Regulation of Estrogenic Effects by Beclin 1 in Breast Cancer Cells

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Abstract

Beclin 1 is an essential mediator of autophagy and a regulator of cell growth and cell death. We examined the effect of Beclin 1 overexpression on the action of estradiol (E2) and two antiestrogens, raloxifene and 4-hydroxytamoxifen, in estrogen receptor α (ERα)-positive MCF-7 breast cancer cells. [3H]-thymidine incorporation studies showed that Beclin 1-overexpressing cells (MCF-7-beclin) had a lower proliferative response to E2 compared with cells transfected with vector control (MCF-7.control). There was only a 35% increase in [3H]-thymidine incorporation, after 24 hours of E2 treatment of MCF-7.beclin cells compared with untreated cells, whereas this increase was 2-fold for MCF-7.control cells. E2-induced changes in the expression of early-response genes were examined by real-time quantitative PCR. There were significant differences in the pattern of expression of E2-induced genes c-myc, c-fos, Erg-1, and Nur77 between MCF-7.beclin and MCF-7.control cells two hours after treatment. Although E2-induced growth of MCF-7.control cells was completely inhibited by 500 nmol/L raloxifene or 500 nmol/L 4-hydroxytamoxifen, these concentrations of antiestrogens had no significant effect on the growth of MCF-7.beclin cells. Confocal microscopic and coimmunoprecipitation studies showed evidence for colocalization and association of Beclin 1 and ERα. In addition, E2 caused a decrease in Akt phosphorylation in MCF-7.beclin cells, compared with a 3-fold increase in MCF-7 cells, five minutes after treatment. These results indicate that Beclin 1 can down-regulate estrogenic signaling and growth response, and contribute to the development of antiestrogen resistance. This observation might be useful to define and overcome antiestrogen resistance of breast cancer. [Cancer Res 2008;68(19):7855–63]

Introduction

Beclin 1 is an essential gene in autophagy, a cell survival pathway that enables use of long-lived proteins as a source of amino acids under conditions of nutritional deprivation (1–4). In autophagy, Beclin 1 interacts with class III phosphatidylinositol-3-OH kinase (vps34) during autophagosomal membrane engulfing of damaged cytoplasmic organelles and long-lived proteins. Autophagy can facilitate cell survival, delaying apoptotic death (5, 6). Alternatively, autophagy can facilitate a form of cell death called autophagic or type II programmed cell death, characterized by autophagic vacuoles in the cytoplasm (7). Autophagy and Beclin 1 are important in the balance of breast cancer cell growth and death (8, 9). The function of Beclin 1 is, in part, defined by its interaction with the antiapoptotic gene products, Bcl-2 and Bcl-xl (8, 9). Beclin 1 is also a tumor suppressor gene because one allele of Beclin 1 is lost in subsets of breast, prostate, and other tumors (10). Overexpression of Beclin 1 in MCF-7 breast cancer cells reduced tumorigenicity in nude mice (11). These results raise questions on molecular pathways by which Beclin 1 modifies estrogenic function in breast cancer.

Estrogens function through the receptors, estrogen receptor (ER)α and ERβ, the ligand-activated transcription factors controlling the growth of ER-positive breast tumors and target tissues (12, 13). ERα mediates the proliferative stimulus of E2 on ER-positive breast cancer cells, whereas ERβ suppresses cell proliferation (14). Estrogenic ligands alter the conformation of ERs such that ERs recruit coregulatory proteins to the promoter/ enhancer sites of responsive genes to facilitate transcription (12, 13, 15). ER-recognition of estrogen response element and associated changes in chromatin stimulate a cell- and tissue-specific network of genes, enabling E2 to exert multiple functions. In addition, estrogenic action includes nongenomic mechanism(s), involving membrane ERs and activation of kinase cascades in association with G protein–coupled receptors or growth factor receptors (15–17).

