and S4L). As a control, we demonstrated that re-introduction of PDEF in the basal tumor cell line SUM159, which lacks endogenous PDEF expression, inhibited anchorage-independent growth and survival (Figure S4M), confirming the reported role of PDEF as a tumor suppressor in basal-like breast cancer (Feldman et al., 2003; Turner et al., 2007). To further test the role of PDEF in luminal tumor formation, NS control or PDEF knockdown MCF7 cells were injected in the inguinal mammary fat pads of NOD-SCID mice that had previously been implanted with an estrogen pellet (Figure 6F). In line with the observed in vitro growth defect, PDEF downregulation reduced tumor growth, with a 40% decrease in final tumor weight compared to tumors formed by control MCF7 cells. Interestingly, histological sections of PDEF knockdown tumors displayed a fragmented appearance and decreased cellularity, in contrast to the suppressive role of PDEF in basal SUM159 cells, because expression of PDEF in these cells diminished tumor formation in vivo (Figure S4N). Western blot analysis of lysates from PDEF knockdown tumors revealed increased detection of cleaved caspase 8, whereas the proliferation marker PCNA was not differentially expressed (Figure 6G), indicating that the reduced tumor size of PDEF knockdown xenograft tumors is predominantly due to increased apoptosis rather than a proliferation defect. TUNEL cell death detection in tumor sections also clearly showed increased cell death in PDEF knockdown tumors (Figure 6H). Collectively, these data support a critical prosurvival role of PDEF in ER⁺ breast cancer cells. Conversely, we confirmed a tumor-suppressive function for GATA3 (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2008), because GATA3 silencing promoted soft agar colony formation in MCF7 cells (Figures S4O and S4P).

PDEF Is Involved in Endocrine Resistance

To better understand the mechanisms underlying acquired endocrine resistance, in vitro models of hormone-independent growth were previously developed through long-term culture of MCF7 cells in media devoid of any estrogens or in the presence of tamoxifen (Ariazi et al., 2011). We hypothesized that PDEF might play a functional role in this hormone-refractory phenotype based on our finding that PDEF downregulation sensitized MCF7 cells to hormone depletion and anti-estrogens. To evaluate this hypothesis, we analyzed two long-term estrogen-deprived clones (MCF7 2A and 5C) (Ariazi et al., 2011) and tamoxifen-resistant cells (TAM-R) isolated from parental MCF7 cells.

PDEF mRNA levels were higher in estrogen-deprived 2A and 5C cells relative to the parental controls (WS8), whereas the levels of GATA3 mRNA were lower (Figure 7A). The inverse correlation between PDEF and GATA3 was confirmed in this model at the protein level (Figure 7B). Similarly, TAM-R cells also displayed higher PDEF levels, concomitant with undetectable levels of GATA3 mRNA and protein (Figures 7A and 7B). To determine whether the higher PDEF expression observed in these models was functionally relevant, we assessed the survival of MCF7 5C cells after PDEF downregulation. PDEF knockdown induced increased cell death and a reduction in soft agar colony formation, suggesting that hormone-refractory cells are partially dependent on PDEF for survival (Figure 7C). In addition, PDEF downregulation in MCF7 TAM-R cells triggered a dramatic decrease in soft agar colony formation (Figure 7D). Interestingly, tamoxifen significantly increased colony numbers of TAM-R cells transduced with a NS shRNA (compare white bars), indicating that tamoxifen functions in TAM-R cells as an agonist to promote anchorage-independent cell growth. In fact, PDEF silencing resulted in a greater fold-reduction in colony formation in the presence of tamoxifen compared to the vehicle condition. These results support the conclusion that PDEF plays an important role in endocrine resistance.

PDEF Gene Expression Signature Reflects a Prosurvival Role and Identifies Cell Death Receptor FAS as an Important Target

Microarray gene expression analysis was performed in MCF7 cells transduced with NS or PDEF-targeting shRNAs after subjection to hormone depletion for 48 hr. Analyses of differentially expressed genes combined with gene ontology revealed a downregulation of cell cycle-related genes and an upregulation of apoptosis-related genes in PDEF knockdown cells (Figure 8A). A complete list of PDEF-regulated genes from this microarray analysis is shown in Table S2. These target genes constitute potential effectors of the prosurvival role of PDEF.

