Pituitary Tumor Transforming Gene Binding Factor: A New Gene in Breast Cancer

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Abstract

Pituitary tumor transforming gene (PTTG) binding factor (PBF; PTTG1IP) is a relatively uncharacterized oncoprotein whose function remains obscure. Because of the presence of putative estrogen response elements (ERE) in its promoter, we assessed PBF regulation by estrogen. PBF mRNA and protein expression were induced by both diethylstilbestrol and 17β-estradiol in estrogen receptor α (ERα)–positive MCF-7 cells. Detailed analysis of the PBF promoter showed that the region −399 to −291 relative to the translational start site contains variable repeats of an 18-bp sequence housing a putative ERE half-site (gcccctcGGTCacgcctc). Sequencing the PBF promoter from 122 normal subjects revealed that subjects may be homozygous or heterozygous for between 1 and 6 repeats of the ERE. Chromatin immunoprecipitation and oligonucleotide pull-down assays revealed ERα binding to the PBF promoter. PBF expression was low or absent in normal breast tissue but was highly expressed in breast cancers. Subjects with greater numbers of ERE repeats showed higher PBF mRNA expression, and PBF protein expression positively correlated with ERα status. Cell invasion assays revealed that PBF induces invasion through Matrigel, an action that could be abrogated both by siRNA treatment and specific mutation. Furthermore, PBF is a secreted protein, and loss of secretion prevents PBF inducing cell invasion. Given that PBF is a potent transforming gene, we propose that estrogen treatment in postmenopausal women may upregulate PBF expression, leading to PBF secretion and increased cell invasion. Furthermore, the number of ERE half-sites in the PBF promoter may significantly alter the response to estrogen treatment in individual subjects.

Introduction

Breast carcinogenesis requires multiple genetic changes, including the altered expression and function of tumor suppressor genes and oncogenes. Most human breast cancers evolve from normal epithelial cells in terminal duct lobular units through a series of increasingly abnormal stages over long periods of time. Key stages in this progression are hyperplasia, atypical hyperplasia, in situ carcinoma, invasive carcinoma, and, finally, metastatic disease (1). Invasion into surrounding stroma defines the transition from in situ to invasive carcinoma. However, most defects responsible for the development and progression of malignant disease remain unknown (1).

Described in only nine publications (2–10), four of which are from our own group (2, 3, 6, 8), Pituitary tumor transforming gene (PTTG) binding factor (PBF) was identified through its ability to interact with PTTG1, the human securin (4). First isolated in 1997 (11), PTTG1 is an estrogen-regulated gene (12, 13) previously implicated in breast cancer, with highest expression in invasive and metastatic breast cancers (14). Its binding partner PBF has not, however, been studied in the context of breast cancer before.

Initially identified in 1998 (10), PBF comprises six exons spanning 24 kb within chromosomal region 21q22.3. The 180-amino acid peptide sequence of PBF shares no significant homology with other human proteins but is highly conserved across a wide diversity of animal species (73% homology to mouse, 67% frog, 60% chicken, 52% zebra fish), suggesting both unique functionality and significant evolutionary importance.

PBF is ubiquitously expressed (15), but a decade after its cloning, very little has been reported concerning its function. We previously characterized PBF expression in thyroid cancers and showed it to be a transforming gene in vitro and to be tumorigenic in vivo (8). Furthermore, high PBF expression was independently associated with poor prognosis in human thyroid cancer. Most recently, we showed that PBF repressed iodide uptake in thyroid cells, both through transcriptional regulation (3) and through altered subcellular trafficking (6).

PBF is thus a relatively uncharacterized transforming gene that plays a part in multiple cellular processes, particularly in the setting of endocrine neoplasia. We now present extensive

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doi: 10.1158/0008-5472.CAN-09-3531
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data, outlined below, that suggest PBF represents an entirely novel gene of direct relevance to breast cancer.