Cell signaling through the ERs is pivotal to the regulation of breast cancer cell growth so that the ERα antagonist, tamoxifen, is the first targeted breast cancer therapeutic agent (18). 4-Hydroxytamoxifen is an active metabolite of tamoxifen, with higher affinity for ERα than tamoxifen (19). Although tamoxifen is initially effective against ERα-positive tumors, resistance develops in most women (20). The agonistic activity of tamoxifen on uterine cells is also a cause for concern (21). Raloxifene is an antiestrogen with mixed agonist/antagonist activity, in a tissue-specific manner (22, 23). Raloxifene is an agonist capable of enhancing bone density, whereas it is an antagonist on breast epithelial cells and the reproductive system. Understanding the basis of agonist activity of antiestrogens is an important aspect of overcoming antiestrogen resistance.

For a subset of ERα-positive tumors, cell growth is totally dependent on ERα (12, 13). Functions of ERα as a transcription factor and its ability to bind to accessory proteins and coregulators are critical to the control of cancer cell growth (12, 13). Because Beclin 1 inhibited E2-dependent MCF-7 tumor growth in immune-deficient mice (10), an interaction between ERα signaling and Beclin 1 function seemed possible. We, therefore, examined the consequences of Beclin 1 overexpression on E2-induced cell growth and cell signaling. We found that E2-induced cell signaling and
Materials and Methods

Cell culture. MCF-7 cell line was obtained from the American Type Culture Collection. We obtained MCF7.beclin cells expressing Beclin 1 from a tetracycline-repressible promoter (pTRE/flag-beclin 1; ref. 11), and a control cell line containing empty vector (MCF7/empty) from Dr. Beth Levine (University of Texas, Southwestern Medical Center, Dallas, TX). DMEM, phenol red–free DMEM, fetal bovine serum (FBS), and anti–β-actin antibody were from Sigma Co. Antibiotics, trypsin, and other additives for cell culture medium were purchased from Invitrogen. Anti-Beclin 1, anti-phospho-Akt, anti-Akt, anti-pERK, and anti–phospho-ERK antibodies were purchased from Cell Signaling Technology. Secondary antibodies conjugated with Alexa Fluor 633 or Alexa Fluor 488 were from Invitrogen.

MCF-7 cells were maintained in DMEM, supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 40 μg/mL gentamicin, 2 μg/mL insulin, 0.5 mmol/L sodium pyruvate, 50 mmol/L nonessential amino acids, 2 mmol/L l-glutamine, and 10% FBS. MCF-7/empty and MCF7.beclin 1 cells were maintained in DMEM supplemented with 10% FBS, 40 μg/mL gentamicin, 5 mg/mL G418, 2 μg/mL insulin, 200 μg/mL hygromycin, and 2 μg/mL tetracycline. Before each experiment, cells were grown for 3 d in phenol red–free DMEM containing serum treated with dextran-coated charcoal (DCC) to remove serum-derived estrogenic compounds (16). MCF-7.beclin cells were grown for 3 d in phenol red–free DMEM without tetracycline, before plating under phenol red–free conditions.

[3H]-Thymidine incorporation. Cells (0.5 × 10^6) were seeded in 6-well culture plates in phenol red–free DMEM supplemented with DCC treated serum and additives. After 24 h of plating, cells were treated with 4 mmol/L E2. DNA synthesis was measured by adding 4 μC/mL of [3H]-thymidine, 1 h before harvest (24). Cells were treated with 5% trichloroacetic acid followed by 1 N NaOH, and 1 N HCl. The radioactive thymidine in cellular DNA was quantified by liquid scintillation counting.

Cell proliferation. MCF-7.beclin cells were seeded at a density of 5 × 10^4 cells per well in 24-well plates, in the presence of tetracycline in phenol red–free medium. A parallel study was set up with MCF-7.beclin cells without tetracycline. Cells were dosed 24 h after plating, with 4 mmol/L E2, and redosed after 48 h. After appropriate treatment periods, live cells were counted using the trypan blue exclusion, using a hemocytometer.

