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HER2 Regulation of Peroxisome Proliferator-activated Receptor γ (PPARγ) Expression and Sensitivity of Breast Cancer Cells to PPARγ Ligand Therapy[1]

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ABSTRACT

Induction of terminal differentiation of cancer cells is an evolving novel therapeutic approach, and accordingly, peroxisome proliferator-activated receptor γ (PPARγ), a ligand-stimulated transcription factor with differentiation-promoting activity and overexpressed in a variety of cancers, has emerged as one of the promising therapeutic targets. Because c-erbB family growth factor receptor 2 (HER2) overexpression is one of the most recognizable molecular dysfunctions in breast tumors, in the studies presented here, we explored the effect of HER2 overexpression on the status of PPARγ expression and on the sensitivity of breast cancer cells to PPARγ-ligand troglitazone-induced growth inhibition. We show that HER2 overexpression in MCF7 breast cancer cells enhanced the expression of PPARγ-mRNA and -protein. Furthermore, PPARγ expression was dramatically increased in 11 of 16 breast tumors as compared with the adjacent normal tissue. In addition, HER2 up-regulation resulted in a partial inhibition of transcriptional activity of the endogenous PPARγ, stimulation to differentiation, and resistance to troglitazone-mediated inhibition of anchorage-independent growth of breast cancer cells. Conversely, down-regulation of HER2 by anti-HER2 monoclonal antibody Herceptin led to a decreased level of PPARγ protein and sensitization of breast cancer cells to the inhibitory effects of troglitazone. In summary, these findings show for the first time that HER2 up-regulates PPARγ expression and modulates the sensitivity of breast cancer cells to PPARγ ligand therapy.

INTRODUCTION

Breast cancer is one of the deadliest cancers and a leading cause of cancer-related mortality in developed countries. Despite aggressive education and screening programs, ~15–20% of all breast cancer patients have invasive disease at the time of diagnosis, which is often no longer surgically resectable (1, 2). There are few treatment options for such patients, because most radiation and chemotherapeutic regimens do little to extend survival. Despite recent advances in therapy for breast cancer, the median survival of breast cancer patients usually does not exceed 2 years, and >43,000 women in the United States die of metastatic breast cancer each year. However, an evolving understanding of the genetic and molecular alterations in breast cancer has led to the development of novel agents that may contribute to an extension of patient survival and eventually a cure for this devastating malignancy.

A number of characteristic patterns of gene expression have been documented in breast cancer and have been collectively responsible for malignant phenotypic alterations. One of the most common alterations is the overexpression of epidermal growth factor receptor-2 (also known as HER2), which begins in early premalignant lesions and is present at increased frequency as cancer progresses. Indeed, HER2 is overexpressed and/or amplified in a number of human malignancies, including advanced breast cancers. HER2 overexpression has been detected in 20–30% of human breast carcinoma (3, 4). HER2 is a receptor without known ligands; instead, it heterodimerizes with other members of the c-erbB family to effectively activate its intracellular tyrosine kinase domain (5–7). Kinase activation leads to a signaling cascade that results in cell growth stimulation, altered cell differentiation, and increased motility and invasiveness of cancer cells. In breast cancer, HER2 overexpression is a significant negative prognostic indicator for a variety of therapies (7–9). The frequency of HER2 overexpression in adenocarcinomas and its importance in mediating cancer cell growth and aggressiveness has led to the development of a humanized mouse monoclonal antibody, Herceptin (trastuzumab). Herceptin blocks receptor signaling and leads to receptor down-regulation (8), resulting in growth inhibition and, importantly, sensitizing cancer cells to other therapeutic agents. This therapeutic antibody has proven effective both as a single agent and in combination with established cytotoxic agents, such as tamoxifen for breast cancer, for patients with tumors characterized by HER2 overexpression (9, 10). Indeed, Herceptin produces synergistic growth inhibition in adenocarcinomas when combined with a variety of agents, including taxanes and gemcitabine. The high frequency of HER2 overexpression in breast cancer and the paucity of effective agents in this setting make Herceptin a strong candidate for testing clinical efficacy. Indeed, early results of a Phase II clinical trial show that combining Herceptin with gemcitabine leads to higher response

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rates than with gemcitabine alone. Despite well-documented role of HER2 in the pathobiology of breast tumors, its potential role in modulation of differentiation pathway remains poorly understood.