We next examined whether PDEF-regulated genes have prognostic value for survival of patients with ER⁺ breast carcinoma (Figure 8B). A semisupervised principal component method was implemented using the PDEF-regulated genes in MCF7 cells; we did not include the PDEF probe because it is prognostic as a single predictor. Using this approach, a 130-probe PDEF signature that is able to predict patient outcome in the Ivshina data set was identified (Figure 8B; Table S3). Importantly, this signature is also significant when applied to independent cohorts of ER⁺ tumors (Figure S5A). Because increased expression of cell cycle genes is a hallmark of cancer, it was necessary to define whether cell cycle genes solely contribute to the prognostic value of the PDEF signature. Survival analysis using a PDEF signature where cell cycle-associated genes were eliminated revealed that non-cell cycle PDEF-regulated genes are prognostic (Figure S5B, left panel). A PDEF signature restricted to genes implicated in response to stress and apoptosis is also prognostic (Figure S5B, right panel).

To further define the clinical relevance of PDEF target genes, we analyzed the overlap between the PDEF signature and existing breast carcinoma gene expression data sets. A large proportion of genes upregulated in PDEF knockdown MCF7 cells were significantly downregulated in tumors versus normal

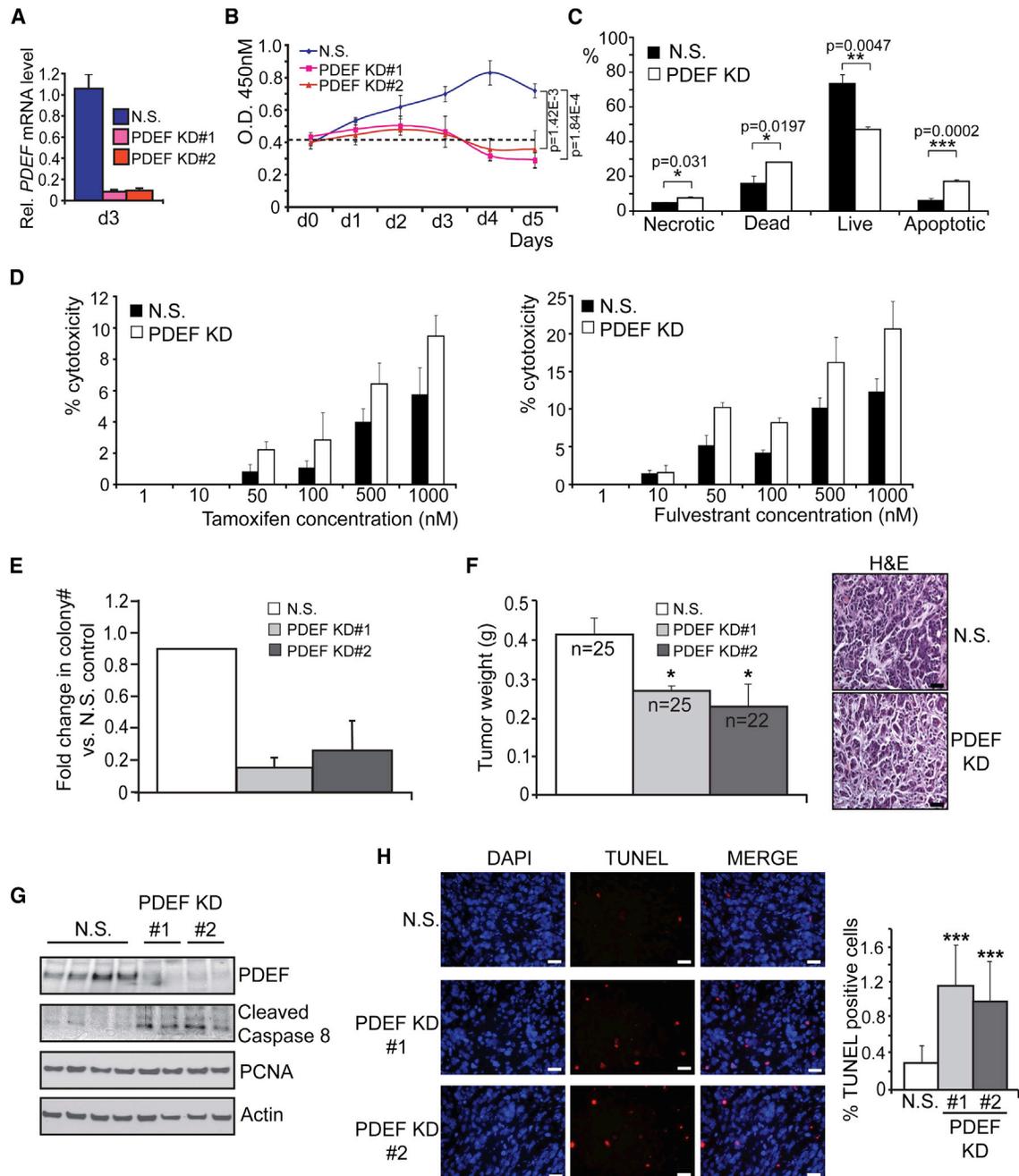


Figure 6. PDEF Knockdown Sensitizes MCF7 Cells to Hormone-Related Stress and Decreases Their Tumorigenic Properties

