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PC Cell-Derived Growth Factor Mediates Tamoxifen Resistance and Promotes Tumor Growth of Human Breast Cancer Cells

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ABSTRACT

PC cell-derived growth factor, also known as progranulin, is an M_r 88,000 growth factor (referred as PCDGF/GP88) overexpressed in human breast cancer. Antisense inhibition of PCDGF/GP88 expression in MDA-MB-468 cells inhibited tumor formation in nude mice. In estrogen receptor-positive cells, PCDGF/GP88 was expressed in response to estradiol and shown to mediate its mitogenic effect. Pathologic studies indicated that PCDGF/GP88 was expressed in 80% of invasive ductal carcinomas in correlation with parameters of poor prognosis. In the present article, the relationship between PCDGF/GP88 expression and tamoxifen resistance was examined in MCF-7 cells. PCDGF/GP88 overexpression rendered MCF-7 cells able to proliferate in the absence of estrogen and in the presence of tamoxifen. The PCDGF/GP88-overexpressing cells formed tumors in ovariectomized nude mice in the absence of estradiol and in its presence, in contrast to MCF-7 cells. Tumor growth of the overexpressing cells was increased significantly when the mice were treated with tamoxifen. PCDGF/GP88 blocked tamoxifen-induced apoptosis by preventing down-regulation of bcl-2 expression and poly(ADP-ribose) polymerase cleavage. In addition, PCDGF/GP88-overexpressing cells presented higher level of the angiogenic factors vascular endothelial growth factor and angiopoietin-1 than MCF-7 control cells. Tamoxifen treatment additionally increased the level of vascular endothelial growth factor. These studies suggest that PCDGF/GP88 plays a critical role in breast cancer tumorigenesis and in the transition to estrogen independence and tamoxifen resistance, a hallmark of poor prognosis. On the basis of the *in vivo* studies, it is postulated that tamoxifen treatment of patients with estrogen receptor-positive breast tumors overexpressing PCDGF/GP88 could have adverse clinical consequences.

INTRODUCTION

Antiestrogen therapy is widely used for the management of breast cancer because it is well tolerated unlike conventional cytotoxic chemotherapy regimens. Tamoxifen has been the major agent used for this purpose until the recent development and clinical application of novel estrogen receptor (ER) antagonists such as ICI 182,780 (1). The inhibitory effect of tamoxifen is observed almost exclusively in breast tumors that are ER^+ because estrogen is the major growth stimulator for these types of tumors. However, after prolonged antiestrogen hormonal therapy, breast cancer often progresses from an estrogen-sensitive state to an estrogen-insensitive state (2). In this case, the growth of breast tumors that was inhibited previously by tamoxifen becomes refractory to tamoxifen treatment. Although the development of tamoxifen resistance may be associated with the acquisition of an ER^- phenotype, tamoxifen resistance also has been observed in ER^+ tumors (3, 4). In these later cases, constitutive overexpression of autocrine growth factor or growth factor receptor by tumor cells has been proposed as one possible mechanism for developing tamoxifen resistance (5). Such increased autocrine or paracrine growth factor signaling network then could bypass the need for ER-mediated growth stimulation in human breast cancer cells and would render antiestrogen therapy ineffective. For example, clinical studies have reported a decreased efficacy of tamoxifen for tumors overexpressing c-erbB2 (6). In addition to inhibiting the growth-promoting effect of estrogen, tamoxifen also has been shown to induce programmed cell death in breast cancer cell lines and clinical samples (7–9). Failure to undergo apoptosis in response to tamoxifen also would confer tamoxifen resistance (10). Therefore, increase in autocrine growth factor signaling that mediates proliferation signals and antiapoptotic signals may induce resistance to tamoxifen therapy.

PC cell-derived growth factor (PCDGF/GP88) is an M_r 88,000 cysteine-rich glycoprotein originally purified as an autocrine growth factor from the conditioned medium of the highly tumorigenic mouse teratoma PC cells (11). PCDGF/GP88, also known as granulin/epithelin precursor or progranulin, is overexpressed in many cancer cells, including teratoma (12), breast cancer (13), ovarian cancer (14), renal carcinoma (15), multiple myeloma (16), and glioblastoma (17). It has been reported that PCDGF/GP88 stimulates proliferation and survival in several cell types, including cancer cells and fibroblasts, endothelial cells, and preimplantation embryos (18). These biological activities are mediated via activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3′-kinase (PI3K) pathways (19). In human breast cancer cells, PCDGF/GP88 expression was stimulated by estradiol in a time- and dose-dependent fashion in ER^+ cells (13). In these cells, PCDGF/GP88 was shown to mediate the mitogenic activity of estrogen by stimulating cyclin D1 expression (20). Inhibition of PCDGF/GP88 expression in ER^- MDA-MB-468 cells by antisense transfection led to a complete inhibition of tumorigenesis in nude mice (21). Pathologic studies in paraffin-embedded breast cancer biopsies have shown that high PCDGF/GP88 expression was found in 60% of ductal carcinomas *in situ* and in 80% of invasive ductal carcinomas (IDCs), whereas normal mammary epithelium and benign tumors tested negative for PCDGF/GP88 (22). In IDCs, PCDGF/GP88 expression correlated with parameters of poor prognosis, such as tumor grade, proliferation index, and p53 positivity. Correlation studies with ER expression in the biopsies indicated that PCDGF/GP88 expression was found in ER^+ and ER^- tumors (22). Interestingly, 20% of ER^+ IDCs expressed high level of PCDGF (21). In the present article, we examined the effect of PCDGF/GP88 overexpression in ER^+ breast cancer cells and investigated the possible correlation between PCDGF/GP88 overexpression and tamoxifen resistance in human breast cancer cells. We used PCDGF/GP88-overexpressing MCF-7 cells as models to determine the effect of PCDGF/GP88 on responsiveness of the cells to tamoxifen *in vitro* and in mouse xenograft models. Our results indicate that the overexpression of PCDGF/GP88 confers tamoxifen resistance, prevents tamoxifen-induced apoptosis in MCF-7 cells, and promotes tumor growth and angiogenesis in the presence of tamoxifen *in vivo*.

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MATERIALS AND METHODS

Materials. 17β-Estradiol (E_2) was purchased from Calbiochem (San Diego, CA). G418, Taq polymerase, and Superscript II were obtained from Life Technologies, Inc. (Rockville, MD). Tamoxifen was purchased from Sigma-Aldrich (St. Louis, MO). The Biopolymer Core Laboratory of the University of Maryland (Baltimore, MD) synthesized the oligonucleotide primers used in the reverse transcription-PCR. All of the pellets were obtained from Innovative Research of America (Sarasota, FL). Enhanced chemiluminescence kit was obtained from Pierce (Rockford, IL). Mouse anti-poly(ADP-ribose) polymerase antibody (anti-PARP) was purchased from Oncogene Research (Boston, MA).

