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PC Cell–Derived Growth Factor Stimulates Proliferation and Confers Trastuzumab Resistance to Her-2-Overexpressing Breast Cancer Cells

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PC Cell – Derived Growth Factor Stimulates Proliferation and Confers Trastuzumab Resistance to Her-2-Overexpressing Breast Cancer Cells

Wes E. Kim[1,2] and Ginette Serrero[1,3]

Abstract

Purpose: Trastuzumab is only effective in 25% to 30% of the administered breast cancer patients who overexpress the erbB2/Her-2 oncoprotein. PC cell – derived growth factor (PCDGF/GP88) is an 88-kDa glycoprotein growth factor overexpressed in 80% invasive ductal carcinomas. Our objective was to determine whether the increased levels of PCDGF/GP88 confers Trastuzumab resistance in erbB2-overexpressing breast cancer cells.

Experimental Design: The ability of PCDGF to induce erbB2 phosphorylation and to confer Trastuzumab resistance was studied in erbB2-overexpressing MCF-7 and SKBR3 breast cancer cell lines.

Results: PCDGF/GP88 added exogenously induced the phosphorylation of erbB2 in a dose-dependent and time-dependent manner in erbB2-overexpressing breast cancer cells. In addition, the overexpression of PCDGF/GP88 conferred Trastuzumab resistance in erbB2-overexpressing cells. Furthermore, overexpression of PCDGF/GP88 in erbB2-overexpressing cells provided a growth advantage over erbB2-overexpressing cells that do not have increased levels of PCDGF/GP88. Lastly, PCDGF/GP88 induced the phosphorylation of mitogen-activated protein kinase in a time-dependent manner in erbB2-overexpressing cells, and pretreatment with Trastuzumab was not able to attenuate the phosphorylation levels of mitogen-activated protein kinase induced by PCDGF/GP88.

Conclusion: These data suggest that PCDGF/GP88 confers Trastuzumab resistance in erbB2-overexpressing cells. Thus, the increase in PCDGF/GP88 levels may indicate Trastuzumab unresponsiveness in breast cancer patients.

Among the several growth factors and their respective receptors that are implicated in breast carcinogenesis, the erbB family of receptor tyrosine kinases plays a critical role. The erbB family consists of four members: erbB1/epidermal growth factor receptor (EGFR), erbB2/Her-2, erbB3/Her-3, and erbB4/Her-4 (1, 2). Except for erbB2/Her-2 that lacks an accessible ligand-binding domain (3), each member of the erbB receptor family has several ligands. Upon ligand binding, these receptors undergo either homodimerization or heterodimerization, leading to transphosphorylation, and initiation of signaling cascades, including both mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, leading to cell proliferation and survival (4, 5). Of all the possible pairings between the family members, the erbB2/erbB3 heterodimers are the most abundant and potent signaling modules formed (6, 7).

ErbB2/Her-2 is overexpressed in numerous human tumors, including breast, ovarian, gastric, colon, and non – small cell lung cancers (8, 9). Approximately 30% of breast carcinoma overexpress erbB2/Her-2. Overexpression of erbB2/Her-2 in breast cancer is associated with poor prognosis and leads to growth deregulation and malignant transformation (10). Cells overexpressing erbB2 are more invasive and resistant to chemotherapy and endocrine therapy (11, 12). Presently, the most common treatment for patients with breast tumors overexpressing erbB2 is the use of the humanized monoclonal antibody Trastuzumab. Derived from the murine monoclonal antibody 4D5, Trastuzumab was engineered to contain the antigen-binding domains of 4D5, fused to the human IgG1 constant domains (13). Trastuzumab binds to the extracellular domain of the erbB2 receptor, and this binding is believed to cause homodimerization, a pairing that is ineffective in the activation of oncogenic downstream signaling, resulting in erbB2 down-modulation (14). Furthermore, as a result of this homodimerization, erbB2 is not able to heterodimerize with its preferred partner erbB3. The Fc portion of the Trastuzumab antibody is also believed to play a role in Trastuzumab antitumor activity through antibody-dependent cell-mediated cytotoxicity and/or through complement-dependent cytotoxicity (15, 16). Treatment with Trastuzumab causes a cytostatic growth inhibitory effect in breast cancer cells overexpressing

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erbB2 (13, 16). However, as a single agent, Trastuzumab is only effective in ~20% of the treated patients (17). Yet, in combination therapy with chemotherapeutic agents, such as taxotere, the response rate is increased to 50% (18).

PC cell–derived growth factor (PCDGF/GP88), also known as progranulin, granulin/epithelin precursor or acrogranin, is an 88-kDa glycoprotein (GP88), composed of 7.5 cysteine-rich tandem repeats known as epithelin/granulin (19). Granulins and epithelins have been initially isolated from inflammatory cells and bone marrow and from kidney extracts and reported to have growth modulatory activities (20). The 88-kDa protein is the largest member of this family and has been shown to play a role in tumorigenesis, including stimulation of proliferation, survival, migration, angiogenesis, and matrix metalloprotease activity (21). In addition, in normal tissues, it plays a role in wound healing and in inflammation (22). The pathways involved in PCDGF/GP88 signaling include both the MAPK extracellular signal-regulated kinase 1/2 (Erk1/2), PI3K, and focal adhesion kinase, leading to the activation of the cell cycle regulatory proteins cyclin D1 and cyclin B (23–26).