CellTiter Glo assay. This assay measures cell viability by level of ATP content (25). Cells (5 × 10^3 per well) were plated in 96-well plates. E2/anti-estrogens were added 24 h after plating. Cells were redosed with medium change at 48 h after the first dose. After 96 h, cells were treated with 0.1 μL of CellTiter Glo (Promega) reagent, incubated for 5 min at 22°C, and luminescence recorded using a GloMax Luminometer (Promega).

qPCR. MCF-7.beclin-1 or MCF-7/empty cells (1 × 10^6) were seeded in 60-mm culture dishes. After 24 h, cells were treated with E2 (4 nmol/L) for 0, 1, or 2 h. RNA was isolated using Trizol reagent (Invitrogen), and 2 μg RNA was reverse transcribed using the first strand cDNA synthesis kit (Fermentas, Inc.) with random hexamers as primers. The expression of TFF1 (pS2), c-Myc, c-fos, Erg-1, Nur77, and Gapdh genes was determined by real-time PCR using the SYBR Green PCR Master Mix (Bio-Rad) with the following primers: 5′-CAATGTGCAACATCGGAGA-3′ and 5′-ACCGTTGCTGACGAAACG-5′ for TFF1 (188 bp); 5′-CTTCCAGCAG-CACTGTCG-3′ and 5′-CTTGGCAGGATGTCTCC-3′ for c-Myc (101 bp); 5′-CCGGCTTCAACAGCAAGT-3′ and 5′-GGTCTCAGGAGAACTTGC-5′ for Erg-1 (167 bp); 5′-ACGTGACGACGATCTCTCC-3′ and 5′-GCCAG-TATAGGTGGAGGGG-5′ for Erg-1 (110 bp); 5′-CCGAGACCAGC-3′ and 5′-TGGTCTGGACGCAACTTCC-3′ for 5′-CAGTTCTGAGAAGC-5′ and 5′-GTGGCTTGAGAACTTCC-3′ for Nur77 (145 bp); and 5′-CATGAGAGTATGATGACACGCTT-3′ and 5′-GGCTAGG-TATTGCTGGGGG-5′ for Gapdh (113 bp). A final volume of 25 μL was used for qPCR in an IQ5 thermocycler (Bio-Rad). Amplification conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and annealing for 30 s (57°C for TFF1 and 55°C for other genes). qPCR products were normalized relative to that of Gapdh to correct for differences in template input. Results are expressed as fold differences in expression of the indicated gene relative to that of Gapdh. Standard curves were generated for every target using six 4-fold serial dilutions.

Confocal microscopy. Cells were plated in Labtek 6-well slide chamber and dosed after 24 h (16). Cells were fixed in 4% paraformaldehyde, blocked in normal goat serum (5%) in PBS, followed by incubation with anti-ErbA antibody (mouse) and anti-Beclin 1 antibody (rabbit) in 2.5% goat serum in PBS. After washing, cells were incubated with appropriate secondary antibodies. Alexa Fluor 633 conjugated anti-mouse IgG was used for ERα (deep red) and Alexa Fluor 488 conjugated anti-rabbit IgG (green) was used for Beclin 1. Experiments were repeated using Alexa Fluor 488–conjugated anti-mouse IgG for detecting ERs (green) and Alexa Fluor 633 conjugated anti-rabbit IgG for detecting Beclin 1 (red). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 nmol/mL). Images were recorded using a Zeiss 510 Laser scanning microscope with a ×60 objective at identical intensity settings for all treatment groups. No fluorescence was detected when cells were treated with fluorescence labeled secondary antibody alone.

Immunoprecipitation. Cells (1.5 × 10^6 per 60-mm dish) were plated in dishes and allowed to attach for 24 h before treatments. After the specified treatments, cells were harvested and cell pellet lysed in buffer containing 50 mmol/L Tris.HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton, 5 mmol/L EDTA, 25 mmol/L sodium fluoride, 25 mmol/L sodium PPI, 2 mmol/L sodium vanadate, 5% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride and 1/10 protease inhibitor cocktail (Calbiochem; ref. 26). Cell lystate (100 μL in 100 μL lysis buffer) was precleared with Protein A/G agarose and incubated with 10 μL of anti-ErbA (mouse monoclonal) antibody for 16 h at 4°C. Reaction mixture was incubated with protein A/G-Sepharose for 1 h at 4°C. Sepharose beads were washed thrice with cold lysis buffer, extracted using Laemmli buffer, and loaded on 12% SDS polyacrylamide gel for immunoblot analysis.

