

# Regulation of human protease-activated receptor 1 (*hPar1*) gene expression in breast cancer by estrogen

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**ABSTRACT** A pivotal role is attributed to the estrogen-receptor (ER) pathway in mediating the effect of estrogen in breast cancer progression. Yet the precise mechanisms of cancer development by estrogen remain poorly understood. Advancing tumor categorization a step forward, and identifying cellular gene fingerprints to accompany histopathological assessment may provide targets for therapy as well as vehicles for evaluating the response to treatment. We report here that in breast carcinoma, estrogen may induce tumor development by eliciting protease-activated receptor-1 (PAR<sub>1</sub>) gene expression. Induction of PAR<sub>1</sub> was shown by electrophoretic mobility shift assay, luciferase reporter gene driven by the *hPar1* promoter, and chromatin-immunoprecipitation analyses. Functional estrogen regulation of *hPar1* in breast cancer was demonstrated by an endothelial tube-forming network. Notably, tissue-microarray analyses from an established cohort of women diagnosed with invasive breast carcinoma exhibited a significantly shorter disease-free ( $P=0.006$ ) and overall ( $P=0.02$ ) survival of patients that were positive for ER and PAR<sub>1</sub>, compared to ER-positive but PAR<sub>1</sub>-negative patients. We propose that estrogen transcriptionally regulates *hPar1*, culminating in an aggressive gene imprint in breast cancer. While ER<sup>+</sup> patients are traditionally treated with hormone therapy, the presence of PAR<sub>1</sub> identifies a group of patients that requires additional treatment, such as anti-PAR<sub>1</sub> biological vehicles or chemotherapy.—Salah, Z., Uziely, B., Jaber, M., Maoz, M., Cohen, I., Hamburger, T., Maly, B., Peretz, T., B.-S., R. Regulation of human protease-activated receptor 1 (*hPar1*) gene expression in breast cancer by estrogen. *FASEB J.* 26, 2031–2042 (2012). [www.fasebj.org](http://www.fasebj.org)

*Key Words:* thrombin • tumor • ERE • tissue microarray

Abbreviations: BAEC, bovine aortic endothelial cell; ChIP, chromatin immunoprecipitation; CM, conditioned medium; DFS, disease-free survival; E<sub>2</sub>, estrogen; EGFR, epidermal growth factor receptor; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; ERE, estrogen response element; GPCR, G-protein coupled receptor; LUC, luciferase; NE, nuclear extract; OS, overall survival; PAR<sub>1</sub>, protease-activated receptor 1; PR, progesterone receptor; TNBC, triple-negative breast cancer; WT, wild type.

BREAST CANCER COMPRISES a heterogeneous group of diseases categorized into 3 major subtypes (1, 2). Among these are tumors that possess estrogen receptors (ERs) and progesterone receptors (PRs), breast tissues that overexpress HER2, and triple-negative breast cancer (TNBC) tissues. While the first 2 may well respond to endocrine therapy or biologically targeted anti-HER2 vehicles, TNBC is considered the most aggressive subtype. Furthermore, TNBC lacks any detectable molecular signature and is the most poorly understood. Consequently, a major challenge in breast cancer is to identify molecular targets to possibly expand current categories and assist in determining treatment.

While the human mammary gland is highly dependent on estrogen (E<sub>2</sub>) for normal growth and maintenance (3–5), ligation of E<sub>2</sub> to ER, a nuclear transcription factor, is the driving force in breast cancer progression (6). E<sub>2</sub>-bound ER serves as a potent transcription factor for the induction of novel genes downstream that are important for normal cellular function and tumor growth, independent of proliferation (7–10). These downstream genes may consequently become central targets in breast cancer therapy (11). The main dogma for transcriptional regulation by E<sub>2</sub> involves the following steps: binding of E<sub>2</sub> to ER to initiate receptor phosphorylation, receptor dimerization, and association of the ligand-ER complex with binding motifs in the promoter of target genes. Concomitantly, E<sub>2</sub>-bound ER favors the recruitment of coactivators that enhance its transcriptional activity. In fact, the coregulators serve as a fine-tuning device for receptor transcriptional activity. Several coregulators have been implicated in cancer, most notably AIB1 (SRC3), which is overexpressed in the majority (two-thirds) of all breast cancers.

The ER signaling pathway in breast cancer is thought to be regulated also outside the nucleus, initiated at the cell membrane *via* crosstalk with receptor tyrosine kinases, including epidermal growth factor receptor

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(EGFR or HER2/Neu) and insulin-like growth factor receptor IR (12–14). This might be a mode of ER activation in the E<sub>2</sub>-resistance pathway. These receptor kinases activate signaling pathways, culminating in the phosphorylation of ER as well as its coactivators and corepressors at multiple sites and influencing their specific final function (15).

Protease-activated receptor 1 (PAR<sub>1</sub>), a G-protein-coupled receptor (GPCR), is the first and prototype member of the mammalian PAR family that comprises 4 genes (16, 17). While a well-known classical observation points to a tight link between hyperactivation of the coagulation system and cancer malignancies, the molecular mechanism that governs procoagulant tumor progression remains poorly defined (18–20). There is correlation between levels of *hPar1* expression and breast cancer progression in both clinically obtained biopsy specimens and a wide spectrum of differentially metastatic cell lines (21–24). PAR<sub>1</sub> is associated with breast cancer malignancy. Its expression correlates with the aggressiveness of human breast carcinoma cell lines, and there is increased expression of the *hPar1* gene and protein in human breast tumors when compared with corresponding normal tissues (21). Mammary gland tumors produced by MCF7 breast carcinoma cells, genetically engineered to overexpress either wild-type (WT) full-length *hPar1* cDNA or a superactive form of *hPar1*, were considerably larger and more vascularized when compared with the truncated form devoid of the cytoplasmic tail or mock-injected cell-producing tumors (25). This observation indicates that PAR<sub>1</sub> is capable of eliciting mammary tumors in a tight association with PAR<sub>1</sub>-driven signaling. Consequently, we wondered whether there is hormonal regulation of *hPar1* gene expression in the context of breast tissues.

In the present study, we investigated E<sub>2</sub> regulation of PAR<sub>1</sub> in breast carcinoma and established its functional effect. A shorter disease-free survival (DFS; *P*=0.006), as well as overall survival (OS; *P*=0.026), were observed in ER<sup>+</sup> and PAR<sub>1</sub><sup>+</sup> women compared with ER<sup>+</sup> but PAR<sub>1</sub><sup>-</sup> patients from a tissue microarray of an established cohort of individuals with invasive breast carcinoma. This outcome identifies PAR<sub>1</sub> as part of an aggressive gene imprint. Collectively, our data suggest transcriptional regulation of *hPar1* by E<sub>2</sub>-bound ER. Noticeably, the presence of PAR<sub>1</sub> may contribute to decisions on treatment. While ER<sup>+</sup> women receive hormone treatment, the presence of PAR<sub>1</sub> identifies a group that may also need either anti-PAR<sub>1</sub> biological vehicles (currently emerging in the market) or chemotherapy.