A second evolving novel therapeutic approach to cancer treatment is the induction of terminal differentiation through ligand activation of nuclear hormone receptors such as PPARγ.[3] Recent reports have shown that PPARγ is overexpressed in a variety of cancers, including breast cancer. Furthermore, it has been shown that treatment of cancer cells with PPARγ ligands including the antidiabetic drug TGZ results in growth inhibition, differentiation, and apoptotic cell death (11–18). PPARγ ligands are also effective when used in combination with other targeted therapeutic agents such as inhibitors of histone deacetylase (19). Thus, PPARγ may represent a new molecular target for effective breast cancer therapy. However, complete growth inhibition was not achieved with TGZ as a single agent even at the highest doses tested; thus, cotreatment with an agent that functions by an independent mechanism might bring about greater growth inhibition and treatment efficacy. This report presents, for the first time, data that indicates HER2 up-regulates PPARγ expression and modulates the sensitivity of breast cancer cells to PPARγ ligand therapy.

MATERIALS AND METHODS

Cell Cultures and Reagents. SKBR3, MCF7, and MCF7/pcDNA, MCF7/HER2 human breast cancer cells were maintained in DMEM supplemented with 10% FCS. The following antibodies were used: anti-PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HER2 (Neomarkers, Fremont, CA), anti-vinculin (Sigma, St. Louis, MO), and antimouse horseradish peroxidase-conjugate (Amersham, Piscataway, NJ). In all of the experiments, Herceptin (Genentech, San Francisco, CA) was used at concentrations of 10 nM. Stock chemicals were from Sigma and Biomol.

Northern Blot Hybridization. Total cytoplasmic RNA was isolated using the Trizol reagent and 20 μg of RNA analyzed via Northern hybridization using a 1.8-kb nucleotide cDNA of PPARγ cDNA sequence corresponding to nucleotide 1–1760 (11, 14). rRNA (28S and 18S) was used to assess the integrity of the RNA, and the blots were routinely reprobed with human GAPDH cDNA for RNA loading and transfer control, as described previously (20).

Tissue Samples and Western Blotting. Human breast tissue samples were obtained from a tissue bank maintained by the University of Texas M. D. Anderson Cancer Center Breast Cancer Core Pathology Laboratory. Tumor estrogen receptor status for Fig. 2*B* was determined by the Surgical Pathology Core Facility. Specimens from patients who had undergone surgery for breast cancer were snap-frozen in liquid nitrogen and stored at −80°C, as described previously (21). Thawed tissue samples were homogenized in Triton X-100 lysis buffer (20 mM HEPES, 150 mM NaCL, 1% Triton X-100, 0.1% deoxycholate (v/w), 2 mM EDTA, 2 mM Na_3VO_4, and protease inhibitor mixture), and an equal amount of protein was analyzed by Western blotting. The protein vinculin was used routinely as a loading control.

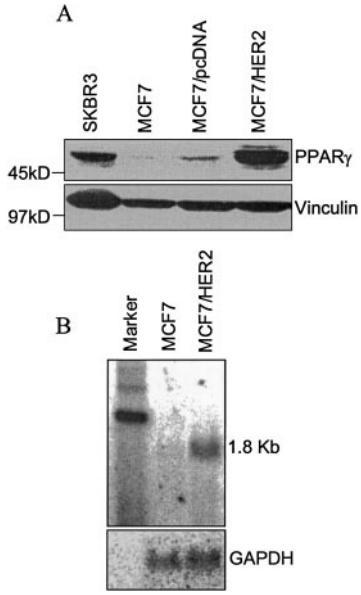

Fig. 1 HER2 induces PPARγ expression. *A,* cell lysates from the indicated cell lines were Western blotted with antibodies against PPARγ, or vinculin (as a control). *B,* expression of PPARγ mRNA and GAPDH mRNA by Northern hybridization analysis. *kD, M_r* in thousands.

PPRE-Luciferase Assays. Cells were split into 6-well tissue culture plates (Falcon) 24 h before transfection. Subconfluent cells were transiently transfected with PPRE-luciferase (14) using the LipofectAMINE method (Life Technologies, Inc.). After 24 h, 10 μM of TGZ or CIG were used to treat cells. Luciferase activity was measured 24 h after PPARγ ligand treatment using a Luciferase assay kit (Promega). Each experiment was repeated three to five times, and transfection efficiency varied between 30 and 50%.

Lipid Droplet Staining with Oil Red O. MCF and MCF7/HER2 cells were seeded into 6-well plates containing a glass coverslip in each well. Cells were cultured for 7 days in the absence or presence of 10 μM TGZ. The cells were fixed in 3.7% formaldehyde and PBS for 10 min at room temperature and then were stained with 1% oil red O and 60% triethylphosphate for 15 min, washed in PBS, followed by 100% Hemalaun staining for 15 min, and were washed in PBS (12).