Materials and Methods

Tissues. Breast tumor paraffin-embedded samples arranged on tissue microarrays (TMA) were available from 146 patients (16). There were 6 cases of ductal carcinoma in situ, 4 cases of medullary carcinoma, 1 atypical medullary carcinoma, 7 cases of mucinous type, 2 cases of tubular carcinoma, 22 cases of lobular carcinoma, 1 lobular-papillary, and 1 benign tumor. A further 101 cases were designated pathologically as being of no special type (breast tumors not fitting the histologic categories above but tending to be invasive ductal carcinomas that could not be further characterized on morphologic grounds; ref. 16). Clinical follow-up, encompassing tumor grade, vascular invasion, lymph node stage, Nottingham prognostic index, and estrogen receptor (ER) status was available for the breast cancer series. Normal breast paraffin-embedded samples were available from US Biomax (n = 6).

Cell lines and hormonal treatments. MCF-7 human Caucasian breast adenocarcinoma cells were obtained from the European Collection of Animal Cell Cultures in April 2008. Low passage number cells obtained from the original stock were maintained in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS). Cells were treated with diethylstilbestrol (DES) and 17β-estradiol (EST) at final concentrations of 10 nmol/L and 20 nmol/L, respectively, and with ICI 182780 (Faslodex/Fulvestrant) at 100 nmol/L and 1 µmol/L in RPMI 1640 phenol red-free medium (Life Technologies) supplemented with 10% charcoal-stripped serum.

RNA extraction, reverse transcription quantitative PCR, and Western blot analysis. Total RNA was extracted from MCF-7 cells using Sigma Trizol kit, as described previously (3). RNA was isolated from paraffin-embedded tissues on microscope slides using the Pinpoint Slide RNA Isolation System kit (Zymo Research). RNA was reverse transcribed using Reverse Transcription System (Promega), as previously described (3). Expression of specific mRNAs was determined using 7500 Real-Time PCR System (Applied Biosystems; ref. 8). Western blot analyses were performed as we have described previously (2, 6, 17, 18).

Cross-linking chromatin immunoprecipitation. Briefly, MCF-7 cells were cross-linked by addition of 1% formaldehyde directly to 1.5 x 10^6 exponentially cells. After sonication samples were immunoprecipitated with Protein A-agarose beads (Upstate Biotech) with 5 µg ER antibody (Santa Cruz), alongside an IgG sample as a background control (Abcam). Immunocomplexes were then sequentially washed, and cross-links were removed before DNA extraction. PBF promoter primers of sequence 5‘-GCA-GCC-CTT-TAG-GTA-GGA-G and 5‘-GAG-GAA-AGG-AGC-CTG-GTA-GC were then used with 5 µL of chromatin immunoprecipitation (ChIP) DNA material for analysis by semiquantitative PCR.

DNA extraction. Genomic DNA from normal and tumorous colorectal samples and normal thyroid specimens had previously been extracted (17, 19). Normal DNA was also obtained from whole blood from patients with normal thyroid function (20). Genomic DNA was isolated from breast tumor and normal tissue, which had been formalin fixed and paraffin embedded using the Pinpoint Slide DNA Isolation System kit (Zymo Research).

PCR. Two primer sets were designed to amplify the region of the PBF promoter, which contains a repeated 18-bp sequence. Forward (F) and reverse (R) sequences were primer set 1(F) 5‘-GCC-CTC-CCC-TAG-TCC-CCT-3‘, primer set 1(R) 5‘-GCG-AGG-AGA-GCG-GCT-GA-3‘; primer set 2(F) 5‘-GCA-GCC-CTT-TAG-GAT-GGA-G-3‘, primer set 2(R) 5‘-GAG-GAA-AGG-AGC-CTG-GTA-GC-3‘. Product sizes were 220 and 286 bp, respectively. Sequencing was carried out to confirm estrogen response element (ERE) repeat numbers using the forward primer of primer set 1 or 2.