PCDGF/GP88 levels have been reported to be higher in multiple types of cancer when compared with corresponding normal tissues. These included primary glioblastoma multiform tumors, renal cell carcinomas, invasive ovarian tumors, multiple myeloma, and prostate and breast cancers (27–31). Our laboratory has focused on investigating the role of PCDGF/GP88 in breast tumorigenesis. It has been shown that in normal mammary epithelial cells and in nontumorigenic MCF-10A cells, the level of PCDGF/GP88 mRNA and protein expression is either absent or low (32). PCDGF/GP88 mRNA and protein expression were expressed in both estrogen receptor–positive MCF-7 and T47D cells, and in estrogen receptor–negative breast carcinomas, such as MDA-MB-468, MDA-MB-453, and MDA-MB-231 (32). PCDGF/GP88 expression positively correlated with the degree of tumorigenicity and estrogen independence in human breast carcinoma (24, 32). In addition, the overexpression of PCDGF/GP88 in MCF-7 breast cancer cells conferred resistance to the antiestrogen tamoxifen both *in vitro* and *in vivo* (33). In addition, inhibition of PCDGF/GP88 expression in MDA-MB-468 breast carcinoma by antisense transfection led to inhibition of tumor formation in nude mice (32).

Pathologic studies in paraffin-embedded human breast cancer biopsies indicated that PCDGF/GP88 was overexpressed in 80% of invasive ductal carcinoma, where it correlated with clinical variables of poor prognosis, such as tumor grade, p53 expression, and Ki67 index (34). Immunohistochemical analysis of PCDGF and erbB2 expression in invasive ductal carcinomas showed no correlation between the two molecular targets. However, interestingly, our studies indicated that 25% of Her-2-overexpressing biopsies (3+ by immunohistochemistry) were strongly positive for PCDGF/GP88 (34). Based on these results, we investigated the effect of PCDGF on the proliferation and Trastuzumab responsiveness of Her-2-overexpressing breast cancer cells.

Materials and Methods

Cell lines. MCF-7 cells and stable transfectants were cultivated in DMEM/F12 supplemented with 5% fetal bovine serum. ErbB2-overexpressing MCF-7 cells and GP-88/ErbB2–overexpressing MCF-7 cells were cultivated in the presence of 500 μg/mL G418 (RPI, Mt. Prospect, IL) alone (MCF-7/erbB2) or with 800 μg/mL Zeocin (GP88/ErbB2). BT-474 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM/F12 supplemented with 10% fetal bovine serum. SKBR3 cells (American Type Culture Collection) and the stable transfectants were grown in McCoy's 5A supplemented with 10% fetal bovine serum. SKBR3/EV and SKBR3/GP88 cells were cultivated in the presence of 800 μg/mL G418. MCF-7/erbB2 cells were obtained by stably transfecting MCF-7 cells with pcDNA3 vector (Invitrogen, Carlsbad, CA), containing the erbB2 cDNA (ref. 6; kindly provided by Dr. Yosef Yarden, The Weizmann Institute of Science, Rehovot, Israel), using LipofectAMINE Plus (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. MCF-7 erbB2 cells were selected with 1.2 mg/mL G418. GP88/erbB2 cells were established by transfection of MCF-7 erbB2-overexpressing cells with a PCDGF/GP88 cDNA expression vector followed by selection in the presence of 800 μg/mL of Zeocin (Invitrogen). SKBR3 cells, which naturally overexpress erbB2, were also stably transfected with the PCDGF cDNA pcDNA3 vector and were selected in the presence of 800 μg/mL G418.

Western blotting. Cells (2×10^5) were plated in the appropriate culture medium as described above. Forty-eight hours later, cells were washed with serum-free medium and incubated in serum-free medium for 24 hours followed by the addition of various treatments. Cell lysates were collected in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mmol/L Na_3VO_4, and 1 mmol/L NaF]. Forty micrograms of total protein lysates were resolved on a SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. After blocking, membranes were incubated with the primary antibody followed by incubation with the appropriate horseradish peroxidase–conjugated secondary antibody. Immunoreactive bands were detected with enhanced chemiluminescence horseradish peroxidase substrate (Pierce, Rockford, IL). For immunoblots and/or immunoprecipitations, the following antibodies were used: phospho-erbB2/Her-2 (Upstate Biological, Inc., Lake Placid, NY); phospho-p42/44, ERK2, phospho-Akt, phospho-EGFR (Cell Signaling, Beverly, MA); actin (Sigma Chemical Co., St. Louis, MO); Trastuzumab (Genentech, South San Francisco, CA). For phosphorylation studies, the following inhibitors were used: genistein (Calbiochem, San Diego, CA), Iressa (Biaffin BmbH & Co., Kassel, Germany), AG825 (Calbiochem), and U0126 (Cell Signaling).

^{3}H-thymidine incorporation. Cells (8×10^4) were plated in 24-well plates in the appropriate media. Forty-eight hours later, cells were washed, and fresh medium was added along with the various treatments. Forty-eight hours later, 1 μCi of ^{3}H-thymidine was added into each well for 24 hours, Cell lysates were collected in 0.5 mol/L NaOH. Samples were measured on a scintillation counter.

Soft agar assay. Cells (1×10^4) in 0.33% agarose in DMEM/F12 supplemented with 10% fetal bovine serum were layered on top of 0.6% agarose in the same medium on six-well plates. Colonies were grown for 21 days, with fresh media being added every 3 days (±40 μg/mL Trastuzumab), after which they were stained with 0.005% crystal violet and counted under a microscope.

Nude mice xenograft studies. MCF-7/erbB2 (5×10^6) and GP88/erbB2 cells were injected s.c. into athymic female nude mice (National Cancer Institute, Frederick, MD). Two sites per animal were injected. Estradiol pellets (1.7 mg, 60-day release; Innovative Research of America, Sarasota, FL) were also implanted s.c. by a 10-gauge trochar on the back of the animals. Five mice were used in each experimental group. Tumor volume was monitored for 42 days and calculated with the following formula: tumor volume = (length^2 × width) / 2.