Soft-Agar Colony Formation Assay. Soft-agar colony growth assays were also performed as described previously (22). Briefly, 1 ml of 0.6% Difco agar in DMEM supplemented with 10% fetal bovine serum and insulin was layered onto tissue culture plates. MCF7 or MCF7/HER2 cells (10^4) were mixed with 1 ml of 0.36% (w/v) Bactoagar solution in culture medium, and layered on the top of 0.6% (w/v) Bactoagar layer. The plates were incubated at 37°C in 5% CO_2 for 21 days.

Immunohistochemical Methods. For immunohistochemical detection of PPARγ and HER2, sections were depar-

[3] The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; TGZ, troglitazone; CIG, ciglitazone; MAPK, mitogen-activated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPRE, PPAR response element.

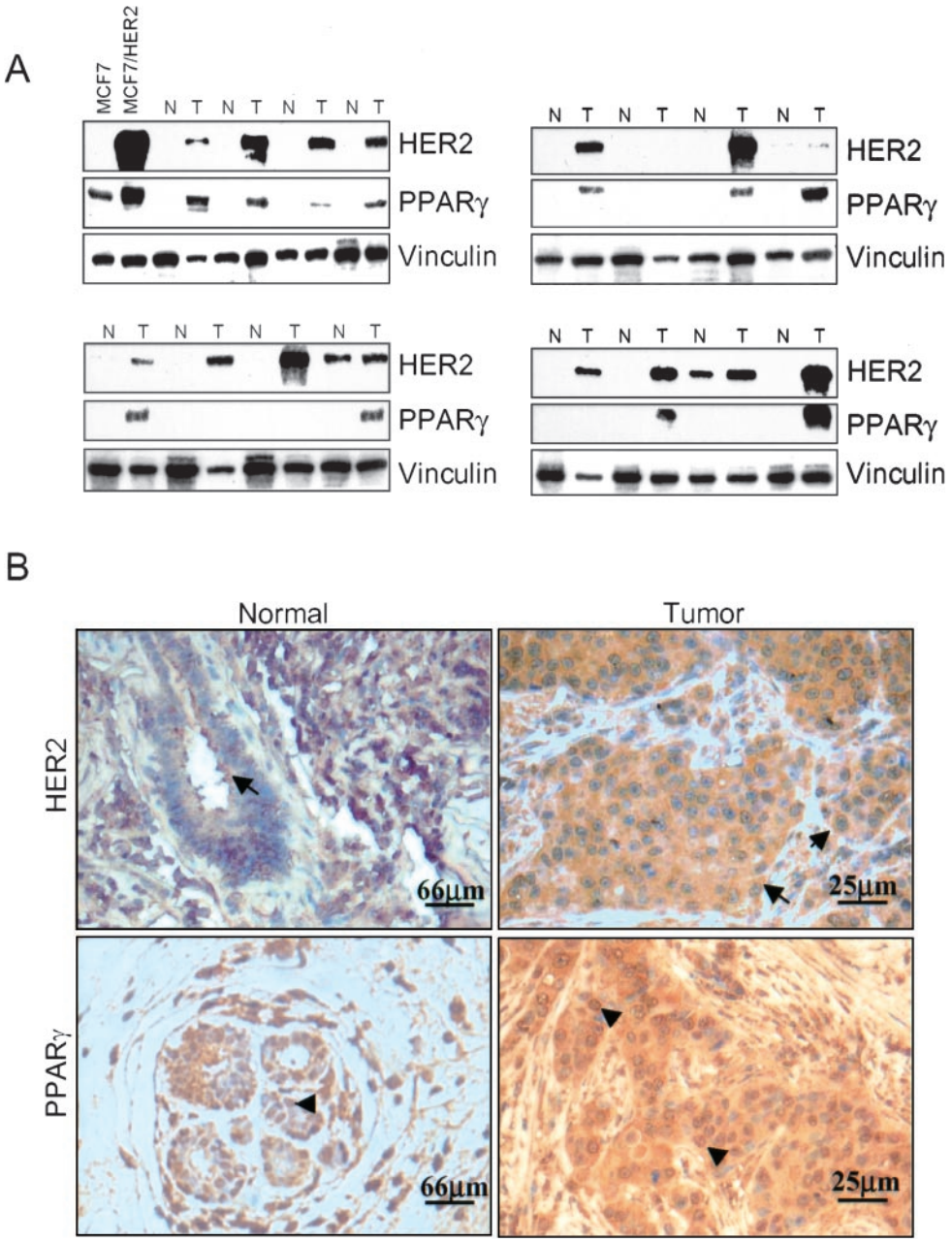

Fig. 2 PPARγ expression in normal tissues and in breast tumors. *A*, Western blots of paired normal breast tissue and breast cancer immunoblotted for PPARγ and HER2 receptor. *B*, representative paraffin-embedded tissue sections from one paired normal and tumor tissue sample were analyzed by immunostaining with antibodies against HER2 and PPARγ receptor. *Arrows*, positive staining (HER2); *arrowheads*, positive staining (PPARγ).

affinized with xylene and rehydrated using graded ethanol. Endogenous peroxidase activity was inactivated by incubating the sections in 0.3% H_2O_2 and methanol for 30 min. The sections were then boiled for 10 min in 0.01 M citrate buffer and were cooled for 30 min at room temperature to expose antigenic epitopes. The sections were then sequentially biotin- and protein-blocked and were incubated with primary antibody overnight at room temperature, followed by incubation with biotinylated secondary antibody, streptavidin-biotin complex, amplification reagent, and streptavidin-peroxidase complex (DAKO Corporation, Carpinteria, CA). The sections were then developed with diaminobenzidine-H_2O_2 and were counterstained with Mayer's hematoxylin. Primary antibody against PPARγ (Santa Cruz Biotechnology) and anti-HER2/neu (Neomarkers, Fremont, CA) were used at a dilution of 1:100.

RESULTS AND DISCUSSION

HER2 Regulation of PPARγ Expression in Breast Cancer Cells. To explore the potential effect of HER2 deregulation on the expression of PPARγ in breast cancer cells, we used MCF7, MCF7/pcDNA, and a well-characterized clone of MCF7/HER2 breast cancer cells with aggressive phenotypes (10, 23). The results shown in Fig. 1*A* demonstrate that HER2