Western blot analysis. Cells (1.5 × 10^6 per 60-mm dish) were plated in dishes and allowed to attach for 24 h before treatments. After the specified treatments and time periods, cells were harvested and cell pellet was lysed in buffer (16). Proteins (25 μg) were separated on a 12% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride Polyscreen membrane and incubated with 1:2000 to 1:10,000 dilution of the primary antibody. Protein bands were visualized using horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology) and SuperSignal West Peco Chemiluminescent Substrate (Pierce). Membranes were stripped in stripping buffer [62.5 mmol/L Tris.HCl (pH 6.8), 2% SDS, 100 mmol/L (fresh) β-mercaptoethanol] and washed thrice in washing buffer [20 mmol/L Tris.HCl (pH 7.6), 150 mmol/L NaCl, and 0.05% Tween 20]. A second primary antibody was then added and the membrane reprobed as needed. To verify equal protein loading, membranes were stripped and reblotted with anti–β-actin antibody. Lightly exposed Kodak XAR Biomax films were scanned using an Epson B4 Scanner and band intensities quantified using the NIH Image J 1.34S program. Fold changes in the intensity of the protein signals reported are the mean of the results from three experiments.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was performed as described previously (27) with minor modifications. Cells (8 × 10^5) in 10-cm dishes were washed once with PBS and cross-linked with 1.5% formaldehyde at 37°C for 10 min. After washing with PBS, cells were collected in 1.6 mL of lysis buffer [0.5% SDS, 5.6 mmol/L L EDTA, 33 mmol/L Tris.HCl (pH 8.1), 0.5% Triton X-100, 84 mmol/L NaCl] and incubated on ice for 30 min. Cell lystate was sonicated using Sonicator 3000 (Misonix). Samples were diluted 5-fold with dilution buffer [0.01% SDS, 1.2 mmol/L EDTA, 16.7 mmol/L Tris.HCl (pH 8.1), 1.1% Triton X-100, 167 mmol/L NaCl] and then precleared with salmon sperm DNA/protein A agarose for 2 h at 4°C. Anti-ERα antibody (BC-20, 5 μg) or rabbit IgG (Santa Cruz) was used to immunoprecipitate from 200 μg of protein. Immunoprecipitated DNA was amplified by PCR using AccuPrime TaqDNA polymerase and visualized with ethidium bromide staining. The following primers from pS2 promoter used were as follows: 5′-CTAGCCAAGGTGGGCCCTTCGT-3′ (forward) and 5′-TCTTTCAACACTGGTAAAT-3′ (reverse).
after the addition of [3H]-thymidine. Cells were harvested for scintillation counting determination by flow cytometry with propidium iodide staining.

DNA synthesis was observed in MCF-7.beclin cells. To verify the DNA synthesis due to E2 treatment. However, only 35% increase in 24-hour time point, MCF-7.control cells showed a 2-fold increase in DNA synthesis in E2-treated cells was indicated time points.

Western blots. We found a decrease in Akt phosphorylation in MCF-7.beclin cells treated with E2, compared with untreated cells. In contrast, only c-myc mRNA level showed a moderate increase at 2 hours after E2 treatment in MCF-7.beclin cells, whereas c-fos, Erg-1, and Nur77 mRNAs decreased, compared with those of untreated cells. All of the tested mRNAs showed lower levels in E2-treated MCF-7.beclin cells, compared with E2-treated MCF-7.control cells. These results indicate that the reduction of E2-induced DNA synthesis in MCF-7.beclin cells is associated with altered expression of early-response genes.

E2 is known to induce Akt phosphorylation as a part of nongenomic mechanism of action of ERα. We, therefore, examined whether E2 response is modified in MCF-7.beclin cells. Cells were treated with 4 nmol/L E2 for 5, 10, 20, and 30 minutes. Cells were harvested and phospho-Akt levels determined by Western blots. We found a decrease in Akt phosphorylation in MCF-7.beclin cells treated with E2, compared with untreated cells.