Luciferase assays. The pGL3_PBFpromoter construct was created by inserting bases –510 to –211 relative to the translational start site into the pGL3 basic vector (Promega). Cells were harvested in passive lysis buffer (Promega). The Dual Luciferase Reporter Assay System (Promega) was used, and data were expressed as a ratio of Renilla luciferase activity.

Biotinylated oligonucleotide pull down assay. Pull-down assays were essentially as described previously (21). Oligonucleotides of a consensus EREs binding sequence (22), 5‘-GTC-CAA-AGT-CAG-GTC-ACA-GTG-ACC-TGA-AAG-TT-3‘, PBF ERE sequence (half-sites in bold), 5‘-CTC-GCC-CTC-GGG-CCA-GGC-CCT-CCT-GGC-CTC-GGC-CTG-GTA-CGC-GAG-GCT-C-3‘ and mutant PBF ERE sequence (base changes underlined), 5‘-CTC-GCC-CTC-GAG-TTA-TTT-CGC-GCC-CCT-AAG-TTT-GTC-GCC-GGC-CCT-GCA-A-TTT-GGC-CCT-GCC-CCT-C-3‘ were 5′-end-labeled with biotin and incubated with recombiant ERe protein (P2187; Invitrogen, Ltd.). In competition reactions, recombinant protein was additionally incubated with 1 to 2 nmol of nonbiotinylated oligonucleotide. DNA/protein complexes were captured with 0.1 mg of magnetic streptavidin beads (Promega). Subsequently, bound proteins were probed with an anti-ERe antibody (Santa Cruz).

Immunohistochemistry. Normal and tumorous breast specimens were immunostained using our specific rabbit polyclonal antibody to PBF (1:200). For negative controls, the primary antibody was replaced by 2% normal serum. Sections were counterstained with Mayer’s hematoxylin, and blinded scoring was carried out according to the intensity (0, not present; +1, least intense; +3, most intense) and percentage (<25, 25–50, 50–75, >75) of PBF staining.

Invasion assays. MCF-7 cells transfected with vector only (V0), hemagglutinin (HA)–tagged PBF (PBFA-HA), or the PBF mutant Δ29-93 were seeded onto BD Falcon cell culture inserts (pore size, 8 µm/L). Subsequently, 800 µL of RPMI 1640 supplemented with 20% FBS or charcoal-stripped serum were added to the well below the BD Falcon cell culture inserts. For knockdown, cells were treated with either 50 nmol/L of scrambled (negative control 1, Ambion) or PBF-specific siRNAs 14399 and 147350 (mixed in equal
quantities; Ambion). After 24 or 48 hours, cells were fixed and stained using Mayer’s H&E (Sigma).

**MTT assays.** Cells were transfected or treated with DES and EST at final concentrations of 10 and 20 nmol/L. At 24 and 48 hours post-PBF transfection or 48 hours post-estrogen treatment, 100 μg of MTT were added to each well, as previously described (23).

**Detection of PBF secretion by Western blotting.** Cell lysates were harvested in radioimmunoprecipitation assay (RIPA) buffer. Cell medium was removed from MCF-7 cells and centrifuged to remove cellular debris. Supernatants were then added to three volumes of 100% ethanol and centrifuged, and the pellet was resuspended in RIPA buffer.

**Detection of PBF secretion by immunoprecipitating radiolabeled PBF.** Cells were maintained in 1 mL of medium containing 2/3 standard medium and 1/3 leucine-free equivalent, along with 10 μCi l-leucine [3,4,5-3H] (MP Biomedicals). Media extracts were centrifuged to remove floating cells. To obtain cell lysate fractions, cells were lysed in RIPA buffer. Rabbit polyclonal anti-PBF antibody (5 μL; ref. 6) or normal rabbit control serum (5 μL) was added to cell medium and lysate fractions. Immunocomplexes were pelleted by centrifugation at 13,000 x g, and dpm was measured. Western blot analysis of PBF immunoprecipitation confirmed that PBF was specifically pulled down.