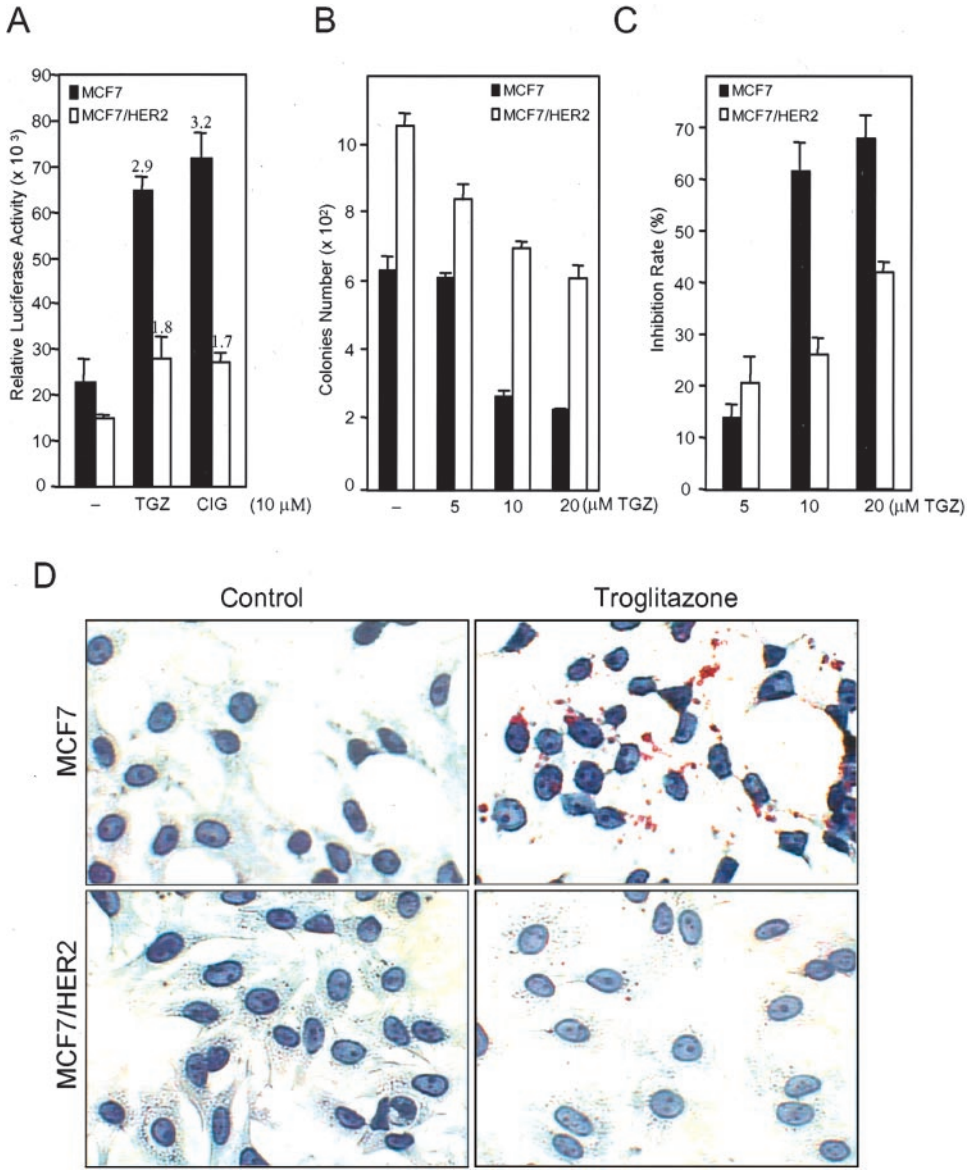

Fig. 3 Effect of HER2 overexpression on transcriptional activity of PPARγ and cell growth. *A*, PPRE-luciferase activity in MCF7 and MCF7/HER2 cells stimulated by TGZ or CIG. *Numbers above each bar*, the induction folds of PPARγ transcriptional activity as compared with the baseline of each cell line. *B* and *C*, effect of HER2 expression on TGZ-mediated inhibition of anchorage-independent growth of breast cancer cells ($n = 3$). *D*, effect of HER2 expression on TGZ-mediated differentiation-associated appearance of lipid droplets (*red*) of breast cancer cells. TGZ, 10 μM for 7 days ($n = 3$).

overexpression in MCF7 cells was accompanied by a significant increase in the steady-state level of PPARγ protein (M_r 50,000) as was the case in HER2-overexpressing SKBR3 breast cancer cells. Consistent with these findings, MCF7/HER2 cells also exhibited an increased expression of the steady-state levels of PPARγ mRNA (Fig. 1*B*). Together, these findings suggested that HER2 up-regulates the expression of PPARγ in breast cancer cells.

HER2 and PPARγ Expression in Human Breast Cancer. To explore the significance of PPARγ in human breast cancer progression, we examined PPARγ protein expression in human breast tumors. Previous reports have indicated that PPARγ may not be ubiquitously expressed. We next investigated whether PPARγ protein expression was altered in paired normal human breast epithelium and breast carcinoma biopsy samples (23). As shown in Fig. 2*A*, PPARγ expression was dramatically increased in 11 of 16 tumors as compared with the adjacent normal tissue with little or no PPARγ expression. Interestingly, these same tumor samples also had elevated levels of HER2 (Fig. 2*A*). There was no relationship between tumor ER status and PPARγ levels in the samples analyzed here (data not shown). To further validate the existence of a close relationship between HER2 and PPARγ levels, we next examined the codistribution of PPARγ and HER2 by immunocytochemistry in three of the paired samples examined by Western blot. As shown in one representative example in Fig. 2*B*, tumor overexpression of membranous and cytoplasmic HER2 (*arrows*), as compared with paired normal tissue, was accompanied by intense nuclear expression of PPARγ (*arrowheads*) in paraffin-embedded sections of the same tumor. Additional studies using a large number of clinical samples are needed to confirm these findings. In brief, these findings suggest that HER2 over-