Cell Proliferation Assay. The ER^+ human breast cancer cell line MCF-7 was obtained from American Type Culture Collection (Manassas, VA). Pools of PCDGF/GP88-overexpressing MCF-7 cells (O4 and O7 cells) were developed by transfection of PCDGF-pcDNA3 expression vector as described previously (20). Control MCF-7 cells (MCF-7 EV) were obtained by transfecting empty pcDNA3 vector and selecting G418-resistant cells as described previously (20). Both cell lines were maintained in DMEM/F12 supplemented with 5% fetal bovine serum (FBS) and 400 μg/ml of G418. For proliferation assay, 5×10^4 cells were plated in six-well culture plates (Costar, Cambridge, MA) in phenol red-free α-MEM (PFMEM) supplemented with 5% charcoal-extracted FBS. Cells were treated with either 1 nM E_2 alone or in combination with 1 μM tamoxifen. Control cells were treated with vehicle alone (0.01% DMSO). Medium was changed on day 4. Cell numbers were counted by hemocytometer. Each time point was performed in triplicate. Doubling time was calculated by nonlinear regression using Sigma Plot version 8.0 software.

[^{3}H]-Thymidine Incorporation Assay. Cells were inoculated at a density of 4×10^4 cells per well on 24-well dishes in DMEM/F12 supplemented with 5% FBS. After 24 h of incubation, the medium was changed to PFMEM and supplemented with 5% charcoal-extracted FBS. After 24 h, cells were washed once with medium, and medium was changed to serum-free PFMEM. The cells were treated for 24 h with vehicle only, 1 nM of E_2 alone, or with increasing doses of tamoxifen. Thymidine incorporation assay was performed in triplicate as described previously (13).

***In Vivo* Tumorigenesis Assay in Nude Mice.** MCF-7 EV or O4 cells (5×10^6 cells per site) were injected s.c. at two sites into 6-week-old ovariectomized athymic female nude mice (National Cancer Institute, Frederick, MD). E_2 pellets (1.7 mg; 60-d release) or placebo pellets were implanted s.c. into the back 1 day before inoculating the cells. Five to 10 nude mice per experimental group were used depending on the experiments. For the *in vivo* tamoxifen resistance experiment, the animals that had received E_2 pellets and been injected with the cells were implanted with tamoxifen base pellets (5 mg; 60-day release) or placebo pellets 10 days after the cell inoculation. The width (W) and length (L) of individual tumors were measured weekly with a caliper. Average tumor volume was calculated with the widely used formula: tumor volume = $(W^2 \times L) \times 0.5$. The Institutional Animal Care and Use Committee of the University of Maryland, Baltimore approved all of the animal studies.

Measurement of ERE-Luciferase Reporter Gene Activity. MCF-7 EV or O4 cells (2.5×10^5 cells) were plated in PFMEM supplemented with 5% FBS in six-well plates. Cells were transfected transiently with pGL2-ERE-luciferase plasmid DNA by Lipofectamine (Life Technologies, Inc.). The pcDNA3 β-galactosidase construct was cotransfected as an internal control to determine transfection efficiency. One nM E_2 and/or 1 μM tamoxifen were added 5 h after transfection. Cell lysates from triplicate dishes were collected using reporter lysis buffer 36 h after transfection. Determination of luciferase and β-galactosidase activities was performed using kits following the manufacturer's protocols (Promega, Madison, WI). Luciferase activities were normalized to the transfection efficiency determined by measuring β-galactosidase activity of each sample.

Determination of mRNA Expression for bcl-2, bcl-x_L, bax, VEGF, Angiopoietin-1, and Angiopoietin-2 by Reverse Transcription-PCR. Five μg of total RNA were reverse transcribed into single-strand cDNA by Superscript II (Life Technologies, Inc.) using 250 ng random hexamer (Life Technologies, Inc.) as primer. The reverse transcription reaction was carried out for 1 h at 42°C in 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 0.01 M DTT, and 0.5 mM deoxynucleoside triphosphate. A total of 30–35 PCR cycles, depending on the gene amplified, was performed, followed by electrophoresis on 1% agarose gel. The specific primer pairs used were

for glyceraldehyde 3-phosphate dehydrogenase: forward primer, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and reverse primer, 5′-CATGTGGGCCATGAGGTCCACCAC-3′;

for bcl-2: forward primer, 5′-GGTGCCACCTGTGGTCCACCTG-3′ and reverse primer, 5′-CTTCACTTGTGGCCCAGATAGG-3′;

for bax: forward primer, 5′-GAGCAGATCATGAAGACAGGGG-3′ and reverse primer, 5′-CTCCAGCAAGGCCCAGCGTC-3′;

for bcl-x_L: forward primer, 5′-CAGTGAGTGAGCAGGTGTTTTGG-3′ and reverse primer, 5′-GTTCCACAAAAGTATCCCAGCCG-3′;

for vascular endothelial growth factor (VEGF): forward primer, 5′-ATGAACTTTCTGCTGTCTTGGGT-3′ and reverse primer, 5′-TCACCGCCTCGGCTTGTCAC-3′;

for angiopoietin-1: forward primer, 5′-TTGCTTTCCTCGCTGCCATTC-3′ and reverse primer, 5′-CAGCATGGTAGCCGTGTGGTTC-3′;

for angiopoietin-2: forward primer, 5′-AGCTACACTTTCCTCCTGCCAG-3′ and reverse primer, 5′-AGCCGTCTGGTTCTGTACTGC-3′.

Western Blot Analysis of PARP Cleavage. Cells were seeded at a density of 7×10^5 cells in a 60-mm dish with DMEM/F12 supplemented with 5% FBS. After 24 h, medium was changed to serum-free phenol red-free DMEM/F12 supplemented with vehicle or purified PCDGF/GP88 (400 ng/ml) for another 24 h. Cells were treated with either vehicle only or factors under investigation for 24 h. Cell lysates were collected in RIPA buffer [50 mM Tris-HCl (pH 7.4), containing 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, and protease inhibitor mixture] containing 6 M urea. One hundred μg of protein from each sample were used for immunoblotting. Intact and cleaved forms of PARP were detected using a mouse monoclonal anti-PARP antibody. The band intensity of the PARP cleaved form was determined by densitometric analysis and normalized to the actin level used as internal standard.

Statistical Analysis. All of the experiments were conducted in triplicate and repeated at least twice. Data were analyzed by Student's *t* test for mean comparison and statistical significance. The values are reported as mean ± SE.

RESULTS

Overexpression of PCDGF/GP88 Promotes Growth and Confers Tamoxifen Resistance *in Vitro*. We first compared the effect of increasing doses of tamoxifen on the proliferation of PCDGF/GP88-overexpressing MCF-7 cells (O4 and O7 cells) and the empty vector transfected MCF-7 EV. As shown in Fig. 1*A*, DNA synthesis in PCDGF/GP88-overexpressing cells (O4 and O7) was not affected by tamoxifen, even at doses (1 μM) that inhibited MCF-7 EV proliferation by 90%. Similar results were observed (Fig. 1*B*). PCDGF-overexpressing cells could proliferate in the absence of E_2 (doubling time O4, 42.1 ± 0.7 h; O7, 39.1 ± 1.95 h; $P > 0.05$) and in the presence of E_2 alone (doubling time O4, 37.3 ± 1.6 h; O7, 35.4 ± 2.2 h; $P > 0.05$) or with 1 μM tamoxifen (doubling time O4, 39.1 ± 1.4 h; O7, 37.4 ± 2.4 h; $P > 0.05$), close to the doubling time of MCF-7 EV cells in the presence of E_2 (37.9 ± 1.0 h). In the absence of E_2, doubling time of MCF-7 was 61.8 ± 1.0 h. On the basis of these data, all of the subsequent experiments were carried out with O4 cells as representative of PCDGF/GP88-overexpressing cells.