Data analysis. All experiments were done in triplicates and repeated at least thrice, unless otherwise noted. The data are presented as the average of the repeated experiments, with the SDs, except for *in vivo* studies, which use SEs. Densitometric analysis was done with

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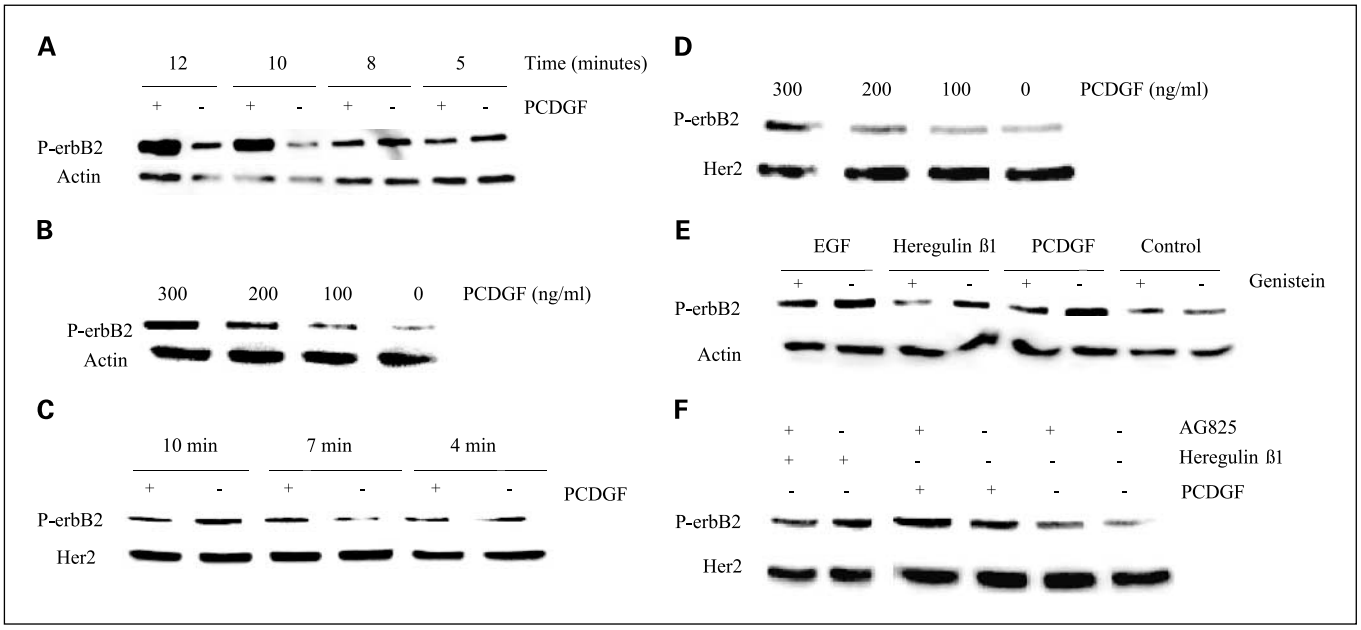

Fig. 1. Time-dependent and dose-dependent activation of erbB2 phosphorylation by PCDGF/GP88 in erbB2-overexpressing breast cancer cells. SKBR3 (*A* and *B*) or MCF-7/erbB2 (*C* and *D*) cells were serum starved for 24 hours in PFMEM followed by treatment with increasing concentrations of PCDGF/GP88 for the indicated times. *E,* cells were pretreated with 30 μmol/L genistein for 24 hours before the addition of either 300 ng/mL PCDGF/GP88, 10 ng/mL Heregulin β1, or 100 ng/mL EGF. *F,* cells were pretreated with 10^{-7} mol/L AG825 for 30 minutes before the addition of either 300 ng/mL PCDGF/GP88 or 10 ng/mL Heregulin β1. Western blot analysis was done with anti-phospho-erbB2 antibody. Membranes were stripped and reprobed with either anti-actin or anti-Her-2 antibodies to ensure equal loading.

ScionImage software. Student's *t* test was used to determine the significance of the data, with $P < 0.05$ considered to be significant.

Results

Effect of PCDGF/GP88 on the phosphorylation of erbB2 in erbB2-overexpressing breast cancer cells. The effect of PCDGF/GP88 on the phosphorylation status of erbB2 was examined using SKBR3 cells that naturally overexpress erbB2 (14). As observed in Fig. 1A and B, PCDGF/GP88 stimulated erbB2 phosphorylation in SKBR3 cells in a time-dependent and dose-dependent fashion. An optimal stimulation of phosphorylation was obtained with 300 ng/mL PCDGF/GP88, compared with lower doses, after 10 minutes of treatment. The level of erbB2 phosphorylation was increased by 2.8 ± 0.4-fold ($P = 0.037$) in cells treated with PCDGF/GP88 when compared with untreated control cells. In addition, erbB2 phosphorylation upon PCDGF/GP88 treatment was also observed in MCF-7/erbB2 cells (Fig. 1C and D) and in BT474 cells, which naturally overexpress erbB2 (data not shown). These observations showed that PCDGF/GP88 could activate Her-2/erbB2 in a dose-dependent and time-dependent fashion in several erbB2-overexpressing cells. The stimulation of erbB2 phosphorylation by PCDGF/GP88 was inhibited by 50 ± 13% ($P = 0.0337$) by 30 μmol/L genistein, a tyrosine kinase inhibitor with broad specificity (Fig. 1E). However, the specific erbB2 inhibitor AG825 had no effect on the ability of PCDGF/GP88 to phosphorylate erbB2, whereas it inhibited Heregulin's effect in erbB2 phosphorylation used as a positive control (Fig. 1F).