(A) PDEF mRNA levels were determined by real-time PCR in MCF7 cells transfected with NS or two independent PDEF-targeting (PDEF KD, KnockDown) siRNAs, and then grown for 3 days in hormone-depleted medium with 10 nM E2. Data represent means \pm SD.

(B) Growth curves of MCF7 cells transfected with NS or PDEF-targeting (KD) siRNAs in hormone-depleted medium with 10 nM E2. Data represent means \pm SD.

(C) AnnexinV/Propidium Iodide cell viability measurement of MCF7 cells transfected with NS or PDEF-targeting (KD) siRNAs, then grown for 24 hr in hormone-depleted medium. Data represent means \pm SD.

(D) Measurement of cytotoxicity after transfection of MCF7 cells with NS or PDEF-targeting (KD) siRNAs, followed by treatment for 48 hr with increasing doses of tamoxifen (left) or fulvestrant (right). Data represent means \pm SD.

(E) Soft agar colony formation assay of MCF7 cells infected with NS or PDEF-targeting (KD) shRNAs. The average fold change \pm SEM in colony number compared to control NS cells across three independent experiments is shown (* $p < 0.02$, *** $p < 0.0001$).

(F) Measurement of MCF7 xenograft tumor weight. Mammary fat pads of NOD/SCID mice were injected with NS or PDEF-targeting shRNA (KD #1 and #2) MCF7 cells. Estradiol release pellets were implanted subcutaneously 2–3 days prior to surgery. Tumors were harvested and weighed after 8 weeks of growth. Data represent mean \pm SEM, * $p < 0.04$. Tumor sections were analyzed with hematoxylin and eosin staining. Scale bars represent 50 μ m.

(G) Western blots of MCF7 xenograft tumor lysates. Tumors expressing either NS or PDEF-targeting (KD) shRNAs were analyzed.