**Statistical analysis.** All experiments were repeated at least thrice. Statistical difference between control and treatment groups was determined by one-way ANOVA followed by Dunnet’s posttest using SigmaStat statistical program. A P value of <0.05 was considered to be statistically significant.

**Results**

We tested the effect of E2 on MCF-7.beclin cells that expressed Beclin 1 from a tetracycline-repressible promoter. MCF-7 cells transfected with control plasmid (MCF-7.control) were also treated with E2 in the same manner. Cells were plated in 6-well plates, and E2 was added 24 hours later at 0.1 or 4 nmol/L concentrations. At the time of dosing, 62% ± 3% of the cells were in the G1 phase, as determined by flow cytometry with propidium iodide staining. We, therefore, examined whether E2 response is modified in MCF-7.beclin cells. Cells were treated with 4 nmol/L E2 after 48 hours of treatment. The level of DNA synthesis in E2-treated cells was ~2.5-fold higher than that of untreated cells at the same time point. In contrast, Beclin 1-transfected cells showed only 75% increase in DNA synthesis at the 48-hour time point in the presence of 4 nmol/L E2. At the 24-hour time point, MCF-7.control cells showed a 2-fold increase in DNA synthesis due to E2 treatment. However, only 35% increase in DNA synthesis was observed in MCF-7.beclin cells. To verify the level of Beclin 1 protein expression in MCF-7.beclin cells, Western immunoblots were conducted using Beclin 1 antibody. Our result (Fig. 1C) showed that MCF-7.beclin cells had a 5-fold higher level of Beclin 1 compared with MCF-7.control cells, as expected from its overexpression due to transfection.

We next examined E2-induced changes in the expression of early response genes in MCF-7.control and MCF-7.beclin cells. As shown in Fig. 1D, there were significant changes in the pattern of expression of E2-induced genes between MCF-7.beclin cells and MCF-7.control cells. As expected from the E2 sensitivity of MCF-7.control cells, there was 2- to 3.5-fold higher levels of transcripts of Tff1, c-myc, c-fos, and Nur77 in these cells, after 2 hours of E2 treatment, compared with those of untreated cells. In contrast, only c-myc mRNA level showed a moderate increase at 2 hours after E2 treatment in MCF-7.beclin cells, whereas c-fos, Erg-1, and Nur77 mRNAs decreased, compared with those of untreated cells. All of the tested mRNAs showed lower levels in E2-treated MCF-7.beclin cells, compared with E2-treated MCF-7.control cells. These results indicate that the reduction of E2-induced DNA synthesis in MCF-7.beclin cells is associated with altered expression of early-response genes.
4-hydroxytamoxifen, on MCF-7.beclin and MCF-7.control cells was no change in the level of total Akt, p-ERK1/2 showed a 3-fold increase in Akt phosphorylation, 5 minutes after its addition. Increase in ERK1/2 phosphorylation remained intact. These results indicate that whereas E2-induced Akt phosphorylation is down-regulated in the presence of Beclin 1 overexpression, we studied MCF-7.beclin cells containing tetracycline have some differences in antiestrogen responses.

We next examined the long-term effects of E2, raloxifene and 4-hydroxytamoxifen, on MCF-7.beclin and MCF-7.control cells using CellTiter Glo assay. Cells were treated with E2 or antiestrogens for 48 or 96 hours. Assay at 96 hours provided maximal responses to E2 and antiestrogens. Our results (Fig. 3) show that the presence of E2 increased the luminescence of MCF-7.control cells by 2-fold, whereas there was only a 33% increase in luminescence in MCF-7.beclin cells. Raloxifene and 4-hydroxytamoxifen inhibited E2-induced growth of MCF-7.control cells at 500 nmol/L. In MCF-7.beclin cells, raloxifene had no significant effect even at 1,000 nmol/L. With 4-hydroxytamoxifen, E2-induced proliferative response of MCF-7.beclin cells was not affected at 500 nmol/L. 4-hydroxytamoxifen, but it was inhibited at 1,000 nmol/L. However, the basal proliferation of MCF-7.beclin cells or MCF-7.control cells in the absence of E2 was not affected by these antiestrogens.