We also investigated whether PCDGF/GP88 could induce erbB1/EGFR phosphorylation. SKBR3 cells, which have elevated levels of EGFR, were treated with 300 ng/mL PCDGF/GP88 at various time points. PCDGF/GP88 was unable to stimulate EGFR phosphorylation in contrast to EGF that induced a rapid and strong EGFR phosphorylation (Fig. 2A). Furthermore, gefitinib (Iressa), an EGFR tyrosine kinase inhibitor, did not inhibit PCDGF/GP88's ability to activate erbB2 (Fig. 2B), whereas it inhibited EGF's effect as expected from published reports (35).

In vivo *tumorigenesis studies of ErbB2- and PCDGF/GP88–overexpressing cells.* We investigated the potential growth advantage of cells that overexpress both PCDGF/GP88 and erbB2 over the cells that overexpress erbB2 only. ErbB2/GP88

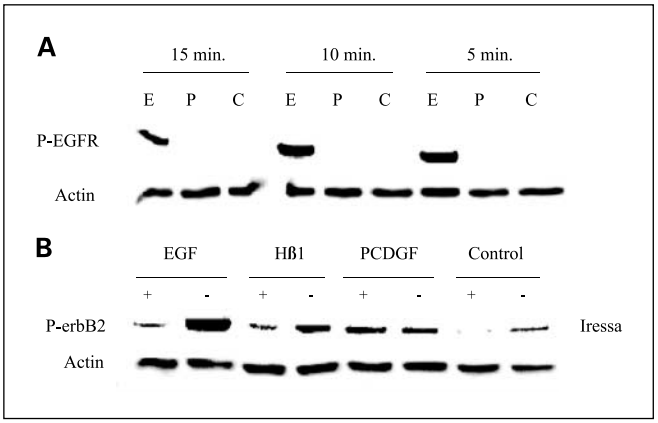

Fig. 2. PCDGF/GP88 does not induce EGFR phosphorylation. *A,* SKBR3 cells were treated with either 300 ng/mL PCDGF or 100 ng/mL EGF for the indicated times. Western blot analysis was done using anti-phospho-EGFR. *B,* 1 μmol/L Iressa (gefitinib) was added 19 hours before the addition of either 300 ng/mL PCDGF/GP88, 10 ng/mL Heregulin β1, or 100 ng/mL EGF. Western blot analysis was done with anti-phospho-erbB2 antibody. Membranes were stripped and reprobed with anti-actin antibody to ensure equal loading. C, control; E, EGF; Hβ1, Heregulin β1; P, PCDGF/GP88.

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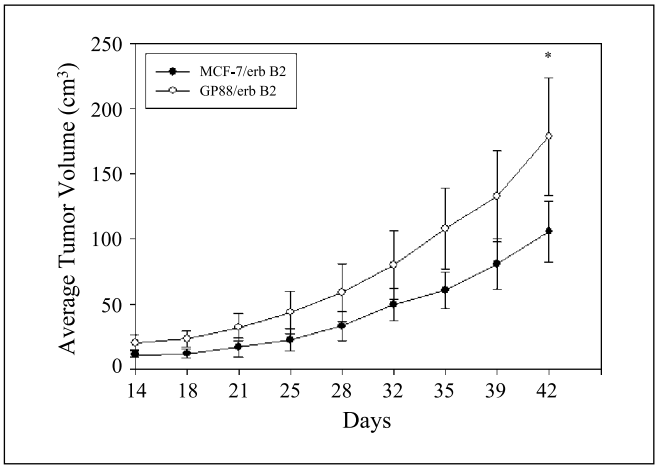

Fig. 3. PCDGF/GP88 overexpression increases tumorigenicity of MCF-7/erbB2–overexpressing cells in nude mice. Cells were resuspended in PBS, and each mouse was injected s.c. at two sites with 5 million cells per site. Five mice were used for each experimental group. The tumor volumes were monitored twice per week for 42 days.

and the MCF-7/erbB2 cells were injected into athymic nude mice to evaluate their tumorigenicity as described in Materials and Methods. We observed a significant increase in the average tumor volumes of mice injected with the GP88/erbB2 cells compared with the MCF-7/erbB2 cells (Fig. 3). The average tumor size of the GP88/erbB2–injected cells had doubled compared with the MCF-7/erbB2–injected cells by day 32, and a significant growth advantage was observed on day 42 ($P = 0.044$).

Overexpression of PCDGF/GP88 confers Trastuzumab resistance in ErbB2-overexpressing breast cancer cells. Based on the above results, we investigated whether the overexpression of PCDGF/GP88 enables this particular growth factor to confer Trastuzumab resistance and to provide a growth advantage. First, we examined the optimal dose of Trastuzumab that inhibits the proliferation of the stably transfected erbB2-overexpressing cells by ^{3}H-thymidine incorporation assay. At a concentration of 40 μg/mL, Trastuzumab was the most effective, inhibiting cell proliferation by 46 ± 6.9%. Higher Trastuzumab concentrations did not have higher inhibitory effect (Fig. 4A). Similar levels of inhibition were observed with SKBR3-EV (Fig. 4C) and BT-474 cells (data not shown). Based on these results, a concentration of 40 μg/mL Trastuzumab was used in all subsequent experiments.