PCDGF/GP88 Overexpression Mediates Estrogen-Independent Tumor Growth *in Vivo*. Because PCDGF-overexpressing cells were able to proliferate in the absence of E_2 *in vitro*, their ability to form tumors in ovariectomized nude mice implanted with either placebo or E_2 pellets was determined. Tumor sizes were determined as described in "Materials and Methods." As shown in Fig. 2, O4 cells formed tumors in placebo- and E_2 pellet-implanted ovariectomized mice, whereas MCF-7 EV cells formed tumors only in mice receiving E_2 pellets (mean tumor volume of MCF-7 with E_2 = 42.4 ± 4.0 mm^3). The incidence of tumor formation for O4 cells in placebo- and E_2-treated ovariectomized mice (70–90%) was higher than for MCF-7 EV cells in E_2-treated mice (33–40%). Interestingly, unlike the tumor incidence, the mean volume of O4 tumors was larger in mice treated with E_2 pellets (148.3 ± 59.3 mm^3; $P < 0.045$) than in mice receiving placebo (62.2 ± 7.0 mm^3). This would suggest that although PCDGF/

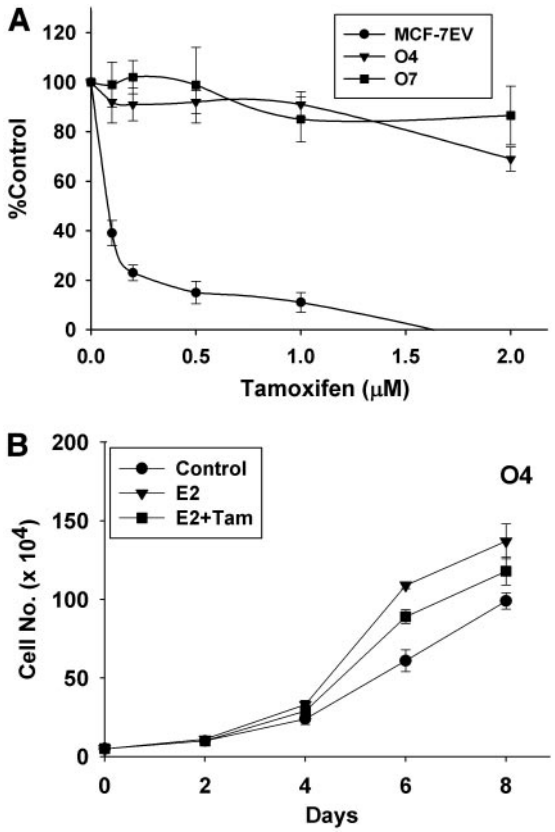

Fig. 1. Effect of 17β-estradiol (E_2) and tamoxifen on the proliferation of O4 and MCF-7 EV cells. *A*, effect of tamoxifen on DNA synthesis. MCF-7 EV (●), O4 cells (▼), and O7 cells (■; 4×10^4 cells/well) were incubated in the presence of 1 nM E_2 alone or in combination with increasing doses of tamoxifen. DNA synthesis was measured by thymidine incorporation, as described in "Materials and Methods." Data are represented as percent of control corresponding to DNA synthesis of cells treated with E_2 only. Experiments were performed in quadruplicate. *B*, long-term growth in estrogen-depleted medium. PCDGF/GP88-overexpressing O4 cells were cultivated in phenol red-free α-MEM containing 5% charcoal-extracted fetal bovine serum supplemented with vehicle (●), 1 nM E_2 alone (▼), or in combination with 1 μM tamoxifen (■).

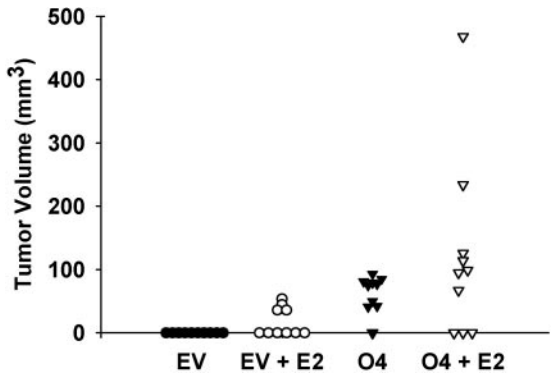

Fig. 2. *In vivo* tumor growth of MCF-7 EV and O4 cells in ovariectomized nude mice in the absence or presence of estradiol. MCF-7 EV and O4 cells were injected s.c. into two sites of ovariectomized athymic nude mice that had received 17β-estradiol (E_2; 60-day release) or placebo pellets. Mice were monitored daily for tumor appearance. Data provided correspond to the tumor volume at 45 days for individual tumors.

GP88-overexpressing cells could proliferate in the absence of E_2, they maintained responsiveness to E_2 *in vivo*. This finding was compatible with the fact that PCDGF/GP88-overexpressing cells presented similar levels of ER expression than MCF-7 EV cells (20). These data indicate that PCDGF/GP88 overexpression in breast cancer cells increased the tumorigenicity of the cells and conferred them the ability to form tumors in the absence of estrogen.

Tamoxifen Treatment Stimulates Growth of PCDGF/GP88-Overexpressing Cells *in Vivo*. Because tamoxifen failed to inhibit O4 cells *in vitro*, its effect on O4 cell tumorigenesis then was examined in mouse xenografts as described in "Materials and Methods." As shown in Fig. 3*A*, tamoxifen inhibited MCF-7 EV tumor formation (85% inhibition; $P < 0.05$) in agreement with the reported inhibitory effect of tamoxifen in ER^+ MCF-7 tumor growth. In contrast, O4 cells formed tumors in mice with or without tamoxifen without any change in tumor incidence (100%). Interestingly, monitoring of tumor volume during the 45 days after tamoxifen implant showed a statistically significant stimulation of tumor growth in the tamoxifen-treated group when compared with placebo control at each time point (Fig. 3*A*). As shown in Fig. 3*B*, the increase in volume of O4 tumors during the 45 days (V^{45}/V^0) was significantly higher in tamoxifen-treated mice (6.69 ± 0.43) than in placebo-treated mice (4.77 ± 0.70; $P < 0.05$). These data demonstrate that PCDGF/GP88 overexpression in breast cancer cells confers estrogen independence and tamoxifen resistance *in vivo* and that tamoxifen potentiates tumor growth in cells that overexpressed PCDGF/GP88.