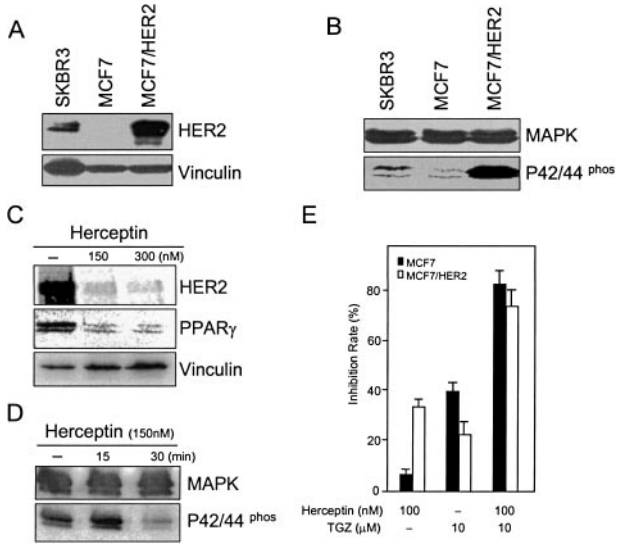

Fig. 4 Effect of HER2 down-regulation on PPARγ expression and functions. *A,* expression of HER2 in exponentially growing cells. *B,* status MAPK phosphorylated on Thr 402/404 residues ($n = 3$). *C* and *D,* MCF7/HER2 cells were treated with Herceptin and Western blotted with the antibodies against indicated proteins ($n = 3$). *E,* effect of Herceptin with or without TGZ on TGZ-mediated inhibition of anchorage-independent growth of breast cancer cells ($n = 3$). *F,* effect of the combination of TGZ with Herceptin HER2 expression on TGZ-mediated inhibition of anchorage-independent growth of breast cancer cells ($n = 3$).

expression might be closely associated with elevated levels of PPARγ in breast tumors.

Effect of HER2 Overexpression on PPARγ Transcriptional Activation and Cell Growth. To determine the influence of HER2 overexpression on transcriptional activity of endogenous PPARγ, we next examined the effect of PPARγ ligands with low TGZ and high CIG affinity for PPARγ on PPRE-luciferase reporter in MCF7 and MCF7/HER2 cells. Results indicated that both PPARγ ligands stimulated the transcriptional activity of PPARγ in the two cell lines (Fig. 3*A*). However, the transcription activities of PPARγ stimulated by TGZ and CIG were significantly lower in MCF7/HER2 cells as compared with MCF7 cells (Fig. 3*A*), raising the possibility that HER2 overexpression suppresses ligand-mediated activation of PPARγ, and may potentially confer resistance to PPARγ ligand-induced growth inhibition. Because both TGZ and CIG appeared equally effective in this assay, we used TGZ in the remaining experiments as a model compound for PPARγ activation.

To examine the possibility that HER2 overexpression suppresses PPARγ activation, we next examined the effect of TGZ on the anchorage-independent growth of breast cancer cells in soft agar. HER2 overexpression in MCF7 cells was indeed accompanied by suppression of PPAR stimulation-associated growth inhibition (Figs. 3, *B* and *C*). Consistent with these findings, HER2 overexpression also resulted in the suppression of differentiation-inducing function of TGZ, as determined by the appearance of lipid droplets in MCF7/HER2 cancer cells (Fig. 3*D*). These observations suggest that HER2 overexpression might antagonize the growth inhibition and differentiation of breast cancer cells by PPARγ stimulation.

Effect of HER2 Down-Regulation on Growth Inhibition by PPARγ Ligand. Elevated expression of PPARγ in HER2-overexpressing cells did not result in hyperresponsiveness to TGZ. In contrast, PPARγ up-regulation was accompanied with resistance to PPARγ activation. These findings suggested that HER2 overexpression may up-regulate cellular pathways with an antagonizing effect on TGZ-mediated growth inhibition. One such pathway that has been up-regulated by HER2 overexpression and also proven to be involved in PPARγ-induced growth inhibition is MAPK (11). Consistent with the previous reports, MCF7/HER2 cells (Fig. 4*A*) have a significant higher level of activated MAPK (Fig. 4, *B* and *C*). Interestingly, blockage and down-regulation of HER2 by anti-HER2 monoclonal antibody Herceptin (20, 21) was associated with a decreased level of activated MAPK (Fig. 4*D*) and PPARγ protein (Fig. 4*E*). In addition, the combination of TGZ with Herceptin resulted in a beneficial growth-inhibitory action as compared with the treatment with individual agents alone (Fig. 4*F*). These findings were consistent with a mechanistic role of MAPK in determining the responsiveness of breast cancer cells to PPARγ ligand therapy (11).

In summary, the results presented here have shown that HER2 up-regulates PPARγ expression and causes resistance of breast cancer cells to PPARγ ligand response. Herceptin, through down-regulation of HER2-mediated growth factor receptor signaling, sensitizes breast cancer cells to the inhibitory effects of TGZ, and that combination of Herceptin and PPARγ ligand therapy may lead to significant anti-growth activity in breast cancer cells. Furthermore, expression of HER2 and PPARγ may also serve as diagnostic indicators to help target particular expression profiles to specific treatment regimens. Additional studies will delineate the molecular pathways involved in HER2 regulation of PPARγ activation.

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