As an additional control for the effects of E2 and antiestrogens in the absence of Beclin 1 overexpression, we studied MCF-7.beclin cells in the presence of tetracycline. E2 response of cells containing tetracycline was similar to that of MCF-7 cells and antiestrogens suppressed the proliferative effect. However, when cells were treated with 4-hydroxytamoxifen and raloxifene in the absence of E2, there was a 30% to 40% increase in proliferation, indicating that MCF-7.control and MCF-7.beclin cells containing tetracycline have some differences in antiestrogen responses.

We also examined E2-induced response of MCF-7.beclin cells by trypan blue exclusion, using a hemocytometer (Fig. 3B). Experiments were conducted in the presence and absence of tetracycline. After 48 hours, E2 treatment induced only 20% to 30% increase in cell number, regardless of the presence of tetracycline. By 4 days, E2 treatment of MCF-7.beclin cells showed a 2.5-fold increase in cell number in the presence of tetracycline. However, there was only ~50% increase in cell number in MCF-7.beclin cells in the absence of tetracycline. This result validates our findings on the influence of Beclin 1 on E2 response, detected by CellTiter Glo assay.

E2α is known to redistribute within the cell after the addition of E2 (16, 30). Therefore, we examined whether E2α and Beclin 1 interact and redistribute in the presence of E2 by confocal microscopy (Fig. 4). In untreated cells, Beclin 1 (green) was found distributed in both cytoplasm and occasionally in the nucleus. In contrast, E2α (red) was mainly found in the nucleus. Addition of E2 caused a movement of Beclin 1 to the perinuclear area. Addition of E2 caused a redistribution of E2α throughout the cytoplasm and the nuclei in the case of mitotic cells (Fig. 4A) and toward cytoplasm in the case of clusters of cells (Fig. 4B). Addition of E2 enhanced colocalization of E2α and Beclin 1, indicated by the yellow color in the merged photographs. Redistribution of Beclin 1 and its accumulation in the perinuclear area was illustrated by the increased colocalization of Beclin 1 with Golgin-97 (Fig. 4C) in E2-treated cells. These results indicate that localization and function of Beclin 1 and E2α are being modified by E2α.

We next examined the effect of raloxifene and 4-hydroxytamoxifen on the localization of Beclin 1 and E2α in MCF-7.beclin cells. Figure 5 shows a representative confocal microscopic study of MCF7.beclin cells with and without raloxifene treatment. In these experiments, green represents E2α and red represents Beclin 1. Although a different set of secondary antibodies were used, accumulation of E2α in the nucleus and distribution of Beclin 1 throughout the cell remained similar to previous studies (11, 16). However, the intensity of Beclin 1 signal was much stronger for Alexa Fluor 633-conjugated (red) antibody (Fig. 5), compared with Alexa Fluor 488-conjugated (green) antibody (Fig. 4). Addition of raloxifene caused a reorganization of E2α or Beclin 1 so that these proteins were found throughout cytoplasm and nucleus, including membrane projections (Fig. 5). Merge pictures showed association of E2α and Beclin 1 with a strong yellow color, indicating colocalization of E2α and Beclin 1 (Fig. 5) in the presence of raloxifene. Colocalization of Beclin 1 and E2α was also observed in the presence of 4-hydroxytamoxifen (data not shown).

Subsequently, we examined the interaction of E2α and Beclin 1 by coimmunoprecipitation. Our results (Fig. 6A) showed that Beclin 1 was immunoprecipitated in association with E2α. Interestingly, addition of E2 caused a 1.5- to 2-fold increase in the level of Beclin 1 associated with E2α, whereas the presence of raloxifene caused a 2-fold increase. The presence of 4-hydroxytamoxifen increased the level of Beclin 1 associated with E2α by 2.5-fold. Taken together, results of immunoprecipitation and subsequent immunoblotting confirmed the association of Beclin 1 with E2α, as observed in confocal microscopic studies. Although coimmunoprecipitation of E2α and Beclin 1 was found in untreated cells as well as in E2- and raloxifene-treated cells, maximal level of coimmunoprecipitated proteins were found in raloxifene-treated cells.