It is known that tamoxifen can act as an ER agonist and antagonist depending on the tissue types and the distribution of specific ER coactivators and corepressors (23). Because tamoxifen appears to have growth-stimulatory activity *in vivo* for PCDGF/GP88-overex-

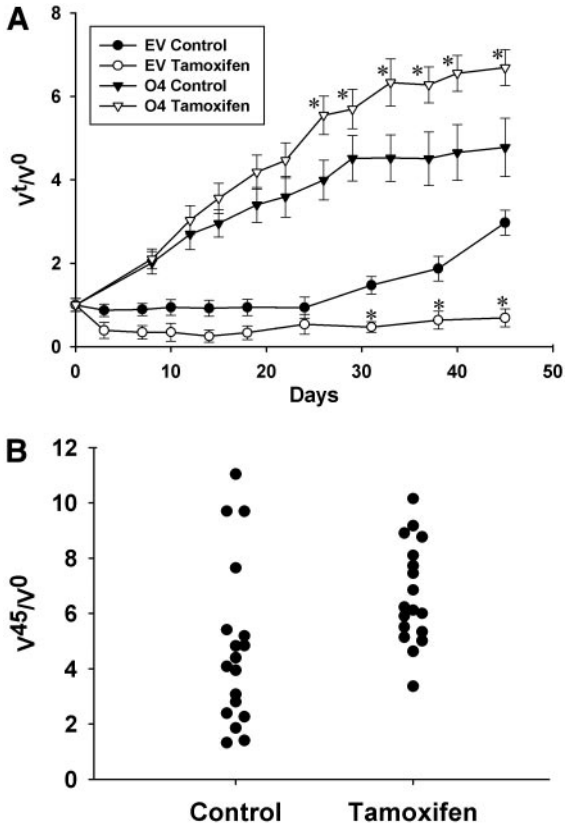

Fig. 3. Effect of tamoxifen on the growth of O4 cells in nude mice. Twenty female nude mice were implanted with 17β-estradiol pellets 1 day before O4 cells were injected (two sites per mouse) as described in "Materials and Methods." After 10 days, when the tumors were visible, the mice were separated in two groups that received either placebo pellets or tamoxifen pellet (referred as day 0). Tumor growth was monitored as described previously for an additional 45 days. At the end of the experiments, the tumors were excised to extract RNA for the reverse transcription-PCR described below. *A*, time course of MCF-7 EV and O4 tumor development in the absence (▼ and ●) or presence (▽ and ●) of tamoxifen. *B*, growth of O4 tumor expressed as V^{45}/V^0 for individual tumors measured at day 45 (V^{45}) after tamoxifen or placebo treatment. The increase in tumor volume (V^t/V^0) was expressed as the ratio of mean tumor volume determined at each time point (V^t) and over the one at day 0 (V^0; time when tamoxifen or placebo pellets were implanted). Values are expressed as mean ± SE. $*P < 0.05$.

pressing cells, we examined whether tamoxifen could act as an ER agonist instead of an ER antagonist in these cells. For this purpose, we compared the effect of tamoxifen on ERE-luciferase reporter gene activity in MCF-7 EV and O4 cells in the presence and absence of E_2. As shown in Fig. 4, E_2 stimulated ERE-luciferase activity in MCF-7 EV and O4 cells by 4.2- and 3.7-fold, respectively, over the control untreated level. Tamoxifen inhibited E_2-mediated stimulation of ERE-luciferase activity by 80% in MCF-7 EV cells and by 56% in O4 cells ($P < 0.05$). In the absence of E_2, tamoxifen stimulated ERE-luciferase activity by 1.6-fold in the PCDGF-overexpressing cells ($P < 0.05$), whereas it slightly inhibited it in MCF-7 EV cells. These data indicate that tamoxifen maintains its ER antagonist effect in PCDGF/GP88-overexpressing cells treated with estrogen. This would suggest that tamoxifen-mediated stimulation of PCDGF/GP88 tumor growth is not simply because of an agonistic effect of tamoxifen and may involve alternative pathways.

PCDGF/GP88 Prevents Tamoxifen-Induced PARP Cleavage. It has been reported that tamoxifen exerts two independent effects on ER^+ breast cancer cells (*i.e.*, inhibition of ER function and activation of apoptosis via down-regulation of bcl-2; Refs. 9, 24). Because PCDGF/GP88 confers tamoxifen resistance, experiments were carried out to examine whether PCDGF/GP88 was acting by preventing tamoxifen-mediated apoptosis. Initial studies examined whether PCDGF/GP88 treatment inhibited PARP cleavage induced by tamoxifen in MCF-7 EV cells. Cleavage of PARP is a hallmark of caspase-dependent apoptosis (25, 26). As shown in Fig. 5, tamoxifen treatment induced apoptosis of MCF-7 EV cells, as seen by the increase of cleaved PARP form (M_r 85,000) when compared with control untreated cells. PARP cleavage induced by tamoxifen in MCF-7 EV cells was inhibited by the addition of PCDGF/GP88 (80% inhibition; $P < 0.05$), similarly to E_2 taken as a positive control.

PCDGF/GP88 Inhibits Apoptosis by Preventing Tamoxifen-Induced bcl-2 Down-Regulation. Apoptosis is controlled by the ratio of proapoptotic and antiapoptotic factors (27), particularly bcl-2, bcl-x_L, and bax (28). Previous reports have suggested that bcl-2 expression was down-regulated by tamoxifen treatment, leading to activation of apoptosis in MCF-7 cells (9) and in tissues from patients treated with tamoxifen (8). Therefore, the status of bcl-2 expression was examined in MCF-7 EV and O4 cells treated with tamoxifen. As shown in Fig. 6, tamoxifen induced the down-regulation of bcl-2 transcript in MCF-7 EV cells in a dose-dependent manner. In contrast, tamoxifen failed to down-regulate bcl-2 in O4 cells even at doses that completely down-regulated bcl-2 expression in MCF-7 EV cells. Interestingly, bax or bcl-x_L expression in MCF-7EV cells or O4 cells remained unchanged at all of the tamoxifen doses tested. These data suggest that prevention of tamoxifen-induced apoptosis and bcl-2 down-regulation by PCDGF/GP88 is one pathway leading to tamoxifen resistance in breast cancer cells that overexpress this growth factor.

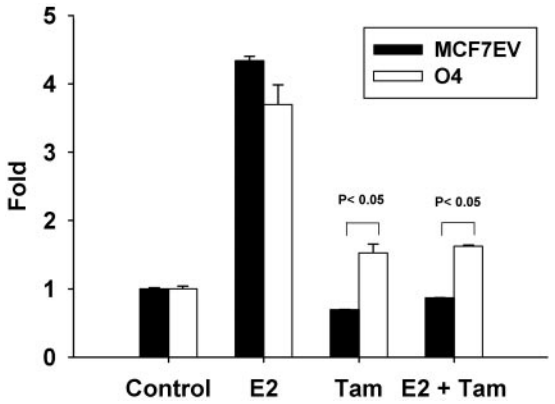

Fig. 4. Effect of 17β-estradiol (E_2) and tamoxifen on ERE-luciferase reporter gene activity in MCF-7 EV and O4 cells. Cells were cotransfected with pGL2-ERE-luciferase and β-galactosidase reporter gene constructs. E_2 (1 nM) and/or tamoxifen (1 μM) were added after transfection. Cell lysates were collected after 36 h to assay luciferase activity. The data for luciferase activities are expressed as fold of activation above the control untreated cells.

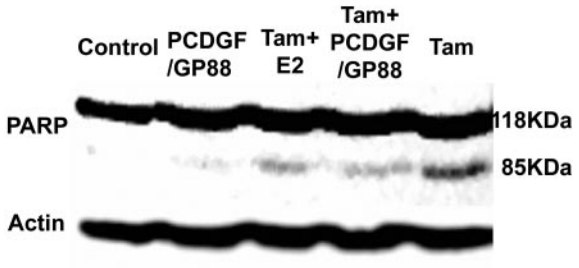

Fig. 5. PCDGF/GP88 prevents poly(ADP-ribose) polymerase (*PARP*) cleavage and inhibits apoptosis induced by tamoxifen in MCF-7 EV cells. MCF-7 EV cells were cultivated in estrogen-depleted medium and treated with tamoxifen (*Tam*), 17β-estradiol (E_2), or PCDGF/GP88 as indicated in "Materials and Methods." The level of PARP cleavage was determined by the presence of M_r 85,000 band (*top*). Level of β-actin was determined as internal control for equal loading (*bottom*). Data are representative of three independent experiments.