**Immunofluorescent analysis of PBF vesicular localization.** MCF-7 cells were transfected with 1 μg PBF-HA and 1 μg chromogranin A-GFP (24) on coverslips. Cells were fixed and permeabilized before blocking. Rabbit polyclonal anti-HA (Y-11) antibody (Santa Cruz Biotechnology) was used as primary antibody, and Alexa Fluor 594–conjugated goat anti-rabbit IgG (Invitrogen) was used as secondary antibody. Hoechst stain was used to visualize nuclei (1:1000).

**Statistical analysis.** Data were analyzed using Sigma Stat (SPSS Science Software UK Ltd.). Normally distributed data were analyzed using a two-sample Student’s t test. The Mann-Whitney rank-sum test was used for comparison between the two groups of nonparametric data. Data containing categorical information were analyzed using the χ² test and Fisher’s exact test.

**Results**

**PBF mRNA and protein expression are induced by estrogen.** The binding partner of PBF (PTTG1) is an estrogen-regulated gene implicated in the etiology of breast cancer.
cancer (14). We therefore examined whether PBF might also be estrogen regulated. PBF mRNA expression was induced 48 hours after treatment by 10 and 20 nmol/L DES and 10 and 20 nmol/L EST in ERα-positive MCF-7 cells (Fig. 1A). PBF protein expression levels were also significantly upregulated 2-fold to 3-fold compared with vehicle controls (Fig. 1B). Furthermore, treatment with the anti-estrogen ICI 182780 at 100 nmol/L and 1 μmol/L concentrations repressed EST stimulation of PBF expression in a dose-dependent manner (Fig. 1C).

To investigate whether EST stimulation of PBF expression occurred directly through the PBF promoter, cross-linking ChIP assays were carried out as described in Materials and Methods. In each case (n = 4 experiments), ERα bound to the human PBF promoter, with most pronounced binding occurring between 24 and 48 hours post-estrogen treatment in MCF-7 cells (Fig. 1D).

**The human PBF promoter is polymorphic for ERE half-sites.**

*In silico* analysis of the human PBF promoter identified the region −399 to −292 relative to the translational start site to be replete with putative EREs (Fig. 1A). Preliminary sequencing of this region revealed that it contained variable repeat numbers of an 18-bp sequence housing a putative consensus ERE half-site (gcccctcGGTCAcgcctc; Fig. 2A). PCR and sequencing of the region were additionally examined with a separate set of primers, confirming the existence of polymorphic numbers of 18-bp repeats (Fig. 2A).

A panel of 92 genomic DNA samples was available to us, DNA being prepared from normal thyroid and colon tissue and from tumorous colon. PCR and sequencing revealed that subjects may be homozygous or heterozygous for between one and six repeats of the 18-bp region housing the ERE (Fig. 2B). We next examined ERE repeat number
in formalin-fixed paraffin-embedded breast TMA samples. Of the ∼60 tumors assessed, 27 tumors gave informative sequencing data for PBF repeat number, and three of the six normals yielded unambiguous sequence data. These studies revealed that breast tumors could either be homozygous (three and three repeats) or heterozygous (three and five repeats), whereas the n = 3 normal breast specimens were all homozygous for three repeats (Fig. 2B). None of the other variants were detected in breast DNA. Overall, as each 18-bp repeat houses a putative consensus ERE half-site, subjects therefore differ in the number of EREs present in their PBF promoter.

The promoter region −399 to −292 is estrogen responsive. Having determined that PBF is regulated by estrogen and that ERα binds to the PBF promoter in ChIP assays and having identified a polymorphic region in the human PBF promoter containing variable numbers of putative ERE half-sites, we next examined the estrogen responsiveness of this fragment of the promoter. DES and EST (20 nmol/L) both induced significant luciferase activity compared with vehicle-treated cells after 24 hours (Fig. 2C). At 48 hours, the effect was more pronounced (DES, 1.8 ± 0.2-fold, P < 0.01 compared with vehicle, n = 3; EST, 1.7 ± 0.3-fold, P < 0.05 compared with vehicle, n = 3; Fig. 2C).