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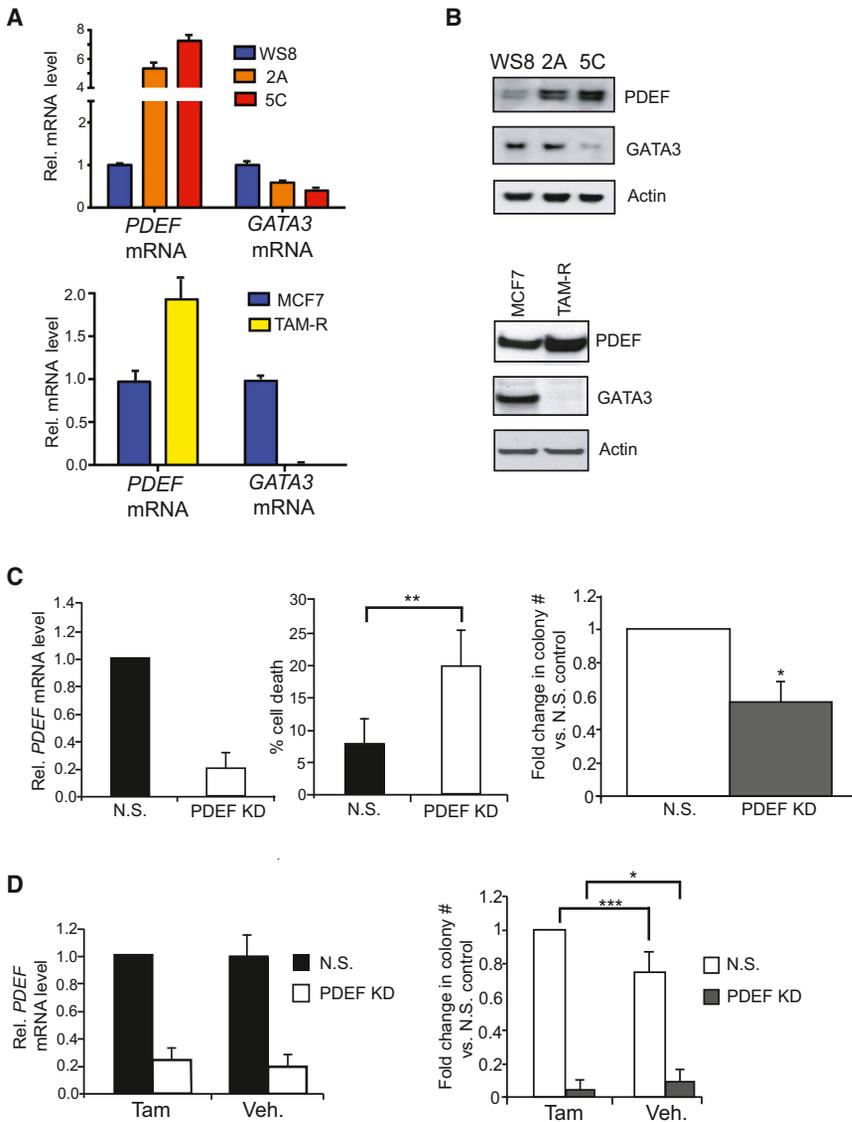


Figure 7. PDEF Expression Is Increased in Long-Term Estrogen-Deprived Cells and Tamoxifen-Resistant Cells Derived from MCF7 Cells

(A and B) PDEF and GATA3 mRNA and protein levels were determined by real-time PCR (A) or western blot (B) in parental MCF7 cells (WS8), clones 2A and 5C (upper panels), and tamoxifen-resistant (TAM-R) cells (lower panels). mRNA levels are presented as means \pm SD and normalized to the housekeeping gene *GAPDH*.

(C) Left: *PDEF* mRNA levels were determined in MCF7 5C cells by real-time PCR after transfection of NS or *PDEF*-targeting (*PDEF* KD, KnockDown) siRNAs. Middle: measurement of MCF7 5C cell mortality (** $p < 0.005$, data represent mean \pm SD). Right: soft agar colony formation assay of MCF7 5C cells infected with NS or *PDEF*-targeting (KD) shRNAs. Data represent the average fold change \pm SEM in colony number compared to control NS cells, * $p < 0.02$.

(D) Left: *PDEF* mRNA levels were determined in MCF7 TAM-R cells by real-time PCR 7 days after transduction of NS or *PDEF*-targeting (KD) shRNAs in the presence of 10^{-7} M tamoxifen or vehicle (EtOH). Right: soft agar colony formation assay of MCF7 TAM-R cells infected with NS control shRNA or *PDEF*-targeting shRNA (KD) in the presence of 10^{-7} M tamoxifen or vehicle (EtOH) (** $p < 0.001$, * $p < 0.05$). Data represent means \pm SD.

samples, whereas the genes downregulated in *PDEF* knockdown MCF7 cells were overexpressed in tumors compared to normal samples (Figure S5C). These findings demonstrate that *PDEF* knockdown triggers a gene expression program that switches cells toward a less tumorigenic phenotype.