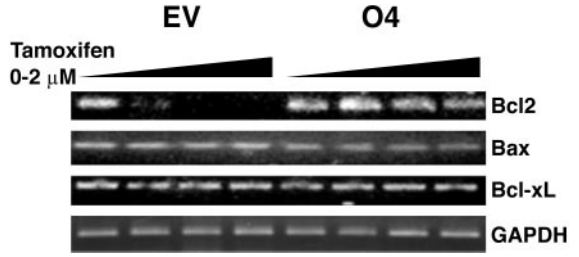

Fig. 6. PCDGF/GP88 prevents down-regulation of tamoxifen-induced bcl-2 expression; bcl-2, bcl-x_L, and bax mRNA expression was determined by semiquantitative reverse transcription-PCR of RNA extracted from MCF-7 EV and O4 cells cultivated with increasing concentrations of tamoxifen (0.5, 1, and 2 μM) as described in "Materials and Methods." Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression was used as internal control for loading.

Tamoxifen Treatment Stimulates Angiogenesis in PCDGF/GP88-Overexpressing Cells. Our *in vivo* studies have shown that the tumor formation of O4 cells in mice treated with E_2 and tamoxifen was increased by tamoxifen (1.4-fold). This indicated that O4 cells not only failed to be inhibited by tamoxifen but also were growth stimulated by tamoxifen *in vivo*. Because tamoxifen did not appear to have a direct growth-stimulatory effect on PCDGF/GP88-overexpressing cell proliferation *in vitro* (Fig. 1), we hypothesized that the stimulation of tumor growth in nude mice could be because of the *in vivo* recruitment of other growth-stimulatory pathways that would provide a growth advantage to the developing tumors. One such possibility was to examine the expression of angiogenic factors in PCDGF/GP88-expressing cells. An angiogenic factor microarray identified VEGFs and angiopoietin-1 and angiopoietin-2 as being expressed by O4 and MCF-7 EV cells (data not shown). Reverse transcription-PCR then was used to compare the expression of these angiogenic factors *in vivo* in MCF-7 EV and O4 cell tumors obtained from mice treated with E_2 alone or with tamoxifen (Fig. 7). Because tamoxifen induced tumor regression in MCF-7 EV cells, MCF-7 EV tumors from tamoxifen-treated mice were not included in these comparative studies. The primer sets selected for VEGF permitted detection of six VEGF transcripts: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$. Only $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ were detectable in these tumors. As shown in Fig. 7, O4 tumors expressed much higher

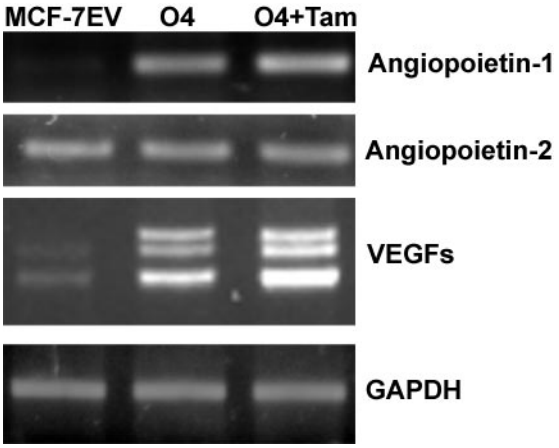

Fig. 7. Up-regulation of angiogenic factors in O4 tumors from tamoxifen-treated mice. Expression of vascular endothelial growth factor (*VEGF*), angiopoietin-1, and angiopoietin-2 was examined by reverse transcription-PCR using RNA extracted from MCF-7 EV tumors developed in mice treated with 17β-estradiol (E_2; *MCF-7 EV*), from O4 tumors in mice treated with E_2 (*O4*), and from O4 tumors in mice treated with E_2 and tamoxifen (*O4 + Tam*; four tumors per experimental condition). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression was included as an internal control.

levels of VEGFs (10.4 ± 2.4-fold) and angiopoietin-1 (9.8 ± 1.4-fold) than MCF-7 EV tumors. Angiopoietin-2 level remained unchanged. The expression of VEGFs and angiopoietin-1 in O4 tumors from tamoxifen-treated mice was 3.2 ± 1.2-fold and 1.3 ± 0.1-fold higher, respectively, than in O4 tumors from placebo-treated mice. These data indicate that PCDGF/GP88 stimulates the expression of angiogenic factors *in vivo* and that tamoxifen potentiates PCDGF/GP88 effect.

DISCUSSION

The present study demonstrates that PCDGF/GP88 overexpression confers estrogen independence for growth and tamoxifen resistance to the human breast cancer MCF-7 cells. This was shown not only by overexpressing PCDGF/GP88 in MCF-7 cells but also by direct treatment of MCF-7 cells with PCDGF/GP88. In addition, it was demonstrated that tamoxifen-resistant MCF-7 cells, selected by culturing the cells in the continuous presence of tamoxifen, expressed higher level of PCDGF/GP88 than tamoxifen-sensitive cells, thereby suggesting a direct relationship between PCDGF/GP88 overexpression and tamoxifen resistance.[4] Estrogen-independent growth of O4 cells was observed *in vitro* and *in vivo*. Notably, PCDGF/GP88-overexpressing O4 cells formed tumors in ovariectomized mice even without E_2 supplementation, unlike MCF-7 EV cells. The tumor incidence and the tumor volume for O4 tumors were significantly higher than for MCF-7 EV cells in the presence of E_2. This indicates that the overexpression of PCDGF/GP88 is important for tumorigenesis of ER^+ breast cancer cells. It has been reported previously that the inhibition of PCDGF/GP88 expression in the ER^- MDA-MB-468 cells by antisense PCDGF/GP88 cDNA transfection prevented tumor formation in nude mice (21). The present data provide evidence that PCDGF/GP88 overexpression also is important for the tumorigenesis of ER^+ breast cancer cells. This conclusion is in agreement with our pathologic studies reporting high PCDGF/GP88 staining in ER^+ and ER^- IDCs (22). In addition, we have found that O4 cells maintained unchanged levels of functional ER expression as shown by the comparable activation of ERE-luciferase reporter gene construct by E_2 in O4 and MCF-7 EV cells (20). These data and the estrogen-independent growth of O4 cells *in vitro* and *in vivo* shown here support the hypothesis that the effect of PCDGF/GP88 in promoting tumor growth is the result of a direct autocrine effect of the growth factor. Although the PCDGF/GP88-overexpressing cells proliferated in estrogen-depleted conditions, they retained growth responsiveness to E_2 *in vitro* and particularly *in vivo*. The mean O4 tumor volume was increased significantly in mice treated with E_2 pellets compared with placebo pellets. This would suggest that PCDGF/GP88 does not mediate all of the E_2 growth-promoting effects and that E_2 and PCDGF/GP88 signaling pathways have additive effects.