Because PCDGF/GP88 stimulates the phosphorylation of erbB2, we examined the effect of overexpression of PCDGF/GP88 on Trastuzumab responsiveness. The GP88/erbB2 cells were treated with Trastuzumab (40 μg/mL); however, these cells continued to proliferate (Fig. 4B). In parallel experiments, expression vector containing PCDGF/GP88 cDNA was stably transfected into the naturally erbB2-overexpressing SKBR3 cells. Whereas the control SKBR3 empty vector transfectants were sensitive to Trastuzumab, the treatment of Trastuzumab had minimal effect on PCDGF-overexpressing SKBR3 cells (SKBR3-GP88; Fig. 4C). These results show that increased levels of PCDGF/GP88 provide resistance to Trastuzumab treatment in erbB2-overexpressing breast cancer cells.

Effect of PCDGF/GP88 on the anchorage-independent growth of erbB2-overexpressing breast cancer cells. The MCF-7/EV (empty pcDNA3 vector), MCF-7/erbB2, GP88/erbB2, SKBR3/EV, and SKBR3/GP88 cells were plated on soft agar to determine their ability to form colonies in an anchorage-independent manner in the presence or absence of Trastuzumab. After 21 days in culture, colonies were stained and counted (Fig. 4D). The MCF-7/EV cells formed the least amount of colonies and were unresponsive to Trastuzumab. This is expected because MCF-7 cells express very little erbB2. The MCF-7/erbB2 cells displayed a high level of growth over their counterpart MCF-7/EV, with a 3.5 ± 0.9-fold ($P = 0.001$) increase in the number of colonies. Similarly, the erbB2-overexpressing SKBR3/EV cells also formed a significantly higher number of colonies compared with the MCF-7/EV cells. Trastuzumab inhibited colony formations of both the MCF-7/erbB2 and SKBR3/EV cells by 45 ± 6.0% ($P = 0.001$) and 40 ± 0.5% ($P = 0.001$), respectively. The GP88/erbB2 cells produced the highest number of colonies, and they were resistant to Trastuzumab. Furthermore, these cells had a slight growth advantage of 1.4 ± 0.1-fold ($P = 0.006$) over their MCF-7/erbB2 parental cell line. Similarly, SKBR3/GP88 cells also showed resistance to Trastuzumab, in addition to having a modest growth advantage of 1.5 ± 0.1-fold ($P = 0.014$) compared with their SKBR3/EV counterpart. These observations further show that the overexpression of PCDGF/GP88 confers Trastuzumab resistance. In addition, we further confirmed that the overexpression of PCDGF/GP88 provides a growth advantage in erbB2-overexpressing breast cancer cells.

Activation of MAPK by PCDGF/GP88 in the presence of Trastuzumab. Although the exact mechanism has yet to be determined, previous studies have shown that treatment of erbB2-overexpressing cells with Trastuzumab decreased the levels of MAPK phosphorylation (36). Based on this, and because PCDGF/GP88 activated the MAPK signaling pathway in erbB2-overexpressing cells (Fig. 5A), we examined the effect of Trastuzumab on PCDGF/GP88's ability to induce the phosphorylation of MAPK. MCF-7/erbB2 cells were pretreated with Trastuzumab for 24 hours and then treated with either PCDGF/GP88 or Heregulin β1. Cells treated with both PCDGF/GP88 and Trastuzumab did not show a reduced level of phosphorylated MAPK compared with cells treated with PCDGF/GP88 alone (Fig. 5B). In fact, PCDGF/GP88 treatment led to a strong phosphorylation of MAPK in Trastuzumab-pretreated cells. In contrast, cells treated with both Heregulin β1 and Trastuzumab showed a 37 ± 5% ($P = 0.051$) decrease in phosphorylated MAPK when compared with cells treated with Heregulin β1 alone (Fig. 5C). These results suggest that PCDGF/GP88 overrides the inhibitory effects of Trastuzumab on erbB2 signaling and activates the MAPK signaling pathway, thus allowing the cells to continue to grow and provide resistance to Trastuzumab in erbB2-overexpressing cells. Previous studies from our laboratory showed the ability of a MAP/ERK kinase inhibitor to attenuate the MAPK phosphorylation levels induced by PCDGF/GP88 (24). To further show that PCDGF/GP88's ability to confer Trastuzumab resistance involves the MAPK pathway, we investigated whether inhibiting MAPK/ERK, with U0126 (a MAPK/ERK inhibitor), could reverse Trastuzumab resistance. MCF-7/erbB2 cells treated with U0126, in addition to both PCDGF and Trastuzumab, had no detectable MAPK phosphorylation levels compared with the cells treated with both PCDGF and Trastuzumab (Fig. 5B). These results show the involvement of the MAPK signaling pathway in Trastuzumab resistance due to increased PCDGF/GP88 levels.