## MATERIALS AND METHODS

### Cells

MCF-7 and T47D cells (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 supplemented

with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin. Steroid-depleted cell culture medium was RPMI 1640 phenol red-free medium supplemented with 10% charcoal-stripped fetal bovine serum. Bovine aortic endothelial cells (BAECs) were cultured in DMEM (with glucose, 1 mg/ml) supplemented with 10% calf serum and bFGF (26).

### RNA extraction and RT-PCR

Total RNA was prepared using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) as described by the manufacturer. PCR primers were as follows: *hPar1*, upstream 5'-GCCAGAATCAAAAAGCAACAA-3' and downstream 5'-GAGATGAATGCAGGAAGTTGTTT-3'; GAPDH, upstream 5'-CCACCCATGGCAAATTCATGGCA-3' and downstream GADPH: 5'-TCTAGACGGCAGGTTCAGGTCCACC-3'.

PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light.

### Activation of PAR<sub>1</sub>

PAR<sub>1</sub> was activated by the TFLLRNPNDK peptide, a selective PAR<sub>1</sub> agonist, or thrombin (1 U/ml).

### Transfection and luciferase (LUC) expression assay

MCF-7 cells, grown for 48 h in steroid-depleted cell culture medium to 60–80% confluency, were transfected with 2 μg of the *hPar1* promoter fragment plasmid DNA Poluc-HTR/-4.1 (F1; kindly provided by Dr. Marschall S. Runge, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA) or empty expression plasmid (2 μg), and CMVβgal (0.3 μg) in FuGene-6 transfection reagent (Roche Diagnostics Co., Indianapolis, IN, USA). After 48 h, the cells were either treated for the indicated periods of time with E<sub>2</sub> (Sigma-Aldrich, St. Louis, MO, USA) or not, and then lysed in 0.1 ml of lysis buffer (Promega, Madison, WI). Luciferase activity was measured as described previously (27).

### Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (NEs) were prepared as described previously (27, 28). Complementary oligonucleotide probes were synthesized (Metabion, Martinsreid, Germany) for the *hPar1* putative estrogen-response element (ERE) sites according to GenBank accession number U63331: P1, 5'-agatgcgaggT-GACCcccg at site -2081; P2, 5'-ccctGTCActacatcataa-3' at site -2150; and P5, 5'-ccctGTCActacatcataa-3' (R) at site -2654. The C consensus sequence ERE is 5'-cagGTCAatcTGACctg-3'. Underscored sequences indicate the seed sequence of ERE (uppercase letters). Lowercase letters indicate a mutation inserted into the sequence. For competition experiments, 1- to 100-fold of unlabeled double-stranded oligonucleotide was added 15 min prior to incubation. The oligos for P3 and P4 are as follows: P3, 5'-ttagcgaaactTGAActttt-3'; P4, 5'-gcgccgtCTcGACCctct-3'. Mutants for each of the oligos are as follows: mutant P1, 5'-agatgcgaggTACCcccg-3'; mutant P2, 5'-tta ttagttagTtACaggg-3'; mutant P3, 5'-ttagcg aaaactT-tAACttt-3'; mutant P4, 5'-gcgccgtCTcGtCCctct-3'; mutant P5, 5'-gaagctgttcagTtACtCac-3'.

### Chromatin immunoprecipitation (ChIP) and PCR analysis

MCF-7 cells were grown for 48 h in serum-free starvation medium and then either left untreated or treated with E<sub>2</sub> (10<sup>-8</sup> M) for 2 h. ChIP was performed as described previously (27, 29). Following treatment, MCF-7 cells were fixed with

formaldehyde (to a final concentration of 1%) at room temperature for 10 min to cross-link histone proteins to DNA. Glycine was then added (to a final concentration of 0.125 M) to the plates to quench formaldehyde. Soluble chromatin was isolated and sonicated. Samples were then centrifuged at 13,000 rpm for 10 min, and the supernatant was collected. For the immunoprecipitation step, 10  $\mu$ g of antibodies, prebound to protein A/G Sepharose beads, was added to 500  $\mu$ l of the purified chromatin sample and incubated overnight at 4°C. Ab-immunocomplexed DNA was then recovered by phenol/chloroform extraction, ethanol precipitation, and resuspension in TE. PCR primer sets were designed to overlap and span the ERE binding sites of the *hPar1* promoter. ERE primer set I: forward, 5'-actgtcgcagctctccacacatc-3', and reverse, 5'-tgtaatccccgcactttaggag-3'; set II: forward, 5'-tgtaatccccgcactttaggag-3', and reverse, 5'-ccactgtcgcagctc tccacatccc-3'. These primers were first evaluated using the *Par1*-Luc construct as a DNA template. Semiquantitative PCR was then performed using the Titanium *Taq*PCR kit (Clontech Laboratories, Palo Alto, CA, USA). Amplification was carried out for 35 cycles (28 cycles for unprecipitated chromatin input lanes) with denaturation at 94°C for 1 min, annealing at 58°C for 30 s, and extension at 72°C for 1 min. PCR products were separated on a 2% agarose gel.

### ELISA

Quantification of the levels of VEGF secreted by *hPar1* cells was carried out by ELISA (Quantikine/human VEGF; R&D Systems, Minneapolis, MN), performed according to the manufacturer's instructions with anti-VEGF neutralizing Abs C20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### Calcium flux assay

Intracellular  $Ca^{+2}$  flux was measured in MCF-7 cells labeled with fura-2AM (a cell-permeable acetoxymethyl ester form of Fura-2; eBioscience, San Diego, CA, USA) by the ratio of fluorescence excitation intensity at 340/380 nm on a Fluro Scan Ascent SL (Thermo Electron Corp., Waltham, MA, USA).

### Three-dimensional (3D) tube-forming assay

The assay was performed as described previously (24). Briefly, type I collagen matrix gel was obtained by simultaneously raising the pH and ionic strength of the collagen solution. Collagen was then used to coat 24-well plates (0.3 ml/well). After polymerization of the collagen at 37°C for 0.5 h, BAECs ( $2 \times 10^4$  cells/0.5 ml/well) were added to each well. Collagen solution (0.4 ml) was carefully placed on top of the cells. After the gel was formed, 0.4 ml of conditioned medium (CM) from the different treatment was added and replaced with fresh medium every other day. Tube formation and alignment of BAECs were visualized by phase microscopy at d 8–10. The morphometric analysis used to evaluate tube formation was performed as follows. Briefly, every well in a given plate (per treatment) was independently evaluated by 2 investigators (M.J. and R.B.). Discrepancies were resolved by simultaneous reexamination of the wells by both investigators using a double-headed microscope. The microscope was calibrated with a micrometer slide before each measurement. Ten fused cells/field were selected, and  $\geq 50$  fused cells/treatment case were assessed. Four microscopic fields were screened. Data are reported as means  $\pm$  SD, and *P* values were determined by  $\chi^2$  test; values of *P* < 0.05 were considered significant. The data are representative of 4 independent experiments performed in triplicate.