In addition, we further analyzed these microarray results to identify potential mediators of the pro-apoptotic phenotype induced by *PDEF* loss. The cell death receptor *FAS* was selected as a candidate effector because *FAS* mRNA was overexpressed in MCF7 cells with *PDEF* knockdown under hormone-depleted conditions (Figures 8C and 8D), suggesting that *PDEF* acts as a repressor of *FAS* expression. Furthermore, analysis of a previously published *PDEF* cistrome established in VCaP prostate cancer cells (Wei et al., 2010) revealed the presence of a *PDEF*

binding region located upstream of the *FAS* gene locus. Direct *PDEF* ChIP in MCF7 cells confirmed *PDEF* recruitment to this binding region (Figure 8E). This result indicates that *PDEF* directly represses *FAS* expression and that this inhibition may promote the survival of MCF7 cells in hormone-depleted media. Interestingly, an inverse correlation between *FAS* and *PDEF* mRNA levels

was observed in multiple ER⁺ breast carcinoma data sets (Figure S5D), thus confirming that *PDEF*-mediated repression of *FAS* expression is also relevant clinically. To determine if *FAS* plays a functional role in the pro-apoptotic phenotype, we treated *PDEF* knockdown MCF7 cells with an anti-*FAS* Ligand (FasL) neutralizing antibody under hormone-depleted conditions (Figure 8F). FasL neutralizing antibody was sufficient to partially rescue the apoptotic phenotype in *PDEF* knockdown cells as compared to a negative control NS antibody. Moreover, *FAS* mRNA and protein expression were enhanced in both MCF7 2A and 5C clones upon *PDEF* downregulation (Figures 8G and 8H). Based on these findings, we hypothesize that increased *PDEF* levels promote the acquired endocrine resistance of estrogen-deprived

(H) Tumor sections were subjected to TUNEL cell death staining (red). Nuclei were stained with DAPI (blue). Three different tumors were stained for each shRNA (NS, *PDEF* KD#1 and #2) and five fields were photographed for each section. Representative images for each tumor type are shown. Scale bars represent 20 μ M. The ratio of TUNEL signal to the total number of nuclei is shown in the histogram. Data represent means \pm SD (** $p < 0.001$). See also Figure S4.