Next, we investigated whether the polymorphic ERE half-sites identified within the promoter region −399 to −292 were capable of binding ERα directly. We performed pull-down assays using oligonucleotides containing either a series of three wild-type (WT) or three mutated PBF ERE half-site repeats. Recombinant human ERα bound specifically to the biotinylated double-stranded PBF ERE oligonucleotide (Fig. 2D), which could be competed out by incubation with increasing excesses of unlabeled PBF oligonucleotide. Furthermore, recombinant ERα bound a biotinylated consensus double-stranded ERE oligonucleotide, and binding was repressed by competition with unlabeled WT PBF ERE oligonucleotide but not by the mutated PBF oligonucleotide in which the EREs had been abolished. As PBF mRNA was induced 1.5-fold to 3-fold at identical time points and at identical doses of estrogen (Fig. 1), these data indicate that the short promoter region −510 to −211 is positively regulated by estrogen and confers most, but not all, of PBF’s responsiveness to DES and EST. In addition, the specific ERE half-site region identified within the promoter region −399 to −292 is capable of binding ERα, suggesting that the main mechanism of estrogen regulation of PBF is directly via the polymorphic EREs of the proximal promoter.

PBF expression and correlation in a breast tumor series. We next investigated whether PBF was expressed in human breast tumors. Initial mRNA investigations used 20 breast TMA specimens and 6 normal breast specimens. PBF mRNA expression was apparent in RNA extracted from 18 of 20 TMA tumor samples, with a mean Taqman reverse transcription–PCR (RT-PCR) ΔCt of −8 to 10, suggesting robustly detectable levels of expression (Fig. 3A). Reverse transcriptase negative controls confirmed that amplification in the tumor samples examined was not an artifact of genomic DNA contamination (data not shown). In contrast, PBF mRNA was not detected in any of the six normal breast specimens analyzed (Fig. 3A).

PBF protein expression was next quantified in a larger series of normal breast specimens (n = 8) and TMA tumor samples (n = 146) through immunohistochemistry, using our rabbit polyclonal antibody (6). Examples of scoring intensities are provided in Fig. 3B. PBF expression was low or absent in normal breast tissue, whereas it was strongly expressed in epithelial cells of all tumor types and grades of breast tumor assessed (Fig. 3C). Specificity of staining was confirmed in negative control experiments (data not shown). Importantly, ERα status positively and significantly correlated with the percentage of PBF protein expression (P < 0.001). However, the remaining phenotypic end-points examined (tumor grade, vascular invasion, lymph node stage, or Nottingham prognostic index) were not associated with PBF staining intensity or percentage expression.

Next, we investigated the relationship between ER status and PBF mRNA expression. Eight fixed tumor specimens and three normal breast samples yielded both mRNA and promoter sequencing data. PBF expression was apparent in 86% of three and five heterozygotes but only 25% of three and three homozygotes (P = 0.044). Thus, PBF is estrogen regulated; its expression is higher in ER-positive than ER-negative tumors, and a greater number of ERE repeats is associated with higher PBF expression.

PBF and estrogen both induce invasiveness in MCF-7 cells. The exact function of PBF in cell transformation is not known (8). Given that PBF is induced in breast cancer and that invasion and metastasis are critical processes in breast cancer progression, we next assessed whether PBF might play a role in cell invasion.