In addition to rendering the growth of breast cancer cells estrogen independent, overexpression of PCDGF/GP88 led to tamoxifen resistance *in vitro* and *in vivo*. Resistance to tamoxifen in PCDGF/GP88-overexpressing cells was observed *in vitro* by thymidine incorporation and long-term proliferation assays. Overexpression of several growth factors or growth factor receptors has been found to be associated with tamoxifen resistance, especially in MCF-7 cells (5). HER-2 receptor overexpression promotes tamoxifen resistance in MCF-7 cells (29). However, in this case, the resistance corresponded to a decreased ability of tamoxifen to inhibit tumor growth *in vivo* rather than a complete loss of tamoxifen response. Blockade of MAPKs in these cells restored tamoxifen sensitivity, suggesting that activation of MAPK was involved in the HER-2-mediated tamoxifen resistance (30). It has been suggested that cross-talk between MAPK and ER enhanced ligand-independent ER activation, resulting in antiestrogen resistance (31, 32). In contrast, other studies have shown that MAPK activation was not sufficient to confer tamoxifen resistance (33). Another signaling pathway, PI3K, also was reported recently to activate ERα in MCF-7 cells (34) and to protect cells from apoptosis (35). Our previous study in MCF-7 cells has shown that PCDGF/GP88 activates MAPK and PI3K signaling pathways (20).[4] Therefore, PCDGF/GP88 overexpression could contribute to tamoxifen resistance by activating either one or both of these pathways. It should be noted that PCDGF overexpression also conferred resistance to the pure ER antagonist ICI 182,780.[4]

Our *in vivo* studies show that PCDGF/GP88-overexpressing cells not only failed to be inhibited by tamoxifen but also formed larger tumors in mice treated with tamoxifen. Growth stimulation by tamoxifen has been reported previously for breast cancer cells transfected with fibroblast growth factor 1 or fibroblast growth factor 4, although the mechanism leading to tamoxifen growth stimulation for fibroblast growth factor 4-overexpressing cells remains unclear (36, 37). The data presented here offer evidence for two possible pathways by which PCDGF/GP88 expression leads to tamoxifen resistance (*i.e.*, inhibition of tamoxifen-induced apoptosis and stimulation of angiogenic factor expression).

It has been reported that the inhibitory effect of tamoxifen on the growth of estrogen-dependent cells involves induction of apoptosis (7, 9, 38). The balance between proapoptotic and antiapoptotic factors determines apoptosis (39). HER-2 overexpression in MCF-7 cells suppresses tamoxifen-induced apoptosis by up-regulating bcl-2 and bcl-x_L proteins (40). Moreover, it has been shown that the down-regulation of bcl-2 is sufficient to induce apoptosis (9). We demonstrate that PCDGF/GP88 overexpression prevented bcl-2 down-regulation induced by tamoxifen, resulting in inhibition of caspase-dependent apoptosis. Tamoxifen had a reduced ability to induce PARP cleavage in O4 cells. In addition, PCDGF/GP88 added exogenously prevented PARP cleavage induced by tamoxifen in MCF-7 EV cells, indicating that it directly prevented tamoxifen-induced apoptosis. The antideath properties of progranulin (PCDGF/GP88) have been reported recently for SW13 tumors (41). In this case, it was shown that overexpression of PCDGF/GP88 prevented anoikis of serum-starved SW13 cells.

[4] Unpublished observations.

As noted previously, only *in vivo* assays in nude mice clearly demonstrated that the tumor growth of PCDGF/GP88-overexpressing cells was increased by tamoxifen treatment. The *in vitro* experiment did not show any increased proliferation of tamoxifen-treated PCDGF/GP88-overexpressing cells. This would suggest that in addition to acting via a direct autocrine stimulation of breast cancer cell growth, PCDGF/GP88 also might promote tumor growth *in vivo* by acting indirectly on the stromal components found in the environment of the tumor, such as the endothelial cells. One likely mechanism would be by stimulating angiogenesis. Angiogenesis is associated with tumor progression. Continuous tumor growth requires new blood vessels to supply nutrients. The stimulation of angiogenic factors promotes tumor growth as new tumor vessels are recruited to the tumor site. Various angiogenic factors have been known to be involved in this process. Among them, VEGF is a major inducer of tumor angiogenesis in breast cancer (42) and is essential for initial *in vivo* growth of human breast carcinoma cells (43). We show an important up-regulation of VEGF and angiopoietin-1 in cells overexpressing PCDGF/GP88 compared with MCF-7 EV cells. The role of angiopoietin-1 and angiopoietin-2 remains unclear (44–47). Angiopoeitin-1 has been shown to act as an angiogenic promoter in embryonic angiogenesis, although its role in tumor neovascularization remains unclear (47). This would suggest that PCDGF/GP88 overexpression leads to stimulation of angiogenesis *in vivo*. In support of its action in angiogenesis, PCDGF/progranulin also was found to potentiate proliferation and promote the formation of tube-like structure in human dermal microvascular endothelial cells (48). No difference in angiopoietin-2 expression could be observed between MCF-7 EV and O4 cells. This is different from MCF-7 cells overexpressing HER-2, in which up-regulation of angiopoietin-2 was reported (49).

Interestingly, our studies indicate that the levels of VEGF and angiopoietin-1 in O4 tumors were stimulated additionally by tamoxifen treatment. Although the tamoxifen-treated MCF-7 tumors were not examined, several published reports have demonstrated that tamoxifen inhibited VEGF expression stimulated by E_2 in MCF-7 cells (50, 51). It is possible that the up-regulation of angiogenic factor expression, particularly VEGF, may be the result of the overexpression of bcl-2 observed in PCDGF/GP88-overexpressing cells. Such a relationship between bcl-2 and VEGF expression has been reported recently in human melanoma via stimulation of VEGF mRNA stability and promoter activation (52). Several cytokines and growth factors, such as tumor necrosis factor α, transforming growth factor β, epidermal growth factor, and insulin-like growth factor I, have been reported to stimulate the expression of angiogenic factors in several cell types (53). Taken together, these data suggest that tamoxifen promotes tumor growth *in vivo* by cooperating with PCDGF/GP88 to stimulate angiogenesis via VEGF.

We have shown previously that inhibition of PCDGF/GP88 expression by antisense transfection or action by treatment with anti-PCDGF/GP88 neutralizing antibodies resulted in inhibition of proliferation *in vivo* and *in vitro* (18). Pathologic studies of PCDGF/GP88 expression in paraffin-embedded biopsies have shown that 80% of IDCs stained positive for PCDGF/GP88 with a high expression (3+) in 60% of IDCs, which is in contrast to normal mammary epithelium and benign tumors. In IDCs, PCDGF/GP88 expression correlated well with prognostic markers such as tumor grade, p53 expression, and high Ki-67 index (22). These data and the fact that PCDGF/GP88 overexpression in breast tumors treated with tamoxifen results in larger tumors in nude mice suggest that tamoxifen treatment may have adverse clinical consequences for patients with PCDGF/GP88-overexpressing breast tumors. Tamoxifen-stimulated phenotype has been observed in 6.6% of patients with ER^+ tumors (54). Whether PCDGF/GP88 overexpression only is seen in these cases or also is implicated in a larger pool of clinical antiestrogen resistance needs to be investigated.

In summary, the studies presented here demonstrate the role of PCDGF/GP88 in the tumorigenesis of ER^+ breast cancer cells. PCDGF/GP88 provides growth and survival advantage by acting as a mitogen for breast cancer cells, inhibiting tamoxifen-induced apoptosis, and promoting tumor angiogenesis *in vivo*. In addition, PCDGF/GP88 overexpression alters cell growth response to estrogen and tamoxifen.