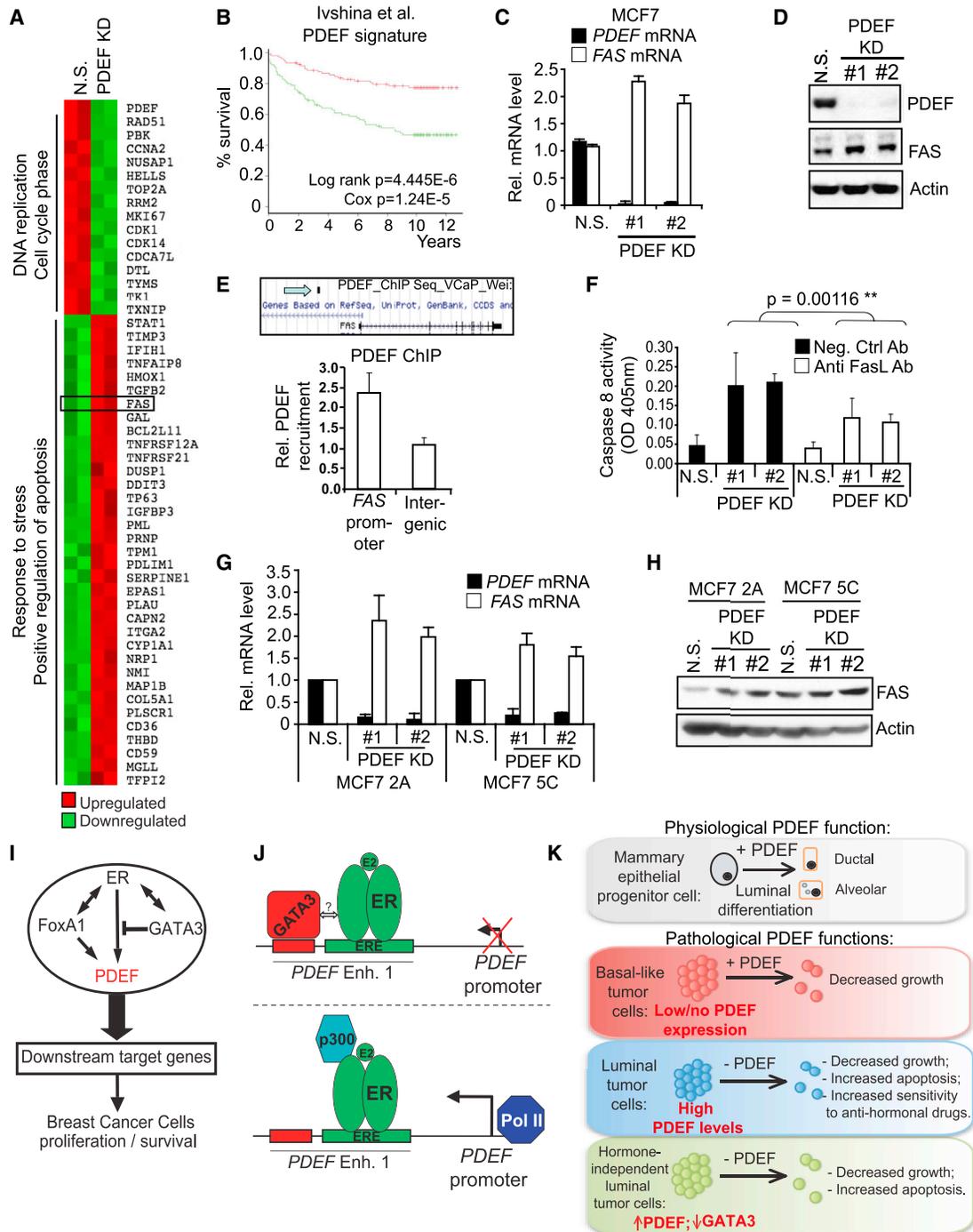


Figure 8. PDEF Promotes a Pro-Survival Gene Expression Program in Hormone-Depleted Conditions and Represses Pro-Apoptotic Death Domain FAS Receptor Expression

(A) Heatmap of a selection of differentially expressed genes generated from microarray analysis of MCF7 cells infected with NS or PDEF-targeting (PDEF KD, KnockDown) shRNAs, then subjected to hormone depletion for 48 hr. These expression changes were statistically significant.

(B) Kaplan-Meier survival analysis of the relationship between survival time and the PDEF signature. The PDEF signature was trained and tested on the Ivshina data set (Ivshina et al., 2006). The log-rank p-value is an indicator of significant differences in patient outcome when patients are dichotomized at the median of the predictor. The Cox p value treats the predictor as a continuous variable.

(C and D) PDEF and FAS mRNA and protein levels were determined by real-time PCR (C) or western blot (D) in MCF7 cells infected with NS or PDEF-targeting (KD) shRNAs, and grown for 48 hr (C) or 72 hr (D) in hormone-depleted medium. mRNA levels are presented as means \pm SD and normalized to the housekeeping gene GAPDH.

(E) Upper: schematic diagram of the PDEF binding region (indicated by arrow) upstream of the FAS gene locus as defined by PDEF ChIP sequencing in VCaP cells (Wei et al., 2010). Lower: direct PDEF ChIP followed by qPCR after culture of MCF7 cells in hormone-depleted medium for 48 hr. Data represent means \pm SD.

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cells by inhibiting the expression of pro-apoptotic genes including *FAS*.

DISCUSSION

In this study, we demonstrated that PDEF is a downstream target of the ER/FOXA1/GATA3 network. In addition to its critical function as a lineage-specific regulator of ER⁺ breast cancer cell survival, PDEF also induces luminal differentiation of nontransformed basal epithelial cells (summarized in Figures 8I–8K).

Gene expression analyses revealed distinct PDEF signatures in two different contexts. PDEF overexpression in nontransformed MCF-10A mammary basal epithelial cells induced a striking luminal differentiation program, whereas PDEF knockdown in transformed luminal MCF7 cells subjected to hormone depletion induced a growth arrest and cell death program. The lack of significant overlap in these signatures suggests distinct roles for PDEF in the context of normal morphogenesis and tumorigenesis. Defining PDEF binding sites in the genome in these two contexts may reveal differential sets of direct target genes and cooperating factors. However, the role of PDEF in promoting survival is likely more important than its role in regulating differentiation in transformed cells because preliminary data suggest that PDEF knockdown induces changes in a subset of differentiation markers in luminal breast cancer cells, but does not appear to result in the generation of bipotent cells as observed in nontransformed COMMA-1D cells.