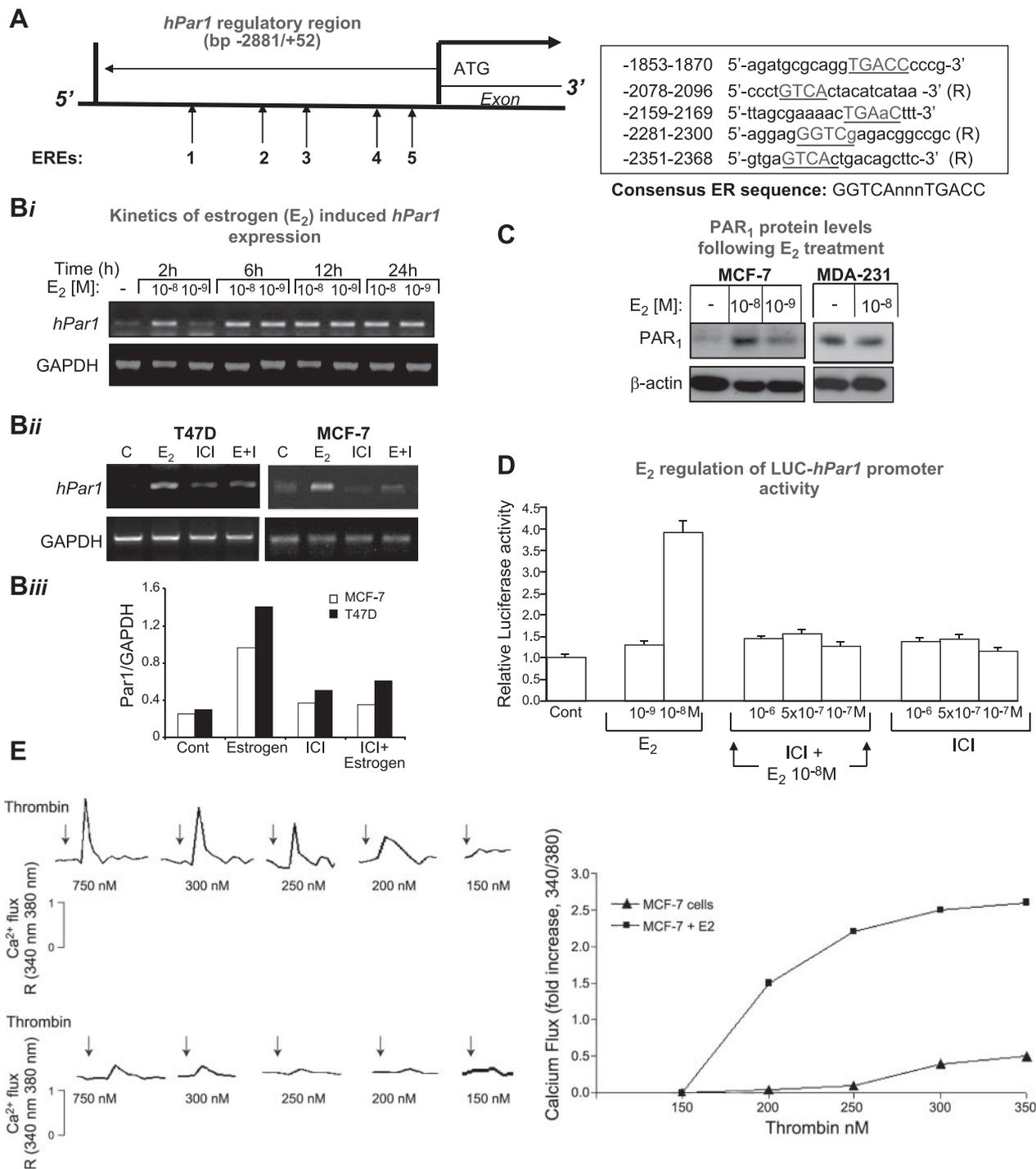
### Tissue microarray construction, immunostaining, and statistics

Formalin-fixed, paraffin-embedded breast carcinoma tissues from 299 nonselected patients with invasive breast carcinoma (220 ductal, 33 lobular, 13 ductal + lobular, 7 medullary, 3 mucinous, 9 DCIS, and 14 DCIS + microinvasion) were obtained from the Department of Pathology, Hadassah Medical Center (Jerusalem, Israel). The use of these specimens and data in research was approved by the Ethics Committee of the Hadassah Medical Center. Sections (5  $\mu$ m) stained with hematoxylin and eosin (H&E) were obtained to confirm the diagnosis and to identify representative areas of the specimen. From these defined areas, 3 tissue cores with a diameter of 0.6 mm were taken with a manual tissue arrayer MTA-1 (Beecher Instruments, Sun Prairie, WI, USA) as described previously (30, 31). From each specimen, 3 tissue cores with a diameter of 0.6 mm were taken from the different regions of the tumor and arrayed in triplicate on a recipient paraffin block (28). Sections of 5  $\mu$ m of the recipient blocks were cut and placed on charged polylysine-coated slides. Immunodetection of PAR<sub>1</sub> was done as described previously (25) with minor modifications. Briefly, sections of the tissue array blocks were deparaffinized and rehydrated. Tissue was then incubated in 3% H<sub>2</sub>O<sub>2</sub>, denatured by boiling (3 min) in a microwave oven in citrate buffer (0.01 M, pH 6.0), and blocked with 10% goat serum in PBS. Sections were incubated with polyclonal anti-PAR<sub>1</sub> antibody raised against a peptide (NH<sub>2</sub>-CLLRNPNDKYEPFWED-COOH) representing an internal sequence downstream of the PAR<sub>1</sub> cleavage site, for activation. We also used polyclonal rabbit anti-PAR<sub>1</sub> antibody directed against a synthetic peptide (NH<sub>2</sub>-KSSPLQKQLPAFISC-COOH) corresponding to an internal sequence of PAR<sub>1</sub> cleavage site, downstream of the above-mentioned peptide. The antibody was diluted 1:100 in 10% goat serum in PBS. Control slides were incubated with 10% goat serum alone. Color was developed using the Zymed AEC substrate kit (Zymed Laboratories, Burlingame, CA, USA) for 10 min, followed by counterstaining with Mayer's hematoxylin. For immunodetection of ER $\alpha$  and PR, monoclonal antibodies NCL-L-ER 6F11 and NCL-PGR-312, respectively, were used (Novocastra, Newcastle upon Tyne, UK). Slides were visualized with a Zeiss axiscope microscope (Carl Zeiss, Oberkochen, Germany) and manually read by an expert pathologist (B.M.). Tumors were considered positive for ER/PR if nuclear staining was seen in  $\geq 10\%$  of the tumor cells. To define a tumor as PAR<sub>1</sub><sup>+</sup>, staining of  $\geq 25\%$  of the tissue was considered positive on the basis of an initial overview of the cases to improve signal-to-noise ratios. We also tested additional, even stricter, cutoffs (*i.e.*,  $\geq 50\%$ ,  $\geq 75\%$ ) of tumor cells staining for PAR<sub>1</sub>.  $\chi^2$  tests were done to study the PAR<sub>1</sub> expression in ER<sup>+</sup> immunohistochemical results, using SPSS software (SPSS Inc., Chicago, IL, USA).