When 1 × 10⁵ cells per well were seeded in invasion assays, PBF overexpression was associated with a 2.5 ± 0.6-fold increased cell invasion compared with VO treatment at 24 hours (n = 6, P < 0.01) and a 6.1 ± 2.9-fold increase at 48 hours (n = 6, P < 0.01; Fig. 4A). At a density of 2 × 10⁵ cells per well, PBF induced a 2.5 ± 0.3-fold increase in cell invasion after 48 hours (n = 6, P < 0.01).

To determine whether the enhanced invasiveness of MCF-7 cells following PBF transfection reflected an increase in cell proliferation, MCF-7 cells were transfected with VO or PBF, and cell number estimated after 24 and 48 hours using MTT assays (Fig. 4B). PBF did not significantly increase the proliferation of MCF-7 cells after 24 hours but did marginally (∼10%) increase cell number after 48 hours. These data suggest that increased proliferation does not explain PBF’s influence upon invasion.

PBF confers estrogen induction of MCF-7 cell invasion. As estrogen induces PBF and MCF-7 cells are well documented as an estrogen-responsive cell line, we next examined the influence of treatment with 10 nmol/L DES on cell invasion. DES treatment induced MCF-7 cell invasion by ∼2.3-fold compared with vehicle treatment (n = 3, P < 0.001; Fig. 5A). DES and EST (10 and 20 nmol/L) treatment did not significantly alter MCF-7 cell proliferation (data not shown).

Subsequently, we validated transient knockdown of PBF in MCF-7 cells using siRNA. PBF-specific siRNA (50 nmol/L) elicited ∼80% to 90% knockdown compared with a scrambled control (Fig. 5B). Cell invasion assays were then repeated...
Figure 3. A, expression of PBF mRNA relative to 18 s rRNA (ΔCt values) in 20 TMA samples of breast tumors compared with normal breast. ND, not detected after 40 cycles of PCR. B, representative immunohistochemical examination of PBF staining in one normal breast sample (US BioMax) and seven tumor samples from TMA sections. Columns 1 to 4 represent the different staining intensities observed, from 0 ( absent) to +3 (intense). Values in the bottom right-hand corners indicate the percentage of PBF expression observed in the whole section, with original magnifications annotated next to tumor type. C, representative immunohistochemical examination of PBF staining in three normal breast samples (N1–N3; US BioMax; ×40 original magnification) and three tumor samples from TMA sections. T1, Grade I; T2, Grade II; T3, Grade III; all ×40 original magnification.
after 24 hours in the presence of 10 nmol/L DES or vehicle. Critically, the increased cell invasion observed after DES treatment in the presence of a scrambled control (103 ± 17 cells, \( P = 0.002, n = 2 \)) was abolished when PBF was simultaneously knocked down using a PBF-specific siRNA (27 ± 14 cells, \( P = \) not significant, \( n = 2 \); Fig. 5C). These data suggest that estrogen induction of MCF-7 cell invasion through Matrigel is mediated via PBF.

**PBF is a secreted protein.** As the induction of invasion by PBF could not be explained by increased cell number and because cell invasion is a process frequently associated with secretion, we assessed whether PBF is a secreted protein. MCF-7 cells were grown in the presence of l-leucine [3,4,5-3H], cell medium fractions were harvested after 24 hours, and immunoprecipitations were carried out with our PBF antibody (Fig. 6). Immunoprecipitation of labeled PBF revealed that PBF is indeed a secreted protein with vector-only transfected MCF-7 cells showing that ~20% of total cellular PBF is secreted over the experimental time frame of 24 hours (Fig. 6A). This was significantly enhanced by transient overexpression
of PBF (49 ± 3% secretion, n = 3, P < 0.05 compared with VO). Furthermore, a mutant of PBF lacking amino acids 29 to 93 (and hence, a functional signal peptide and two potential putative glycosylation sites; ref. 6) showed reduced secretion into the cell medium compared with WT (28 ± 7%, n = 3) but did not differ statistically from VO (Fig. 6A).