The dichotomous functions of PDEF in the context of normal and tumor cells distinguish it from other prodifferentiation factors that function as tumor suppressors. For example, GATA3 is required for luminal cell differentiation (Asselin-Labat et al., 2007); however, GATA3 downregulation enhances invasive activity and tumor cell dissemination to the lung, whereas GATA3 overexpression suppresses metastasis (Kouros-Mehr et al., 2008). In addition, GATA3 is mutated in 17.7% of ER⁺ breast carcinoma samples (Stephens et al., 2012). Interestingly, GATA3 silencing increased ER-binding intensity in a high proportion of recruiting regions (Theodorou et al., 2013). We hypothesize that PDEF could be an important target of the tumor-suppressive action of GATA3. The analysis of estrogen-deprived or tamoxifen-resistant cells provides supportive evidence for an inverse correlation between PDEF and GATA3, which may represent a marker of hormone-refractory breast cancers.

Studies of PDEF function in different cancers, including prostate and ovarian tumors, suggests a context-dependent role in tumorigenesis (Johnson et al., 2010; Sood et al., 2007; Ghadersohi et al., 2008; Rodabaugh et al., 2007). In breast cancer, PDEF cooperates with the oncogenes *ERBB2* and *CSF1R* to promote cell motility, invasion, and anchorage-independent growth of mammary epithelial cells (Gunawardane et al., 2005). In contrast,

other studies showed that ectopic expression of PDEF in highly malignant, ER⁻, basal-like breast tumor cell lines (Feldman et al., 2003; Turner et al., 2007) resulted in reduced proliferation, motility, and invasiveness, suggestive of a tumor suppressor role. However, the relevance of ectopic expression in tumor cell lineages in which PDEF is not normally expressed is difficult to interpret. Exogenous expression of ER in ER⁻ cells led to similar paradoxical results, in which ER appeared to function as a tumor suppressor (Levenson and Jordan, 1994). Luminal epithelial-specific transcription factors, such as ER and PDEF, may reduce the mesenchymal properties of these cell lines, rendering them less invasive and migratory. Thus, loss of expression studies in ER⁺ cells represents a more appropriate context to examine the role of PDEF in tumorigenesis. Our data support a role for PDEF in breast cancer progression, at least in part by maintaining a proliferative state and enabling a prosurvival gene expression program.

Our results identify the FAS receptor, a member of the tumor necrosis family involved in the extrinsic apoptosis pathway (Peter and Krammer, 2003), as a direct PDEF transcriptional target. Nontransformed mammary epithelial cell lines express high levels of FAS, and its expression is decreased in several breast cancer cell lines (Keane et al., 1996). In addition, disease-free survival was significantly longer in patients with FAS-positive breast tumors compared to those with FAS-negative tumors (Mottolese et al., 2000). PDEF represses FAS gene expression, specifically under stress conditions such as hormone depletion. Several other ETS factors also display transcriptional-repressive activities, including Net, Yan, ERF, and Tel (Mavrothalassitis and Ghysdael, 2000). The mechanism of PDEF-mediated repression is not yet elucidated, and the identification of potential coregulators will provide new insights into its transcriptional activity.

Taken together, this study identifies PDEF not only as an essential factor within the ER-associated transcriptional network, but also as a potential therapeutic target for patients with ER⁺ breast carcinoma.

EXPERIMENTAL PROCEDURES

Reagents, Cells, and Tissue Culture Conditions

The antibodies, siRNAs, primers, and cells and tissue culture conditions are listed in the Supplemental Experimental Procedures. Reduction mastectomy tissues were collected from patients who have provided informed consent under the DFCI IRB approved protocol 93-085. See the Supplemental Experimental Procedures for more details.

Gene Expression Microarray Analysis

Total RNA was isolated either from MCF-10A cells expressing pBabe/pBabe-PDEF or from MCF7 expressing NS/PDEF-targeting shRNA and subjected to reverse transcription, labeling, and hybridization to hgu133plus2 gene chip arrays (Affymetrix). See the Supplemental Experimental Procedures for more details.