## RESULTS

### The *hPar1* gene regulatory region contains 5 consensus EREs

A bioinformatic search of the regulatory region of *hPar1* (U63331, 4.1 kb in size; ref. 27) revealed 5 candidates for EREs. The sequences of these putative ERE are -1853 to -1870, 5'-agatgcgagcagcTGACCcccg-3'; -2078 to -2096, 5'-ccctGTCActacatcataa-3' (R); -2159 to -2169, 5'-ttagcgaaaacTGAaCttt-3'; -2281 to -2300, 5'-aggagGGTCgagacggccgc-3' (R); -2351 to -2368, 5'-ccctGTCActacatcataa-3', shown in Fig. 1A. To exam-



**Figure 1.**  $E_2$  treatment induces *hPar1* promoter activity and increases its expression. *A*) Scheme of *hPar1* regulatory region and the potential ERE sequences identified. *Bi*) Kinetics of  $E_2$ -treated MCF-7 cells. Two concentrations ( $10^{-8}$  and  $10^{-9}$  M) of  $E_2$  were applied for various time periods as indicated, followed by RNA isolation and RT-PCR analysis for levels of PAR<sub>1</sub>. While a marked enhancement in PAR<sub>1</sub> level is seen by 2 h of  $10^{-8}$  M treatment and remains up to 24 h, no effect is seen by 2 h at  $10^{-9}$  M. At this concentration, an elevated level of PAR<sub>1</sub> is observed after 4 h treatment and continuously up to 24 h. Similar data were obtained when T47D cells were analyzed (data not shown). Data are representative of  $\geq 5$  experiments. *Bii*) Specificity of  $E_2$  treatment on PAR<sub>1</sub> levels. RT-PCR analysis of *hPar1* performed on RNA isolated from MCF-7 and T47D cells before and after  $E_2$  treatment (2 h,  $10^{-8}$  M) shows induced *hPar1* expression (as demonstrated in *Bi*). This induction was specifically inhibited in the presence of ICI182,780, a potent  $E_2$  inhibitor, while application of ICI alone had no effect on PAR<sub>1</sub> levels. Data are representative of 3 experiments. *Biii*) Histogram represents band intensity after normalization with the GAPDH housekeeping gene. *C*) PAR<sub>1</sub> protein levels following  $E_2$  treatment: Western blot analysis of MCF-7 and MDA-231 cells before and after  $E_2$ . Level of cell lysate loading is verified by  $\beta$ -actin levels. An increase in PAR<sub>1</sub> levels in MCF-7 cells is obtained at  $10^{-8}$  M  $E_2$ . In contrast, no effect on PAR<sub>1</sub> levels is observed when MDA-231 cells lacking ER were similarly treated. Data are representative of  $\geq 3$  experiments. *D*)  $E_2$  regulation of *hPar1*-LUC-promoter activity. MCF-7 cells were transiently transfected with *hPar1*-LUC reporter (continued on next page)

ine whether E<sub>2</sub> modulates *hPar<sub>1</sub>* expression, we used semiquantitative reverse-transcription PCR, Western blot, and *hPar<sub>1</sub>*-driven LUC promoter activity to estimate *hPar<sub>1</sub>* mRNA expression in ER<sup>+</sup> breast carcinoma cells. As shown in Fig. 1*Bi*, a marked increase in *hPar<sub>1</sub>* RNA levels in MCF-7 cells was obtained following 2 and 6 h of E<sub>2</sub> treatment with 10<sup>-8</sup> and 10<sup>-9</sup> M, respectively. This high level was observed up to 24 h at both concentrations. The specificity for E<sub>2</sub> regulation was demonstrated in 2 cell lines (T47D and MCF-7; Fig. 1*Bii*), in which the increased *hPar<sub>1</sub>* level was inhibited by applying ICI 182,780, an E<sub>2</sub> antagonist (32). While 1 × 10<sup>-7</sup> M ICI 182,780 alone had no effect on the endogenous *hPar<sub>1</sub>* level, in the presence of E<sub>2</sub> it completely abolished the induced *hPar<sub>1</sub>* mRNA expression in both MCF-7 and T47D cell lines (Fig. 1*Bii*). Western blot analyses verified the increase in PAR<sub>1</sub> protein levels following E<sub>2</sub> treatment (10<sup>-8</sup> M & 10<sup>-9</sup> M, 2 h) in ER<sup>+</sup> MCF-7, as well as in T47D cells (data not shown), but not in MDA-231 cells, which lack ER (Fig. 1*C*). The responsiveness of the *hPar<sub>1</sub>* promoter to E<sub>2</sub> was shown following transient transfection of *hPar<sub>1</sub>*-driven LUC promoter and subsequent analysis of its activity. As seen in Fig. 1*D*, LUC-*hPar<sub>1</sub>* promoter activity was increased 4-fold (*P* < 0.005) by E<sub>2</sub> treatment (2 h, 10<sup>-8</sup> M). A similar increase in LUC-*hPar<sub>1</sub>* promoter activity was obtained at 10<sup>-9</sup> M E<sub>2</sub> treatment for longer periods of incubation (*e.g.*, 6 h; data not shown). This elevated LUC-*hPar<sub>1</sub>* promoter activity was completely blocked by the E<sub>2</sub> inhibitor, ICI (Fig. 1*D*). MCF-7 cells treated with E<sub>2</sub> (representing elicited PAR<sub>1</sub> levels) exhibit greater sensitivity to thrombin as compared with cells prior to E<sub>2</sub> treatment. This is demonstrated by the Ca<sup>+2</sup> flux assay performed in MCF-7 activated by thrombin before and after E<sub>2</sub> application. As shown in Fig. 1*E*, a marked elevation of nearly 5-fold in the Ca<sup>+2</sup> response was obtained in the E<sub>2</sub>-treated cells in thrombin concentration range analyzed (*i.e.*, 100–750 nM).