To examine whether band intensities in the immunoprecipitation study have contributions from changes in the level of E2α and...
Beclin 1, we conducted direct Western blot analysis. Representative blots from 24-hour treatment are shown in Fig. 6B. Beclin 1 level did not undergo any major change due to treatment with E2 or antiestrogens. ERα level decreased (10–25%) in the presence of E2 and slightly increased in the presence of antiestrogens (1.5- to 2-fold) after normalizing to β-actin level. These changes in ERα are consistent with reports on the effects of E2 and antiestrogens on ERα stability (16, 28).

We next conducted ChIP assay to elucidate the mechanistic differences in the action of E2 and antiestrogens in MCF-7 and MCF-7.beclin cells. A comparison of the effects of E2, 4-hydroxytamoxifen, and raloxifene on MCF-7 cells and MCF-7.beclin cells is presented in Fig. 6C. ChIP assay showed comparable E2 responses on ERα occupation of pS2 promoter in both MCF-7 and MCF-7.beclin cells. Thus, the partial inhibition of the growth of MCF-7.beclin cells is not associated with ERα binding to pS2 promoter. In the presence of 4-hydroxytamoxifen and raloxifene, ERα occupation on pS2 promoter was lower in MCF-7.beclin cells than that in MCF-7 cells. This result argues against a role for Beclin 1 as a repressor that binds to both ERα and DNA. Thus, the lack of antiestrogen response in the presence of Beclin 1 might involve the interaction between ERα and Beclin 1, physically sequestering ERα away from promoter sites.

Discussion

In this study, we examined the effect of Beclin 1 on E2 function and found that Beclin 1–overexpressing cells had reduced proliferative response to E2. qPCR analysis of gene expression showed that several E2-responsive genes were down-regulated in cells expressing Beclin 1. E2 caused a decrease in Akt phosphorylation in Beclin 1–transfected cells. Growth inhibitory responses of antiestrogens, raloxifene, and 4-hydroxytamoxifen were also limited by Beclin 1 transfection. In contrast, E2-induced growth of MCF-7.control cells was totally inhibited by antiestrogens.
The presence of E2 caused a reorganization and accumulation of Beclin 1 in the perinuclear areas of cells. Importantly, we found a colocalization of ERα and Beclin 1 by confocal microscopy in MCF-7.beclin cells. An interaction between Beclin 1 and ERα was also evident from the results of coimmunoprecipitation, followed by Western immunoblotting. ChIP assay results suggest that interaction of ERα and Beclin 1 in the presence of antiestrogens leads to decreased promoter occupancy. Our results show that Beclin 1 can down-regulate estrogen-induced signaling events and cell growth in ERα-positive breast cancer cells. Our results further suggest that Beclin 1 may alter sensitivity of antiestrogens to breast cancer cells.

There have been extensive efforts to understand tamoxifen resistance in breast cancer cells (31). Although a primary mechanism of estrogen insensitivity involves the loss of ERα, tamoxifen resistance often occurs in the presence of ERα. Because ERβ has a down-regulatory effect on many estrogenic responses, it is the growth stimulatory responses of ERα that are generally targeted by tamoxifen (20, 32). A basic mechanism for acquired tamoxifen resistance is that ERα becomes insensitive, as the essential growth regulatory circuits are taken over by growth factor receptors, including members of the HER-2 family of receptors, or G protein–coupled receptors (20, 33). Our results show that overexpression of Beclin 1 provides a context under which ERα does not transmit cell signaling responses such as Akt phosphorylation. Down-regulation of E2-responsive genes, such as c-fos, Erg-1, and Nur77, is indicative of an altered molecular environment for estrogenic function. ChIP assay results showed decreased
receptor occupancy of pS2 promoter due to increased Beclin 1 expression, especially in the presence of tamoxifen.