REFERENCES

1. Cummings, F. J. Evolving uses of hormonal agents for breast cancer therapy. Clin. Ther., *24* (Suppl. C)*:* C3–25, 2002.
2. Davidson, N. E. Biology of breast cancer and its clinical implications. Curr. Opin. Oncol., *4:* 1003–1009, 1992.
3. Johnston, S. R., Saccani-Jotti, G., Smith, I. E., Salter, J., Newby, J., Coppen, M., Ebbs, S. R., and Dowsett, M. Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. Cancer Res., *55:* 3331–3338, 1995.
4. Naundorf, H., Jost-Reuhl, B., Becker, M., Reuhl, T., Neumann, C., and Fichtner, I. Differences in immunoreactivity of estrogen receptor (ER) in tamoxifen-sensitive and -resistant breast carcinomas: preclinical and first clinical investigations. Breast Cancer Res. Treat., *60:* 81–92, 2000.
5. Clarke, R., Skaar, T. C., Bouker, K. B., Davis, N., Lee, Y. R., Welch, J. N., and Leonessa, F. Molecular and pharmacological aspects of antiestrogen resistance. J. Steroid Biochem. Mol. Biol., *76:* 71–84, 2001.
6. Carlomagno, C., Perrone, F., Gallo, C., De Laurentiis, M., Lauria, R., Morabito, A., Pettinato, G., Panico, L., D'Antonio, A., Bianco, A. R., and De Placido, S. c-erb B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. J. Clin. Oncol., *14:* 2702–2708, 1996.
7. Kang, Y., Cortina, R., and Perry, R. R. Role of c-myc in tamoxifen-induced apoptosis estrogen-independent breast cancer cells. J. Natl. Cancer Inst. (Bethesda), *88:* 279–284, 1996.
8. Cameron, D. A., Keen, J. C., Dixon, J. M., Bellamy, C., Hanby, A., Anderson, T. J., and Miller, W. R. Effective tamoxifen therapy of breast cancer involves both antiproliferative and pro-apoptotic changes. Eur. J. Cancer, *36:* 845–851, 2000.
9. Zhang, G. J., Kimijima, I., Onda, M., Kanno, M., Sato, H., Watanabe, T., Tsuchiya, A., Abe, R., and Takenoshita, S. Tamoxifen-induced apoptosis in breast cancer cells relates to down-regulation of bcl-2, but not bax and bcl-X(L), without alteration of p53 protein levels. Clin. Cancer Res., *5:* 2971–2977, 1999.
10. Lilling, G., Hacohen, H., Nordenberg, J., Livnat, T., Rotter, V., and Sidi, Y. Differential sensitivity of MCF-7 and LCC2 cells, to multiple growth inhibitory agents: possible relation to high bcl-2/bax ratio? Cancer Lett., *161:* 27–34, 2000.
11. Zhou, J., Gao, G., Crabb, J. W., and Serrero, G. Purification of an autocrine growth factor homologous with mouse epithelin precursor from a highly tumorigenic cell line. J. Biol. Chem., *268:* 10863–10869, 1993.
12. Zhang, H., and Serrero, G. Inhibition of tumorigenicity of the teratoma PC cell line by transfection with antisense cDNA for PC cell-derived growth factor (PCDGF, epithelin/granulin precursor). Proc. Natl. Acad. Sci. USA, *95:* 14202–14207, 1998.
13. Lu, R., and Serrero, G. Stimulation of PC cell-derived growth factor (epithelin/granulin precursor) expression by estradiol in human breast cancer cells. Biochem. Biophys. Res. Commun., *256:* 204–207, 1999.
14. Jones, M. B., Michener, C. M., Blanchette, J. O., Kuznetsov, V. A., Raffeld, M., Serrero, G., Emmert-Buck, M. R., Petricoin, E. F., Krizman, D. B., Liotta, L. A., and Kohn, E. C. The granulin-epithelin precursor/PC-cell-derived growth factor is a growth factor for epithelial ovarian cancer. Clin. Cancer Res., *9:* 44–51, 2003.
15. Donald, C. D., Laddu, A., Chandham, P., Lim, S. D., Cohen, C., Amin, M., Gerton, G. L., Marshall, F. F., and Petros, J. A. Expression of progranulin and the epithelin/granulin precursor acrogranin correlates with neoplastic state in renal epithelium. Anticancer Res., *21:* 3739–3742, 2001.
16. Wang, W., Hayashi, J., Kim, W. E., and Serrero, G. PC cell-derived growth factor (granulin precursor) expression and action in human multiple myeloma. Clin. Cancer Res., *9:* 2221–2228, 2003.
17. Liau, L. M., Lallone, R. L., Seitz, R. S., Buznikov, A., Gregg, J. P., Kornblum, H. I., Nelson, S. F., and Bronstein, J. M. Identification of a human glioma-associated growth factor gene, granulin, using differential immuno-absorption. Cancer Res., *60:* 1353–1360, 2000.
18. Serrero, G. Autocrine growth factor revisited: PC-cell derived growth factor (granulin precursor), a critical player in breast cancer tumorigenesis. Biochem. Biophys. Res. Commun., *308:* 409–413, 2003.
19. He, Z., and Bateman, A. Progranulin (granulin-epithelin precursor, PC-cell-derived growth factor, acrogranin) mediates tissue repair and tumorigenesis. J. Mol. Med., *81:* 600–612, 2003.
20. Lu, R., and Serrero, G. Mediation of estrogen mitogenic effect in human breast cancer MCF-7 cells by PC-cell-derived growth factor (PCDGF/granulin precursor). Proc. Natl. Acad. Sci. USA, *98:* 142–147, 2001.
21. Lu, R., and Serrero, G. Inhibition of PC cell-derived growth factor (PCDGF, epithelin/granulin precursor) expression by antisense PCDGF cDNA transfection inhibits tumorigenicity of the human breast carcinoma cell line MDA-MB-468. Proc. Natl. Acad. Sci. USA, *97:* 3993–3998, 2000.