### Binding properties of *hPar<sub>1</sub>*-ERE consensus motifs

To determine which of the candidate E<sub>2</sub>-consensus sites are functional and capable of binding protein (*e.g.*, ligated ER), we prepared NEs of ER<sup>+</sup> MCF-7 cells. In parallel, we end-labeled the oligonucleotides representing EREs in the *hPar<sub>1</sub>* promoter corresponding to *hPar<sub>1</sub>* ERE 1, 2, and 5 (designated as P1, P2, and P5; see Fig. 3*C*). As shown in Fig. 2, incubation of <sup>32</sup>P-labeled oligos with E<sub>2</sub> resulted in specific DNA-protein complexes. Significant binding was observed in the oligo

representing C (*i.e.*, 5'-caggtcatcatgacctg-3'), the known consensus binding motif for ERE, serving as a positive control (Fig. 2, lane 4), as well as in P1 (Fig. 2, lane 8), P2 (Fig. 2, lane 10), and P5 (Fig. 2, lane 12). The binding is specific, since no binding was observed when the NE was not present in the assay (Fig. 2, lanes 1, 3), when NE of cells that do not express ER (*e.g.*, DU-145 prostate cancer cells; Fig. 2, lane 14) was applied, or prior to E<sub>2</sub> treatment (Fig. 2, lane 2). Significant binding was observed after E<sub>2</sub> treatment (Fig. 2, lanes 4, 8, 10, 12). Competition for binding between protein and DNA following E<sub>2</sub> treatment, in the presence of excess unlabeled oligos representing P1, P2, or P5 (Fig. 2, lanes 9, 11, 13), showed effective inhibition of the ligated ER. Mutated oligonucleotides representing the 5 ERE motifs found in the *hPar<sub>1</sub>* promoter were prepared as described in Materials and Methods. EMSA revealed that excess cold amounts (×1000) of the mutated *hPar<sub>1</sub>*-EREs failed to compete with the corresponding WT oligonucleotide of each of the individual ERE site (data not shown). Mutants of P3 and P4 did not have any effect, since their labeled oligos did not bind in the first place (see below for nonfunctional ERE sites). Moreover, utilizing excess cold amounts of P1, P2, or P5 ERE oligos effectively inhibited the binding of the consensus C-ERE (Fig. 2, lanes 5, 6, 7). No binding of P1, P2, or P5 was observed prior to E<sub>2</sub> treatment (data not shown). No specific interaction was seen when oligos representing ERE 3 and ERE 4 (termed P3 and P4, respectively; outlined sequence shown in Fig. 3*C*) were applied, regardless of E<sub>2</sub> treatment (data not shown). Application of anti-ER antibodies (anti-ERα, D-12; Santa Cruz Biotechnology) to the binding assay and following E<sub>2</sub> treatment efficiently abrogated the binding of ER to each of the oligos tested, regardless of whether the consensus motif or the *hPar<sub>1</sub>*-representing ERE motifs were tested (Fig. 2, lanes 15, 17, 19, 21). This outcome further demonstrates that the anti-ER antibody recognizes the epitope in ER that interacts with the EREs present in the *hPar<sub>1</sub>* promoter, thereby interfering with these associations. While it is common to obtain a bandshift effect, we repeatedly observed band abrogation, which is not often obtained. Control IgG had no effect on the binding properties (Fig. 2, lane 23). This outcome strongly suggests that the 3 ERE motifs located within the *hPar<sub>1</sub>* regulatory sites are functional and capable of interacting in a sequence-specific manner with the cognate ER.

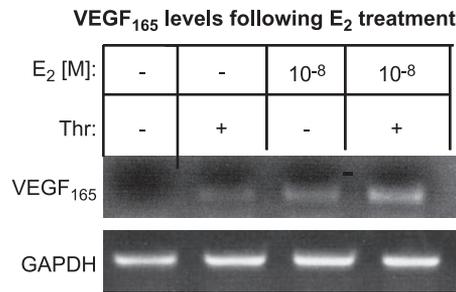
We next asked whether the E<sub>2</sub>-responsive regions identified in the *hPar<sub>1</sub>* promoter physically interact *in*

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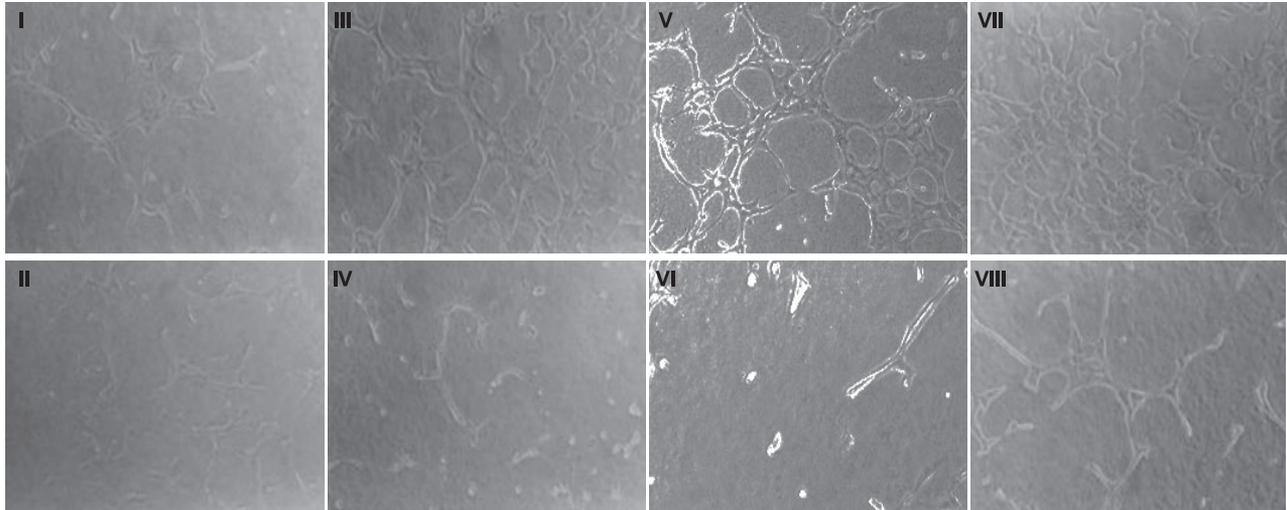
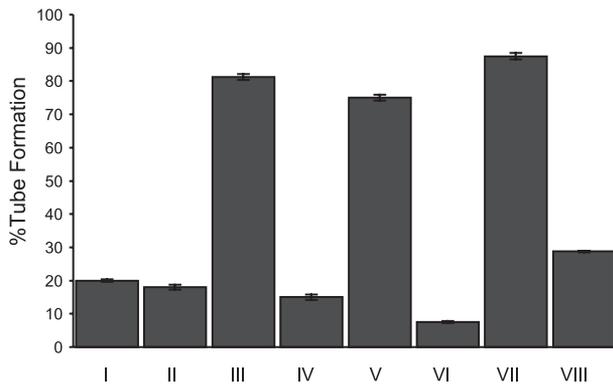
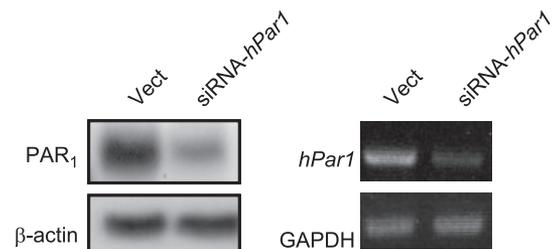
or empty vector plasmids and either treated or not with 10<sup>-8</sup> M E<sub>2</sub> for a period of 2 h. A marked up-regulation in LUC activity was obtained during this time frame at a concentration of 10<sup>-8</sup> M, but not at 10<sup>-9</sup> M. The specificity toward E<sub>2</sub> regulation is shown by application of the inhibitor, ICI. While ICI itself did not have any effect on *hPar<sub>1</sub>*-LUC activity, it potentially inhibited the E<sub>2</sub>-elicited *hPar<sub>1</sub>*-LUC activity. Luciferase activity was normalized to β-gal activity as a control for transfection efficiency. Values are means ± SD (*n* = 6). Data are representative of ≥5 experiments. *E*) Ca<sup>+2</sup> flux signaling of thrombin-treated MCF-7 cells before and after E<sub>2</sub> treatment. MCF-7 cells were seeded onto 96-well plates and were serum deprived for 24 h before E<sub>2</sub> treatment. E<sub>2</sub>-treated cells (2 h) were loaded with Fura2AM (5 μM, 60 min) in Na-HEPES-buffered saline at pH 7.4. Cells were then treated with thrombin at the indicated concentrations and monitored every 30 s. Fluorescence was measured by a dual wavelength excitation fluorimeter at 340 and 380 nm (510 nm for emission) in a Fluro Scan Ascent SL (Thermo Electron).