(F) Measurement of caspase 8 activity in MCF7 cells transfected with NS or PDEF-targeting (KD) siRNAs, then subjected to hormone depletion (48 hr) in the presence of a negative control antibody or anti-Fas Ligand antibody (FasL, 5 ng/ml). Data represent means \pm SD.

(G and H) PDEF and FAS mRNA and protein levels were determined by real-time PCR (G) or western blot (H) in MCF7 2A and 5C clones infected with NS or PDEF-targeting (KD) shRNAs. mRNA levels are presented as means \pm SD and normalized to the housekeeping gene *GAPDH*.

(I) PDEF belongs to a network of ER-associated factors and is positively regulated by ER and FOXA1, whereas GATA3 acts as a repressor of PDEF expression.

(J) Mechanism of GATA3-mediated repression of PDEF.

(K) Physiologic and pathologic functions of PDEF.

See also Figure S5 and Tables S2 and S3.

Lentiviral Infection and siRNA Transfection

Lentivirus was produced in 293T cells and retrovirus in 293GPG cells. For luminal progenitor cells, PDEF-targeting lentiviral particles were obtained from Santa Cruz Biotechnology (sc-45845-V). For PDEF overexpression, either the retroviral pBabe or lentiviral pBob vectors containing human or murine PDEF cDNA were used. See the [Supplemental Experimental Procedures](#) for more details.

Chromatin Immunoprecipitation

ChIP was performed as previously described (Carroll et al., 2006). See [Supplemental Experimental Procedures](#) for more details.

Growth Curves and Cytotoxicity Assay

Growth of MCF7 cells and cytotoxicity of tamoxifen and fulvestrant were measured using the cell proliferation WST-1 assay (Roche). See the [Supplemental Experimental Procedures](#) for more details.

Annexin V Analysis of Apoptosis and Caspase 8 Activity Assay

The Vybrant annexin V Kit #3 and the ApoTarget Caspase 8 colorimetric assay (Invitrogen) were used. See the [Supplemental Experimental Procedures](#) for more details.

Immunofluorescence

MCF-10A cells were stained with antibodies against cytokeratin-8 (Covance HK-8) and keratin-14 (Covance AF64). COMMA-1D colony assays were stained with antibodies against murine cytokeratin-8 (DSHB, University of Iowa; TROMA-1) and keratin-14 (Covance AF641). See the [Supplemental Experimental Procedures](#) for more details.

Flow Cytometry Analyses

MCF-10A and HMEC cells were trypsinized and resuspended in flow buffer (PBS containing 1% BSA, 2 mM EDTA, and 5% FBS) for 10 min. Cells were stained for 30 min with antibodies for PE-conjugated EpCAM and APC-conjugated CD10 (both eBioscience). Analysis was performed on a FACS Calibur machine (BD).

Soft Agar Assays

Six-well plates were coated with an underlay of 0.5% agarose (Sigma, type VII low melting). Cells were then plated in 0.35% agarose. Colonies were allowed to form for approximately 2 weeks. See the [Supplemental Experimental Procedures](#) for more details.

Fat Pad Tumor Assays

All animal studies were performed with the approval of the IACUC of Harvard Medical School. Eight-week-old female NOD/SCID mice were implanted with estrogen pellets (Innovative Research of America; 0.72 mg, 60 day release) prior to injection to support tumor growth; 2×10^6 MCF7 cells (expressing control or PDEF-targeting shRNAs, resuspended in Matrigel) were injected into the number 4 fat pad. Tumors were harvested after 8 weeks of growth. See the [Supplemental Experimental Procedures](#) for more details.

Survival Analysis

Breast tumor data sets were downloaded from <http://www.ncbi.nlm.nih.gov/geo> (lvhsina; GSE4922), (Desmedt; GSE7390) and <http://www.ebi.ac.uk/arrayexpress> (Chin; E-TABM-158). See the [Supplemental Experimental Procedures](#) for more details.

ACCESSION NUMBERS

The GEO accession number for the raw and processed microarray data presented in this paper is GSE40987.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2013.04.026>.

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