To confirm that PBF is secreted into the cell medium, we further carried out Western blotting for PBF-HA. Cell lysates showed successful transfection of PBF-HA (Fig. 6B). Wild-type PBF is a putative glycoprotein (10), which runs as a doublet at around 25 to 30 kDa. Mutant Δ29-93 was, as anticipated, smaller and ran at ~20 kDa (Fig. 6B). Whereas WT PBF was easily detectable in cell media, confirming that PBF is a secreted protein, mutant Δ29-93 was not apparent.

To investigate the mechanism by which PBF is secreted, MCF-7 cells were cotransfected with PBF-HA and chromogranin A-GFP. Chromogranin A-GFP is a chimeric photoprotein that is transported via the regulated pathway for exocytosis (24) and has recently been used as a marker for secretory granules in MCF-7 cells (25). As shown previously in other cell lines, PBF-HA was found within intracellular vesicles, often detected toward the periphery of cells (6), in keeping with a secretory phenotype (Fig. 6C). Although these vesicles were localized in a similar pattern to the chromogranin A-GFP–labeled secretory granules, no colocalization with PBF was observed. This suggests that PBF is secreted via the constitutive pathway of secretion rather than via regulated secretion. Overall then, PBF is secreted by MCF-7 cells, higher expression results in increased secretion, and deletion of the amino acid region Δ29-93 results in significantly attenuated secretion into the cell medium.

**Induction of MCF-7 cell invasion is modulated by PBF secretion.** Having shown that PBF is secreted by MCF-7 cells, we investigated the relationship between secretion and invasion. As before, PBF induced significant cell invasion when overexpressed (269 ± 52 invading cells, n = 10 experiments, P = 0.018 compared with VO control; Fig. 6C). However, mutant Δ29-93 failed to induce cell invasion compared with VO (161 ± 45 invading cells, n = 10 experiments, P = 0.518 compared with VO). Thus, secretion of PBF contributes significantly to its induction of cell invasion.

In summary, PBF is a relatively uncharacterized protooncogene that is induced by estrogen in MCF-7 cells, shows increased expression in breast cancer, and stimulates cell invasion, at least in part through secretion. On this basis,
Figure 6. Effect of WT and mutant PBF overexpression on the secretion and invasiveness of MCF-7 cells. A, the percentage of total PBF secreted into the medium extracted from MCF-7 cells. Mutant Δ29-93 lacks amino acids 29 to 93. B, Western blot of cell medium and whole-cell lysate extracted from MCF-7 cells transfected with VO, WT PBF, and the Δ29-93 PBF mutant. PBF constructs were HA-tagged. Whereas WT PBF is detectable in the cell medium, mutant Δ29-93 is not. C, immunofluorescent subcellular analysis of MCF-7 cells cotransfected with PBF-HA and chromogranin A-GFP. i and iv, vesicular PBF-HA expression (red); ii and v, chromogranin A-GFP expression; iii and vi, merged image of PBF-HA and chromogranin A-GFP. D, cell invasion assays. In contrast to WT PBF, mutant Δ29-93 failed to induce statistically significant cell invasion. Columns, mean values; bars, SEM. n = 10.
PBF warrants further and intensive study in the context of breast cancer initiation and progression.

Discussion

Numerous genetic changes governing the initiation, progression, and metastasis of breast cancer have already been described, but new genetic markers and therapeutic targets are vital for continued progress in addressing the approximately half-million global deaths annually from the disease. Based on our investigations, we propose four principle lines of evidence suggesting a critical role of PBF in breast cancer. First, PBF is highly expressed in breast tumors, where its expression correlates with ER positivity. Second, it is regulated by DES and EST, both at the mRNA and protein level. Third, PBF upregulation results in significant MCF-7 cell invasion through Matrigel, a phenomenon highly dependent upon PBF secretion. Fourth, repressing PBF expression in the face of estrogen stimulation prevents estrogen-mediated induction of cell invasion. Hence, estrogen regulates PBF expression in MCF-7 cells, tumors show increased levels of the protein, and its functional property in promoting invasion can be ameliorated both through mutation and knockdown.