**A**

- I. CM of MCF7 cells
- II. CM of MCF7 cells following E<sub>2</sub> treatment
- III. CM of MCF7 cells following E<sub>2</sub> treatment and PAR<sub>1</sub> activation
- IV. CM as in III but following infection with siRNA-*hPar1*
- V. CM as in III but infected with empty viral vector
- VI. CM as in III but incubated with neutralizing anti VEGF Abs
- VII. CM of MDA-435 cells of high PAR<sub>1</sub> levels following PAR<sub>1</sub> activation
- VIII. CM of MDA-435 cells infected with siRNA-*hPar1* following PAR<sub>1</sub> activation

**Bi****Bii****Histogram of tube formation****C**

**Figure 4.** E<sub>2</sub> treatment of MCF-7 cells elicits PAR<sub>1</sub>-induced functional VEGF. **A)** Semiquantitative RT-PCR for VEGF<sub>165</sub>. Analyses of RNA isolated from MCF-7 cells demonstrate increased levels of VEGF<sub>165</sub> following E<sub>2</sub> treatment (*i.e.*, 2 h, 10<sup>-8</sup> M) alone, and long-term PAR<sub>1</sub> activation (6 h) by thrombin or the ligand-activating peptide, TFLLRNPNDK (data not shown). No expression of VEGF<sub>165</sub> is seen in MCF-7 cells prior to E<sub>2</sub> treatment. **B)** Endothelial cell tube-forming assay. CM from either MCF-7 cells before and after E<sub>2</sub> treatment and PAR<sub>1</sub> activation (I–III), or activated MDA-435 cells (VII, VIII). The importance of PAR<sub>1</sub> in the process is demonstrated following silencing of *hPar1* after infection with siRNA-*hPar1* either in MCF-7 cells (IV, V) or MDA-435 cells (VIII). The effect of VEGF is shown by incubating CM from E<sub>2</sub> treatment as well as PAR<sub>1</sub> activation with neutralizing anti-VEGF Abs C20 (VI; Santa Cruz Biotechnology). **Bii)** Morphometric analysis used to evaluate tube formation was performed as explained in Materials and Methods. Briefly, each well in a given plate (per treatment) was independently evaluated by 2 investigators (M.J. and R.B.). Discrepancies were resolved by simultaneous reexamination of the wells by both investigators using a double-headed microscope. The microscope was calibrated with a micrometer slide before each measurement. Ten fused cells/field were selected, and ≥50 fused cells/treatment case were assessed. Four microscopic fields were screened. Error bars = means ± SD; difference between groups was determined by  $\chi^2$  test. Data are representative of 4 independent experiments performed in triplicate. **C)** Silencing of *hPar1* by siRNA construct. Infection of siRNA-*hPar1* in MDA-435 cells leads to the inhibition of PAR<sub>1</sub> protein and RNA. These data are representative of ≥3 experiments.

TABLE 1. Characteristics of study population (n=299 subjects): clinical and pathological aspects

Parameter	Value
Criteria	
Age (yr)	49 (20–82)
Tumor size (cm)	2 (0.2–9)
Tumor type [n (%)]	
IDC	220 (73.5)
ILC	33 (11)
IDC + ILC	13 (4.3)
DCIS	9 (3.0)
DCIS + microinvasion	14 (4.6)
Mucinous	3 (1.0)
Medullary	7 (2.3)
Lymph node status [n (%)]	
LN-negative	159 (53.1)
LN-positive	120 (40.1)
Grade [n (%)]	
1–2	161 (54.5)
3	134 (45.4)
Total valid	295
ER [n (%)]	
Negative	105 (37.2)
Positive	177 (62.7)
Total valid	282
PR [n (%)]	
Negative	235 (84.2)
Positive	44 (15.7)
Total valid	279
TN [n (%)]	95 (34.79)
Total valid	273

Values are median and range for criteria; number of cases and percentage for all other parameters. DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; TN, triple negative.

PAR<sub>1</sub> (Fig. 5) and ER/PR (data not shown). While the frequency of ER positivity was 62% (176 tumors), and PR was expressed in 44 (15%) tumors, PAR<sub>1</sub> expression was found in 76% of all the tumors. No PAR<sub>1</sub> expression was detected in any of the 5 control specimens of nonmalignant breast tissues (Fig. 5, representative control staining).

$\chi^2$  analysis of DFS of the ER<sup>+</sup> patients (5 yr retrospectively) among the tissue microarray cohort of patients that overexpress or do not overexpress PAR<sub>1</sub> showed the following outcome. A markedly significant ( $P=0.006$ ) difference in the DFS in women that overexpress both ER<sup>+</sup> and PAR<sub>1</sub> represented a distinctly shorter DFS (median not reached) compared with patients who were ER<sup>+</sup> but negative for PAR<sub>1</sub> (Fig. 5B). Likewise, a shorter OS was observed in ER<sup>+</sup> and PAR<sub>1</sub><sup>+</sup> patients, compared with ER<sup>+</sup> and PAR<sub>1</sub><sup>-</sup> ( $P=0.021$ ) patients. These observations strengthen the concept that PAR<sub>1</sub> may account for part of a highly malignant gene signature in breast carcinoma.