22. Serrero, G., and Ioffe, O. B. Expression of PC-cell-derived growth factor in benign and malignant human breast epithelium. Hum. Pathol., *34:* 1148–1154, 2003.
23. Shang, Y., and Brown, M. Molecular determinants for the tissue specificity of SERMs. Science (Wash. DC), *295:* 2465–2468, 2002.
24. Diel, P., Smolnikar, K., and Michna, H. The pure antiestrogen ICI 182780 is more effective in the induction of apoptosis and down regulation of BCL-2 than tamoxifen in MCF-7 cells. Breast Cancer Res. Treat., *58:* 87–97, 1999.
25. Simbulan-Rosenthal, C. M., Rosenthal, D. S., Iyer, S., Boulares, H., and Smulson, M. E. Involvement of PARP and poly(ADP-ribosyl)ation in the early stages of apoptosis and DNA replication. Mol. Cell. Biochem., *193:* 137–148, 1999.
26. Duriez, P. J., and Shah, G. M. Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. Biochem. Cell Biol., *75:* 337–349, 1997.
27. Binder, C., Marx, D., Binder, L., Schauer, A., and Hiddemann, W. Expression of Bax in relation to Bcl-2 and other predictive parameters in breast cancer. Ann. Oncol., *7:* 129–133, 1996.
28. Bargou, R. C., Daniel, P. T., Mapara, M. Y., Bommert, K., Wagener, C., Kallinich, B., Royer, H. D., and Dorken, B. Expression of the *bcl-2* gene family in normal and malignant breast tissue: low bax-á expression in tumor cells correlates with resistance towards apoptosis. Int. J. Cancer, *60:* 854–859, 1995.
29. Pietras, R. J., Arboleda, J., Reese, D. M., Wongvipat, N., Pegram, M. D., Ramos, L., Gorman, C. M., Parker, M. G., Sliwkowski, M. X., and Slamon, D. J. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene, *10:* 2435–2446, 1995.
30. Kurokawa, H., Lenferink, A. E., Simpson, J. F., Pisacane, P. I., Sliwkowski, M. X., Forbes, J. T., and Arteaga, C. L. Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. Cancer Res., *60:* 5887–5894, 2000.
31. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., et al. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science (Wash. DC), *270:* 1491–1494, 1995.
32. Liu, Y., el-Ashry, D., Chen, D., Ding, I. Y., and Kern, F. G. MCF-7 breast cancer cells overexpressing transfected c-erbB-2 have an *in vitro* growth advantage in estrogen-depleted conditions and reduced estrogen-dependence and tamoxifen-sensitivity *in vivo*. Breast Cancer Res. Treat., *34:* 97–117, 1995.
33. Atanaskova, N., Keshamouni, V. G., Krueger, J. S., Schwartz, J. A., Miller, F., and Reddy, K. B. MAP kinase/estrogen receptor cross-talk enhances estrogen-mediated signaling and tumor growth but does not confer tamoxifen resistance. Oncogene, *21:* 4000–4008, 2002.
34. Sun, M., Paciga, J. E., Feldman, R. I., Yuan, Z., Coppola, D., Lu, Y. Y., Shelley, S. A., Nicosia, S. V., and Cheng, J. Q. Phosphatidylinositol-3-OH kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor α (ERα) via interaction between ERα and PI3K. Cancer Res., *61:* 5985–5991, 2001.
35. Campbell, R. A., Bhat-Nakshatri, P., Patel, N. M., Constantinidou, D., Ali, S., and Nakshatri, H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor α: a new model for anti-estrogen resistance. J. Biol. Chem., *276:* 9817–9824, 2001.
36. McLeskey, S. W., Zhang, L., El-Ashry, D., Trock, B. J., Lopez, C. A., Kharbanda, S., Tobias, C. A., Lorant, L. A., Hannum, R. S., Dickson, R. B., and Kern, F. G. Tamoxifen-resistant fibroblast growth factor-transfected MCF-7 cells are cross-resistant *in vivo* to the antiestrogen ICI 182,780 and two aromatase inhibitors. Clin. Cancer Res., *4:* 697–711, 1998.
37. Kurebayashi, J., McLeskey, S. W., Johnson, M. D., Lippman, M. E., Dickson, R. B., and Kern, F. G. Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor 4 and LacZ. Cancer Res., *53:* 2178–2187, 1993.
38. Perry, R. R., Kang, Y., and Greaves, B. R. Relationship between tamoxifen-induced transforming growth factor β 1 expression, cytostasis and apoptosis in human breast cancer cells. Br. J. Cancer, *72:* 1441–1446, 1995.
39. Reed, J. C., Miyashita, T., Takayama, S., Wang, H. G., Sato, T., Krajewski, S., Aime-Sempe, C., Bodrug, S., Kitada, S., and Hanada, M. BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J. Cell Biochem., *60:* 23–32, 1996.
40. Kumar, R., Mandal, M., Lipton, A., Harvey, H., and Thompson, C. B. Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. Clin. Cancer Res., *2:* 1215–1219, 1996.
41. He, Z., Ismail, A., Kriazhev, L., Sadvakassova, G., and Bateman, A. Progranulin (PC-cell-derived growth factor/acrogranin) regulates invasion and cell survival. Cancer Res., *62:* 5590–5596, 2002.
42. Zhang, H. T., Craft, P., Scott, P. A., Ziche, M., Weich, H. A., Harris, A. L., and Bicknell, R. Enhancement of tumor growth and vascular density by transfection of vascular endothelial cell growth factor into MCF-7 human breast carcinoma cells. J. Natl. Cancer Inst. (Bethesda), *87:* 213–219, 1995.
43. Yoshiji, H., Harris, S. R., and Thorgeirsson, U. P. Vascular endothelial growth factor is essential for initial but not continued *in vivo* growth of human breast carcinoma cells. Cancer Res., *57:* 3924–3928, 1997.
44. Sfiligoi, C., de Luca, A., Cascone, I., Sorbello, V., Fuso, L., Ponzone, R., Biglia, N., Audero, E., Arisio, R., Bussolino, F., Sismondi, P., and De Bortoli, M. Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. Int. J. Cancer, *103:* 466–474, 2003.
45. Tian, S., Hayes, A. J., Metheny-Barlow, L. J., and Li, L. Y. Stabilization of breast cancer xenograft tumour neovasculature by angiopoietin-1. Br. J. Cancer, *86:* 645–651, 2002.
46. Currie, M. J., Gunningham, S. P., Han, C., Scott, P. A., Robinson, B. A., Harris, A. L., and Fox, S. B. Angiopoietin-1 is inversely related to thymidine phosphorylase expression in human breast cancer, indicating a role in vascular remodeling. Clin. Cancer Res., *7:* 918–927, 2001.
47. Hayes, A. J., Huang, W. Q., Yu, J., Maisonpierre, P. C., Liu, A., Kern, F. G., Lippman, M. E., McLeskey, S. W., and Li, L. Y. Expression and function of angiopoietin-1 in breast cancer. Br. J. Cancer, *83:* 1154–1160, 2000.
48. He, Z., Ong, C. H., Halper, J., and Bateman, A. Progranulin is a mediator of the wound response. Nat. Med., *9:* 225–229, 2003.
49. Carter, W. B., and Ward, M. D. HER2 regulatory control of angiopoietin-2 in breast cancer. Surgery, *128:* 153–158, 2000.
50. Buteau-Lozano, H., Ancelin, M., Lardeux, B., Milanini, J., and Perrot-Applanat, M. Transcriptional regulation of vascular endothelial growth factor by estradiol and tamoxifen in breast cancer cells: a complex interplay between estrogen receptors α and β. Cancer Res., *62:* 4977–4984, 2002.
51. Takei, H., Lee, E. S., and Jordan, V. C. *In vitro* regulation of vascular endothelial growth factor by estrogens and antiestrogens in estrogen-receptor positive breast cancer. Breast Cancer, *9:* 39–42, 2002.
52. Iervolino, A., Trisciuoglio, D., Ribatti, D., Candiloro, A., Biroccio, A., Zupi, G., and Del Bufalo, D. Bcl-2 overexpression in human melanoma cells increases angiogenesis through VEGF mRNA stabilization and HIF-1-mediated transcriptional activity. FASEB J., *16:* 1453–1455, 2002.
53. Robinson, C. J., and Stringer, S. E. The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J. Cell Sci., *114:* 853–865, 2001.
54. Clarke, R., Liu, M. C., Bouker, K. B., Gu, Z., Lee, R. Y., Zhu, Y., Skaar, T. C., Gomez, B., O'Brien, K., Wang, Y., and Hilakivi-Clarke, L. A. Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. Oncogene, *22:* 7316–7339, 2003.

PC Cell-Derived Growth Factor Mediates Tamoxifen Resistance and Promotes Tumor Growth of Human Breast Cancer Cells

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