Although highly conserved, the 180–amino acid peptide sequence of PBF shares no significant homology with other human proteins. Its exact mechanisms of action have not been defined, but it has been shown to induce tumors in nude mice (8), to regulate expression and function of the sodium iodide symporter (3, 6), and to interact with the human securin PTTG (4). Our experiments are the first to report a role for PBF in the breast and to elucidate a function in cell invasion. We now propose that estrogen regulates PBF mRNA and protein expression and suggest that this is mediated predominantly by a cluster of ERE half-sites ∼300 bp upstream of the translational start site.

One surprising finding of our preliminary sequence analysis was that the human PBF promoter is polymorphic for an 18-bp repeat housing a putative ERE half-site. Importantly, a higher number of putative EREs were statistically associated with greater PBF mRNA expression in the breast and ER-positive tumors having significantly increased PBF protein expression compared with ER-negative tumors. Although these findings would need to be confirmed in a larger series of samples, a higher number of EREs in a breast tumor would therefore predict a greater response to circulating estrogen and hence increased expression of a known transforming gene.

Estrogen has previously been shown to enhance expression of PTTG, the binding partner of PBF (12, 13), and PTTG upregulation has been described in breast cancer (14). Whereas it was not the focus of the current investigation, it would be interesting to correlate PTTG and PBF expression in individual breast tumors. The functional implications of simultaneous overexpression are hard to gauge, given that both proteins are inherently multifunctional and likely to have dependent and independent modes of action. However, future studies may delineate the individual contributions of each gene to breast cancer initiation and progression.

Our in vitro experiments were predominantly carried out in MCF-7 cells, which remain the “gold standard” ERα-positive and estrogen-sensitive breast cell line (26). Furthermore, MCF-7 cells are weakly invasive (27), allowing us to perform physiologically relevant invasion assays.

Our wider clinical associations did not reveal striking associations between PBF expression and tumor phenotype, which was unexpected. Clinical associations were hampered by a lack of a matched normal/tumor cohort, which would allow a more detailed interrogation of the association between promoter polymorphism and clinical outcome. Furthermore, fresh tissue would have allowed us to perform Western blotting for PBF expression, which would have provided more quantitative expression data than through immunohistochemistry on FFPE slides.

The mechanism by which PBF induces cell transformation is not known. Our current study suggests that this might be via the induction of cell invasion. MCF-7 cells showed potent increases in invasion through Matrigel in response to PBF transfection. A mutant PBF, which was not secreted into the cell medium, lost this phenotype. Furthermore, a specific siRNA entirely blocked estrogen induction of cell invasion. Thus, we hypothesize that estrogen induces PBF, which in turn drives breast cell invasion, at least in part through secretion. As estrogen stimulation of PBF expression was abrogated by cotreatment with the anti-estrogenICI 182780, ER antagonists might be useful in treating tumors with high PBF expression to block potential cell invasion mediated by PBF.

In summary, we present evidence that the poorly characterized proto-oncogene PBF has particular relevance to breast tumorigenesis, particularly with respect to progression. Based on our in vitro and ex vivo findings, we predict that estrogen treatment in postmenopausal women may upregulate PBF expression, leading to increased PBF secretion. Given that individuals have different numbers of ERE repeats, it is likely that estrogen will stimulate PBF expression variably, with a potentially critical effect upon breast cell invasion.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Prof. Richard Cheney (University of North Carolina) for providing the chromogranin A-GFP construct.

Grant Support

IDA Cooper Foundation and Medical Research Council.

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Received 09/28/2009; revised 02/05/2010; accepted 02/13/2010; published OnlineFirst 4/20/2010.
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Cancer Res 2010;70:3739-3749. Published OnlineFirst April 20, 2010.

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doi:10.1158/0008-5472.CAN-09-3531

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