## DISCUSSION

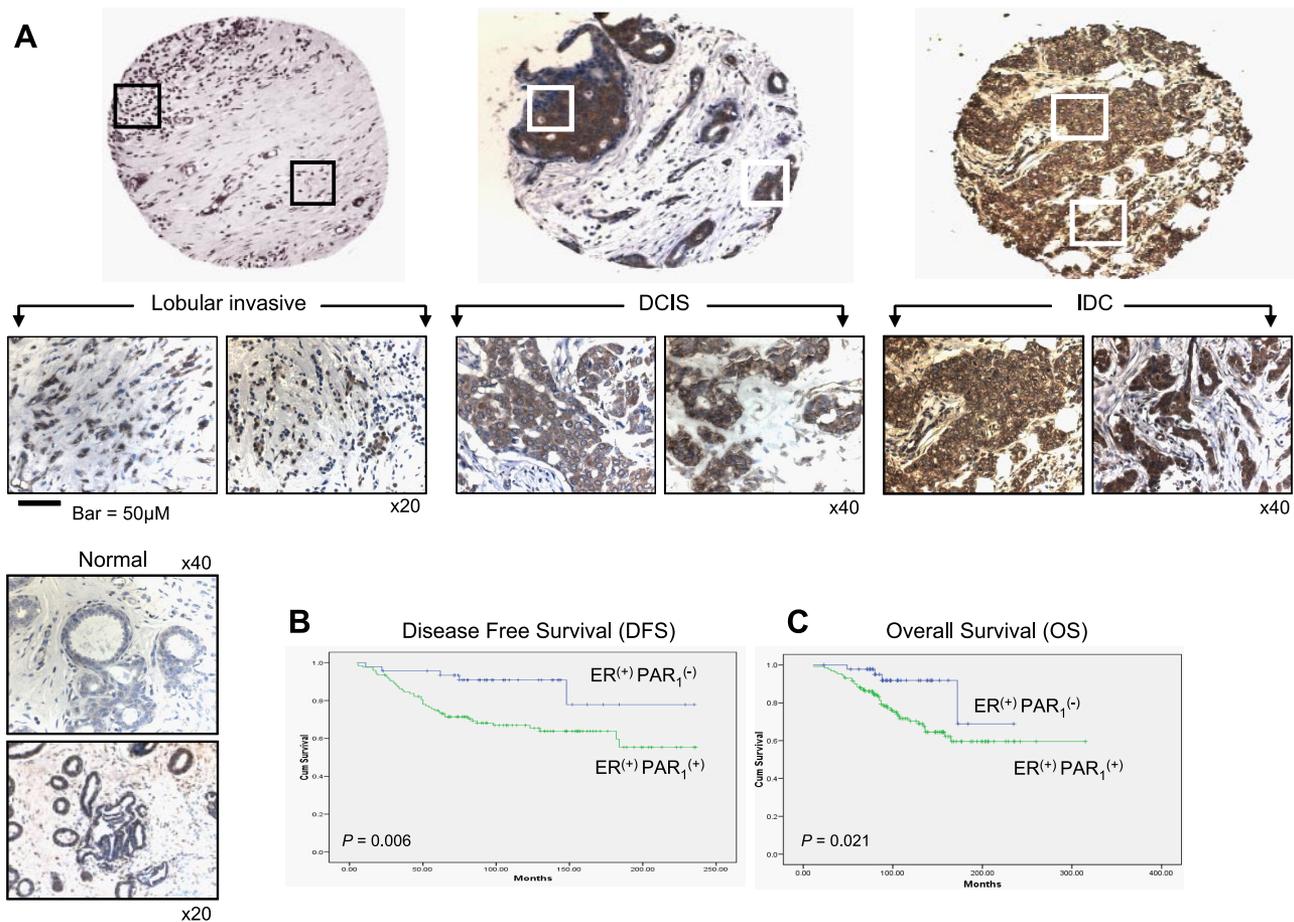
With the goal of gaining mechanistic insights into PAR<sub>1</sub> and E<sub>2</sub> action in the etiology of breast cancer progres-

sion, we examined functional consequences of PAR<sub>1</sub> expression in clinical tissue microarray settings and biochemically characterized E<sub>2</sub> regulation of *hPar*<sub>1</sub> expression. An ongoing challenge is to identify molecular means to assist in evaluating the degree of response to a given therapy. Five-year retrospective analyses of ER<sup>+</sup> patients that express PAR<sub>1</sub> resulted in a significantly shorter DFS and OS compared with ER<sup>+</sup> but PAR<sub>1</sub><sup>-</sup> patients. This could aid in optimizing our approach to personalized care whereby genetic print/s may improve the prediction of the course of disease and response to treatment. Indeed, gene signature might complement the traditional pathological assessment in evaluating tumor behavior. Similarly, Oncotype Dx, a gene profile that has been recently developed for women with hormone receptor-positive disease, is a good example of such refinement and is part of an oncologist's practice today (34). Accordingly, while a low score is consistent with hormone therapy, a high score indicates that the addition of treatment such as chemotherapy may be required. The presence of PAR<sub>1</sub> may tilt the score or stand on its own when making a decision on treatment. In addition, despite early detection that is more common today, there are still small tumors (*e.g.*, early detection) that can cause death. In these tumors, newly emerging biological biomarkers may greatly assist in making a treatment decision.

The regulatory sequence of *hPar*<sub>1</sub> has been cloned (35), enabling the identification of specific transcription factors (*i.e.*, Sp1, *Egr-1*), as well as tumor suppressor genes (*i.e.*, AP<sub>2</sub> and p53), as potent regulators of the *hPar*<sub>1</sub> gene (27, 29, 36). Yet the molecular pathway responsible for enhanced *hPar*<sub>1</sub> overexpression in developing tumors as compared with normal tissues of the same origin remains poorly understood.

Transcriptional regulation plays a central role in *hPar*<sub>1</sub> tumor-associated gene overexpression. We have demonstrated that *Egr-1* acts at the transcriptional level to noticeably induce *hPar*<sub>1</sub> gene levels in prostate cancer (27). Here p53, a nuclear transcription factor and a well-studied tumor suppressor gene, was also shown as a regulator of *hPar*<sub>1</sub> gene expression and function (29). While an inverse correlation exists between WT p53 expression and *hPar*<sub>1</sub>, the mutant p53 forms, representing either loss of p53 or impaired function, directly correlate with *hPar*<sub>1</sub> overexpression in cancer (29). Likewise, Tellez *et al.* (36) demonstrated an inverse correlation between the expression of activator protein-2 $\alpha$  (AP-2) and the overexpression of PAR<sub>1</sub> in metastatic melanoma. In contrast, a direct correlation has been established between the expression of specificity protein 1 (Sp1) and aggressive melanoma (36). Altogether, these observations strengthen the notion that specific transcription factors and tumor suppressors may delicately regulate the overexpression of the *hPar*<sub>1</sub> gene in a given cancer context.

Promoter analyses have identified the functional ERE regions in the *hPar*<sub>1</sub> regulatory region by employing EMSA examinations, which point to the regions corresponding to P1, P2, and P5 capable of specifically