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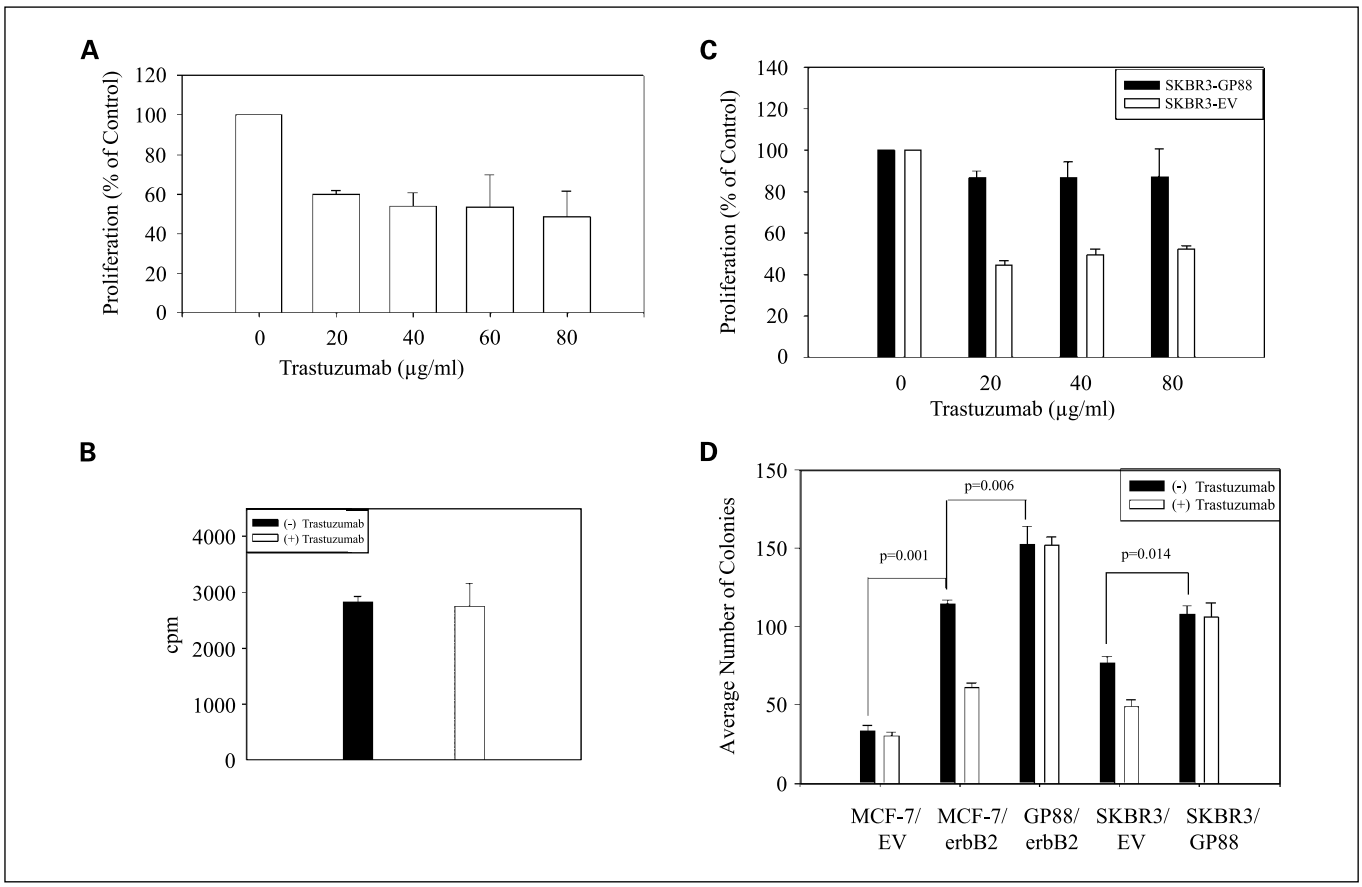

Fig. 4. Trastuzumab resistance by PCDGF/GP88 and erbB2-dual overexpressing cells: (*A*) 8×10^4 MCF-7/erbB2 cells, (*B*) GP88/erbB2 cells, and (*C*) SKBR3/GP88 and SKBR3/EV were plated in the appropriate medium on 24-well plates. Forty-eight hours later, cells were washed, and fresh PFMEM (±1% CSS) was added along with 40 μg/mL Trastuzumab. ^{3}H-thymidine incorporation was carried out as described in Materials and Methods. *D*, 1×10^4 cells in 0.33% agarose were layered on top of 0.6% agarose in DMEM/F12 (+5% serum; 10% serum was used for SKBR3 cells). Colonies were grown for 21 days with or without 40 μg/mL Trastuzumab, after which they were stained with 0.005% crystal violet. Colonies were counted using a microscope.

Effect of PCDGF/GP88 on the activation of Akt. Overexpression of erbB2/Her-2 confers a PI3K-dependent invasiveness in erbB2-overexpressing human breast cancer cells (37). It has been reported that PCDGF/GP88 activates Akt in a variety of cell lines (25, 29). To examine if PCDGF/GP88 could activate Akt in erbB2-overexpressing cells, SKBR3 cells were treated with 300 ng/mL of PCDGF/GP88. After 2 minutes, a 2 ± 0.26-fold induction (P = 0.02) of Akt phosphorylation was observed (Fig. 6A). The level of phosphorylation returned to basal level after 5 minutes of treatment.

Interestingly, PCDGF/GP88 was not able to stimulate Akt phosphorylation in the presence of Trastuzumab (Fig. 6B). The level of Akt phosphorylation induced by PCDGF/GP88 was decreased to the control levels in the presence of Trastuzumab. This was in contrast to PCDGF/GP88's ability to induce p42/p44 MAPK phosphorylation in the presence of Trastuzumab as described above. These results provide an insight into PCDGF/GP88 signaling mechanisms in erbB2-overexpressing breast carcinomas.

Discussion

PCDGF/GP88, also known as epithelin/granulin precursor, progranulin, or acrogranin, is overexpressed in human breast carcinomas, and its expression levels positively correlated with the degree of tumorigenicity. Overexpression of this autocrine 88-kDa glycoprotein in human breast carcinomas is involved in increased cell proliferation, survival, and tumorigenesis as well as tamoxifen resistance. Furthermore, recent reports have linked PCDGF/GP88 to other important tumorigenic processes, such as metastasis, invasiveness, and angiogenesis (21).