Both tamoxifen and raloxifene are selective estrogen receptor modulators, dependent on the presence of coactivator/corepressor proteins (34). Interaction of ligands with ERα and ERβ induces subtle conformational changes that amplified by the binding of tissue-specific proteins (35, 36). Raloxifene was first approved for the prevention of osteoporosis because it enhanced bone density (37, 38). After large comparative study of tamoxifen and raloxifene (STAR trial), raloxifene was found to decrease the incidence of breast cancer and was less prone to induce endometrial cancer, compared with tamoxifen (39). Cell culture studies have shown raloxifene resistance (40). The observation that both 4-hydroxytamoxifen and raloxifene showed a lack of sensitivity in MCF-7.beclin cells, indicates that a common factor in antiestrogen resistance might be the functional availability of ERα, when the receptor is complexed with proteins such as Beclin 1. Such an interaction might allow sequestering of Beclin 1, until conditions of nutritional deprivation or stress arise.

Beclin 1–overexpressing cells were first investigated by Liang and colleagues (11). Although these cells had decreased clonogenicity and showed a different morphology from that of wild-type MCF-7 cells, nutritional deprivation was necessary to produce a large number of autophagosomes (11). Although Beclin 1 was localized in the endoplasmic reticulum, mitochondria, perinuclear membrane as well as the nucleus, Beclin 1 mutants deficient in nuclear export were unable to facilitate autophagy (8). Interestingly, ERα also shuttles between nucleus, cytoplasm, and peripheral membrane and interacts with multiple proteins in these locations.
in regulating E2-induced expression of hundreds of proteins (29). Thus, the interaction of ERα and Beclin 1 may affect the function of both Beclin 1 and ERα.

Our results on DNA synthesis and CellTiter Glo assays showed that MCF-7/beclin cells had a reduced growth response in the presence of E2 compared with vector control cells. However, E2-response was still present, yielding 30% to 75% increase in DNA synthesis at 24 and 48 hours, respectively. Phospho-Akt levels in MCF-7/beclin cells showed a decrease after treatment with E2. Because E2-induced phosphorylation of Akt is dependent on active ERα (15), the association of ERα and Beclin 1 may be involved in decreased Akt phosphorylation. In contrast, ERK1/2 was activated by E2 in Beclin 1–overexpressing cells.

ER-dependent nongenomic functions are dependent on many coregulators, including modulators of nongenomic action of receptors (41) and growth factor receptors (42, 43). E2 facilitates an interaction between ERα and epidermal growth factor receptor member, ERBB4 (44). Cross-talk also exists between ERα and ERBB2 (HER-2) signaling and insulin-like growth factor pathways, contributing to tamoxifen resistance (42, 44).

Divergent protein–coupled receptors and G proteins (such as Gαi and Gβγ) may also be involved in E2 responses (45).

Recent studies suggest that Bcl-2 binding domain of Beclin 1 serves as a point of cross-talk between autophagic and apoptotic pathways (46). Bcl-2 down-regulation by an antisense oligonucleotide provoked autophagic cell death in HL-60 cells (47). Interestingly, crystallographic studies identified Beclin 1 as a Becl-2 homology domain 3–only (BH3-only) protein (9, 48). This observation would classify Beclin 1 along with other proapoptotic BH3-only proteins, such as Bim, Bid, and Bok, suggesting a proapoptotic function that might be activated under unique tissue/ cellular context (49). Whether the interaction of ERα with Beclin 1 modulates proapoptotic function by sequestering essential growth stimulatory proteins needs to be investigated.

In summary, our results reveal an interaction between ERα and Beclin 1 in breast cancer cells. This interaction may modulate the function of ERα and Beclin 1. In the context of ERα function, we found that Beclin 1–transfected cells were less sensitive to E2–induced growth stimulation and to the growth inhibitory effects of antiestrogens. Thus, a novel function for Beclin 1 might involve down-regulation of the action of ERα, contributing to resistance of breast cancer cells to antiestrogens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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