**Figure 5.** Immunohistological staining of PAR<sub>1</sub> in breast cancer tissue microarray. *A*) Representative images of positive PAR<sub>1</sub> immunostaining in breast carcinoma tissue microarray (left, invasive lobular carcinoma; middle, ductal carcinoma *in situ* [DCIS]; right, invasive ductal carcinoma [IDC]), as well as negative staining on sections of normal breast tissues. Histological evaluation was carried out utilizing anatomical compartments of an ocular micrometer. Slide review was independently performed by an expert pathologist (B.M.). The microscope was calibrated with a micrometer slide before each measurement. All measurements were performed on the monitor screen using a  $\times 40$  objective. On examining the sections, fields representing tumor cells from the most cellular area at the center of the tumor were selected. Necrotic and inflammatory areas were avoided. Five microscopic fields were screened. Ten cells/field were selected, and  $\geq 50$  cells/tumor case were assessed. Definition of a tumor as PAR<sub>1</sub><sup>+</sup> was based on  $\geq 25\%$  staining of tumor cells (cutoff point of 25%). Immunostained tumor cells were chosen on the basis of an initial overview of the cases to improve signal-to-noise ratios. Discrepancies were resolved by simultaneous reexamination of the slides by both investigators (B.M. and Z.S.) using a double-headed microscope. Histological scoring was based on the following: +1,  $< 25\%$  positive cells (weak positive); +2, between 25 and 75% positive cells (moderate); +3,  $> 75\%$  positive cells (strong). All controls were negative (0–5% positive cells). Extent of expression classified by score (1–3), number of positive cells/field ( $x=5$ ). *B*) Immunohistological staining of PAR<sub>1</sub> in breast cancer tissue microarray. Retrospective (5-yr) analysis of ER<sup>+</sup> tumors in women represented by the tissue microarray revealed that patients that express both ER and PAR<sub>1</sub> exhibit a significantly shorter DFS ( $P=0.006$ ; median not reached) compared with ER<sup>+</sup> but PAR<sub>1</sub><sup>-</sup> patients. *C*) OS of women according to ER and PAR<sub>1</sub> levels. Retrospective (5-yr) analysis of ER<sup>+</sup> tumors in women represented by the tissue microarray revealed a significantly shorter OS ( $P=0.021$ ; median not reached) compared with ER<sup>+</sup> but PAR<sub>1</sub><sup>-</sup> women.

associating with ER present within the NEs in the assay. ChIP analysis confirmed physical *in vivo* association occurring between the functional ERE motifs and ER. It remains to be determined whether the observed E<sub>2</sub> stimulation of *hPar*<sub>1</sub> may be attributed in part to interplay between the ER and the *hPar*<sub>1</sub>-inducing transcription factors found to regulate *hPar*<sub>1</sub> transcriptional activity.

PAR<sub>1</sub> is tightly associated with breast cancer tumorigenesis, as demonstrated by the orthotopic inoculation of cells expressing *hPar*<sub>1</sub> into mouse mammary glands *in vivo*. Large and highly vascularized tumors were generated when MCF-7 cells were genetically engi-

neered to overexpress either WT *hPar*<sub>1</sub> or Y397Z *hPar*<sub>1</sub>, with persistent signaling. In contrast, cells overexpressing the truncated form of *hPar*<sub>1</sub>, which lacks the cytoplasmic tail, developed small or no tumors, similar to cells expressing empty vector or control untreated cells (25). This outcome highlights the role of signaling in PAR<sub>1</sub>-induced breast cancer growth and development. Indeed, antibody-array membranes revealed essential *hPar*<sub>1</sub> partners, including Etk/Bmx and Shc. The hierarchy of binding, as well as the minimal binding region in PAR<sub>1</sub> C-tail, was established, rendering this region a potential platform for therapeutic vehicles in breast cancer.

Here we have demonstrated E<sub>2</sub> responsiveness of the *hPar<sub>1</sub>* gene promoter and have found that exposure to physiological concentrations of E<sub>2</sub> induces *hPar<sub>1</sub>* expression in breast carcinoma cells. The E<sub>2</sub> effect on *hPar<sub>1</sub>* expression occurred in an ER<sup>+</sup>, but not in an ER<sup>-</sup> breast carcinoma cell line, and was inhibited by the anti-E<sub>2</sub> ICI 182,780, known to block responses mediated through ER signaling (32). These data indicate that the classic ER pathway is involved in transcriptional activation of *hPar<sub>1</sub>* rather than numerous alternative mechanisms of E<sub>2</sub>-regulated cell responses (34, 37, 38). Tamoxifen, the most widely used anti-E<sub>2</sub> in endocrine therapy of breast cancer, plays a dual role. It can act as an antagonist at a high concentration, inhibiting E<sub>2</sub>-induced *hPar<sub>1</sub>* levels, whereas at physiological concentrations it acts as an agonist for the ER-inducing levels of *hPar<sub>1</sub>* (unpublished results).

While we describe here a canonical mode of E<sub>2</sub>-bound ER transcriptional activity (*via* binding to ERE motifs present on the *hPar1* promoter), noncanonical E<sub>2</sub> activation has also been described. ER can interact with other transcription factors, through a process referred to as transcription factor crosstalk. In this case, ER can modulate the activities of other transcription factors such as activator protein (AP)-1, nuclear factor-κB (NF-κB), or SP-1, by stabilizing their DNA binding and/or recruiting coactivators to the complex (39–41). Another mechanism by which E<sub>2</sub> and ER affect gene expression has been termed the nongenomic pathway. In this pathway, E<sub>2</sub> binds to the ER localized outside the cell nucleus (in the cytoplasm or membrane). This, in turn, activates signal transduction pathways in the cytosol, for example, MAPK signaling. The AP-1 transcription factor, which acts through GC-rich regions in the promoter, affects many growth factor receptors, including IGF-1R, erbB 2, and EGFR, leading to MAPK activation, which acts to phosphorylate ER (Ser118) or ER coactivator phosphorylation, thereby leading to increased ER activity (42, 43). PAR<sub>1</sub>, once expressed, is known to activate the MAPK pathway, hence contributing to improved ER transcriptional activities. AP-binding motifs are in fact present in 4 different locations throughout the *hPar1* promoter. It is possible that once PAR<sub>1</sub> is expressed by the canonical ERE pathway, other mechanisms may also be involved. These include transcription-factor crosstalk with AP-1, or *via* the nongenomic activation signaling of the MAPK pathway, which will enhance and improve ER transcriptional activity (44–46).

E<sub>2</sub>-induced *hPar<sub>1</sub>* could be particularly important in the initial stages of breast cancer, when the majority of breast tumors are reportedly ER<sup>+</sup> (47, 48). Overexpression of *hPar<sub>1</sub>* has also been observed in cases of ER<sup>-</sup>, more violent tumors, whereby its level is controlled by selective transcription-factors that come into view as critical regulators. What these specific transcription factors that regulate *hPar<sub>1</sub>* overexpression in breast cancer are is yet unknown. These aggressive tumors that are E<sub>2</sub> independent are resistant to traditional anti-E<sub>2</sub> therapy. In these tumors, high *hPar<sub>1</sub>* levels, at

least in part, account for the more aggressive phenotype and poor prognosis. Consequently, while targeted therapeutics toward PAR<sub>1</sub> [*e.g.*, SCH 530348, an oral anti-PAR<sub>1</sub> antagonist under phase 3 clinical evaluation in patients with atherothrombotic disease (48, 49), or pepducin, which inhibits PAR<sub>1</sub> intracellular function and the cell-penetrating inhibitory peptides, pepducins] are not part of the oncologists' list of protocols, the presence of PAR<sub>1</sub> identifies patients who may require chemotherapy in addition to hormone treatment. In addition, it may provide the basis for broadening an oncologist's therapeutic procedures. These studies provide a guide to treatment decisions on individual-based care. Furthermore, these inhibitors may be considered for future therapeutic modality in breast cancer. EJ

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