The data presented here show that PCDGF/GP88 increases the levels of erbB2 phosphorylation in a time-dependent and dose-dependent fashion in several human breast cancer cell lines that overexpress erbB2, including MCF-7/erbB2, SKBR3, and BT474 cells. Genistein, a broadly specific tyrosine kinase inhibitor, attenuated erbB2 phosphorylation levels induced by PCDGF/GP88. However, interestingly, the erbB2 tyrosine kinase inhibitor AG825 had no effect on erbB2 phosphorylation induced by PCDGF/GP88, whereas it inhibited Heregulin's effect. In addition, we show that the overexpression of PCDGF/GP88 in erbB2-overexpressing cells results in higher proliferation *in vitro* because breast cancer cells MCF-7 or SKBR3 that overexpress both PCDGF/GP88 and erbB2 formed more colonies in soft agar compared with their control counterparts that did not overexpress PCDGF/GP88. Lastly, the GP88/erbB2 cells formed larger tumors in athymic, nude mice compared with the MCF-7/erbB2 cells, further confirming a growth advantage when PCDGF/GP88 is overexpressed in ErbB2-overexpressing breast cancer cells.

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These results show that PCDGF/GP88 expression confers a growth advantage to erbB2-overexpressing cells. In addition, PCDGF/GP88 confers Trastuzumab resistance as shown by the fact that cells overexpressing the growth factor continued to proliferate in the presence of Trastuzumab. Similarly, the colony-forming ability of the cells that overexpress both PCDGF/GP88 and erbB2 was unaffected by the addition of Trastuzumab, whereas cells that only overexpress erbB2 exhibited a 50% reduction in the number of colonies.

To gain a better understanding on how PCDGF/GP88 promotes proliferation, we investigated its signaling properties. First, we showed that PCDGF/GP88 induced the phosphorylation of MAPK/ERK in erbB2-overexpressing breast cancer cells in a time-dependent fashion. In addition, we observed that PCDGF/GP88 induced MAPK/ERK phosphorylation more rapidly in MCF-7/erbB2 cells than in MCF-7/EV cells (data not shown). Because our laboratory had previously shown that PCDGF/GP88 phosphorylates MAPK regardless of the erbB2 expression levels, PCDGF/GP88 is not necessarily dependent on erbB2's own ability to use the particular pathway. Thus, these results suggest a crosstalk in signaling pathways between PCDGF/GP88 and erbB2 that leads to an amplification in the MAPK signaling pathway. In addition, we have shown that PCDGF/GP88's ability to induce MAPK phosphorylation was unaffected by Trastuzumab treatment, perhaps because PCDGF/GP88 does not necessarily rely on erbB2 to stimulate MAPK. However, we showed that the combination of Trastuzumab and U0126 (a MAPK/ERK inhibitor) completely inhibited PCDGF/GP88's ability to phosphorylate MAPK. This suggests that targeting the MAPK pathway could reverse the Trastuzumab resistance induced by PCDGF/GP88.

Agus et al. showed that Trastuzumab could not block heregulin activation of MAPK phosphorylation (38). We, however, were able to show the exact opposite. We attribute this discrepancy to the difference in the incubation time of Trastuzumab. Agus et al. used a 30-minute preincubation, whereas we pretreated our cells for 24 hours. In fact, Yakes et al. showed that the effect of Trastuzumab in MAPK phosphorylation levels is not seen until at least an 8-hour incubation in BT-474 cells and 24 hours in SKBR3 cells (36).

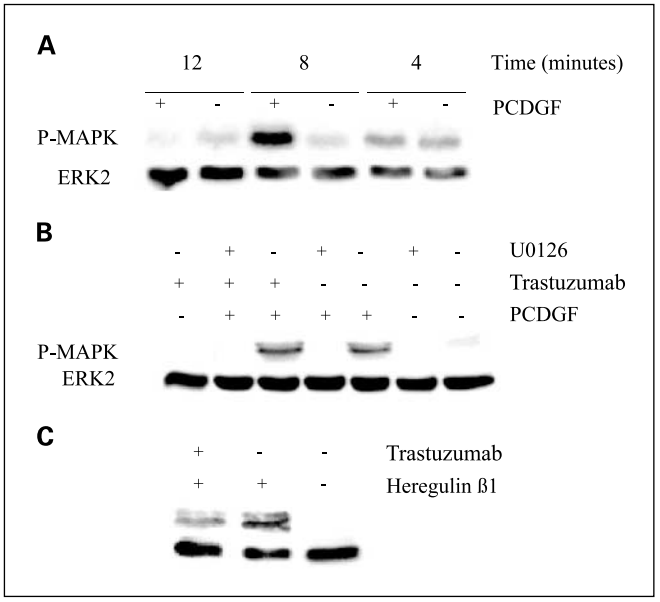

Fig. 5. Activation of MAPK by PCDGF in erbB2-overexpressing cells. MCF-7/erbB2 cells were serum starved for 24 hours in the presence of 40 μg/mL Trastuzumab. The cells were then treated with (*A* and *B*) 300 ng/mL PCDGF/GP88 or (*C*) 10 ng/mL Heregulin β1. *B,* cells were pretreated with U0126 (20 μmol/L) for 30 minutes. Western blot analysis was done with anti-phospho-p42/p44 MAPK antibody. Membranes were reprobed with anti-ERK2 to ensure equal loading.

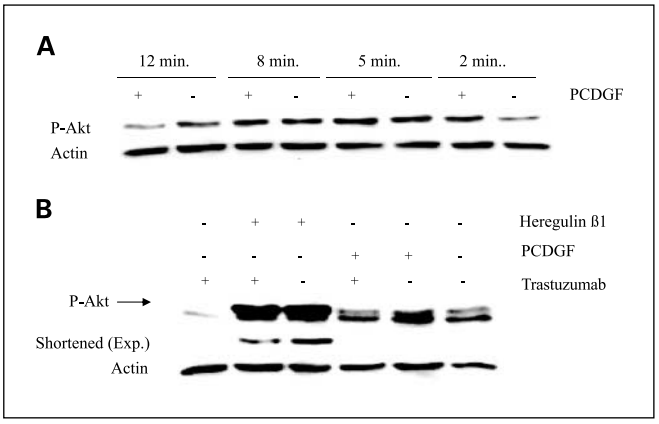

Fig. 6. Activation of Akt by PCDGF/GP88 treatment. *A,* SKBR3 cells were serum starved for 24 hours in PFMEM, after which the cells were treated with 300 ng/mL PCDGF for the indicated times. *B,* MCF-7/erbB2 cells were serum starved for 24 hours and pretreated with 40 μg/mL Trastuzumab. Cells were treated with either 300 ng/mL PCDGF/GP88 or 10 ng/mL Heregulin β1 for 8 minutes. Western blot analysis was done with anti-phospho-Akt antibody. Membranes were reprobed with anti-actin to ensure equal loading.

Another signaling pathway used by erbB2 is the PI3K/Akt pathway. The erbB2/erbB3 heterodimer efficiently couples to the Akt pathway, through the kinase domain of Her3, which contains six docking sites for the p85 subunit of PI3K (39, 40). Our studies showed that PCDGF/GP88 rapidly induces Akt activation in erbB2-overexpressing cells. However, this phosphorylation was inhibited by Trastuzumab. This suggests that when erbB2 is overexpressed, PCDGF/GP88 uses erbB2's ability to signal through Akt in a surrogate mechanism, allowing PCDGF/GP88 to activate Akt as well. Therefore, PCDGF/GP88 cannot activate Akt in the presence of Trastuzumab.

Although a handful of models for Trastuzumab resistance implicates the PI3K/Akt pathway, there are paradigms that suggest MAPK as the integral signaling module in erbB2-overexpressing breast cancer cell survival. Montgomery et al. showed that endogenous anti-Her-2 antibodies attenuate the phosphorylation of MAPK and correlates well with decreased colony formation (41). Interestingly, they found that the phosphorylation levels of Akt remained unchanged (41). Our results would be in agreement with these findings. Trastuzumab effectively suppressed phospho-MAPK levels in erbB2 overexpressing cells, but not when PCDGF/GP88 levels were increased, leading to Trastuzumab resistance. However, PCDGF/GP88 had no effect on the Trastuzumab-mediated attenuation of Akt phosphorylation levels.

There have been previous reports proposing possible mechanisms for Trastuzumab resistance (42–44). One recently showed mechanism showed that Trastuzumab induces the activation of PTEN, the phosphatase controlling PIP3 dephosphorylation, and thus antagonizing the PI3K/Akt pathway in erbB2-overexpressing breast cancer cells (45). Nagata et al. showed that patients with PTEN-deficient breast cancers had a significantly poorer response to Trastuzumab compared with

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patients with normal levels of PTEN (45). Another relevant mechanism is based on the fact that the overexpression of growth factor and growth factor receptor leads to Trastuzumab resistance. For example, increased levels of insulin-like growth factor-1 receptor (IGF-1R) signaling abrogated Trastuzumab's efficacy in cells overexpressing erbB2 (46). Co-targeting both receptors by using Trastuzumab and a dominant-negative IGF-1R led to a synergistic inhibition of erbB2-overexpressing cells (47). This scenario may possibly apply to the Trastuzumab resistance caused by PCDGF/GP88. It can also be assumed that PCDGF/GP88 binds to its receptor, and that the ligand/receptor complex directly interacts with erbB2, providing a steric hindrance towards Trastuzumab. In agreement with this latter possibility, a previous report has proposed that overexpression of Muc4/SMC and the subsequent formation of a complex with erbB2 provided a specific steric block to Trastuzumab binding (44).

Her-2 is not strictly limited to binding to its own family members but rather displays promiscuity with respect to its binding partner. It has previously been shown that erbB2 can form a heteromeric complex with the IGF-1 receptor when induced with heregulin and IGF-1 in MCF-7 cells, and increased levels of IGF-1R signaling in cells overexpressing erbB2 abrogated Trastuzumab's efficacy (46, 48). In addition, erbB2 can directly interact with the cell membrane–bound estrogen receptor in BT-474 cells and thus provide resistance to tamoxifen-induced apoptosis (49). Our own findings suggest that erbB2 interacts with the PCDGF/GP88 receptor similar to the way it interacts with the IGF-1R. This suggests that erbB2 could indeed dimerize with the PCDGF/GP88 receptor, leading to the transphosphorylation of erbB2 by the PCDGF/GP88 receptor. Whether the PCDGF/GP88 receptor interacts with the erbB2/erbB3 heterodimer remains to be seen. However, our results from Figs. 1F and 2A imply that there are no direct interactions between EGFR and the PCDGF/GP88 receptor. Direct demonstration of these possibilities will be examined with the current molecular characterization of the PCDGF/GP88 receptor.

PCDGF/GP88's own signaling ability is potent enough to promote proliferation and cell survival even in the presence of Trastuzumab. PCDGF/GP88 signals through the MAPK/ERK pathway, regardless of the levels of erbB2. Even if Trastuzumab binds to erbB2, PCDGF/GP88 signaling could compensate for any diminished proliferation signals and override the signaling blockade by Trastuzumab as observed with IGF-1 and Trastuzumab (50).

Our pathologic studies have indicated that about 25% of ErbB2-overexpressing invasive ductal carcinomas investigated also expressed PCDGF/GP88 (34). Based on this observation, the results presented here are significant as they provide a new mechanism for Trastuzumab resistance that could apply to a certain percentage of the erbB2-overexpressing population. Based on this, it is hypothesized that PCDGF/GP88 represents an important therapeutic target for breast cancer, and that co-targeting both PCDGF/GP88 and erbB2 could prove to be highly effective in treating erbB2-overexpressing breast cancer patients.

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