Coregulation of Estrogen Receptor by ERBB4/HER4 Establishes a Growth-Promoting Autocrine Signal in Breast Tumor Cells

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Abstract

Although crosstalk between cell-surface and nuclear receptor signaling pathways has been implicated in the development and progression of endocrine-regulated cancers, evidence of direct coupling of these signaling pathways has remained elusive. Here we show that estrogen promotes an association between extranuclear estrogen receptor α (ER) and the epidermal growth factor receptor (EGFR) family member ERBB4. Ectopically expressed as well as endogenous ERBB4 interacts with and potentiates ER transactivation, indicating that the ERBB4/ER interaction is functional. Estrogen induces nuclear translocation of the proteolytic processed ERBB4 intracellular domain (4ICD) and nuclear translocation of 4ICD requires functional ligand-bound ER. The nuclear ER/4ICD complex is selectively recruited to estrogen-inducible gene promoters such as progesterone receptor (PgR) and stromal cell–derived factor 1 (SDF-1) but not to trefoil factor 1 precursor (pS2). Consistent with 4ICD-selective promoter binding, suppression of ERBB4 expression by interfering RNA shows that 4ICD coactivates ER transcription at the PgR and SDF-1 but not the pS2 promoter. Significantly, ERBB4 itself is an estrogen-inducible gene and the ERBB4 promoter harbors a consensus estrogen response element (ERE) half-site with overlapping activator protein-1 elements that bind ER and 4ICD in response to estrogen. Using a cell proliferation assay and a small interfering RNA approach, we show that ERBB4 expression is required for the growth-promoting action of estrogen in the T47D breast cancer cell line. Our results indicate that ERBB4 is a unique coregulator of ER, directly coupling extranuclear and nuclear estrogen actions in breast cancer. We propose that the contribution of an autocrine ERBB4/ER signaling pathway to tumor growth and therapeutic response should be considered when managing patients with ER-positive breast cancer. (Cancer Res 2006; 66(16): 7991-8)

Introduction

Breast cancer is the most commonly diagnosed cancer in North American women and is second to lung cancer as the leading cause of cancer-related deaths in these women (1). The development and progression of breast cancer to a lethal metastatic disease involve, in part, the complex interplay between growth factor and steroid receptors. For example, patients with estrogen receptor α (ER)–positive tumors have a favorable prognosis and can be effectively treated with a selective estrogen receptor modulator such as tamoxifen. On the other hand, overexpression/amplification of oncogenic members of the epidermal growth factor receptor (EGFR) family, including EGFR, ERBB2, and ERBB3, in breast cancer inversely correlates with ER expression and these patients have poor prognosis (2, 3). When coexpressed with ER in preclinical models of breast cancer, ERBB2 promotes cellular proliferation and tamoxifen resistance (4). Interestingly, expression of the final EGFR family member to be identified, ERBB4, in primary breast tumors strongly correlates with ER expression (5–8). Furthermore, patients with tumors coexpressing ERBB4 and ER have fewer recurrences (9) and improved survival (10) when compared with patients expressing ER alone. These clinical observations imply a unique relationship between ERBB4 and ER signaling in breast cancer. Attempts to decipher the molecular contributions of ERBB4 signaling to the biology of ER(+) breast tumors, however, have led to the identification of several divergent ERBB4-regulated cellular responses. On the one hand, we and others have shown that ectopic ERBB4 expression in breast cancer cell lines induces cellular differentiation (11) and apoptosis (12). In contrast, overwhelming evidence suggests that ERBB4 contributes to estrogen-induced proliferation of ER(+) breast cancer cells (6, 13, 14).

The complexity of ERBB4 signaling in breast cancer may be explained by novel ERBB4 proteolytic processing events that result in the release of an independently signaling ERBB4 intracellular domain (4ICD). Ligand activation of ERBB4 results in tumor necrosis factor-α (TNFα)–converting enzyme–mediated shedding of the ligand binding extracellular domain (15). The membrane-associated TNFα-converting enzyme cleavage product (ERBB4 m80) serves as a substrate for presenilin-dependent γ-secretase activity and γ-secretase cleavage of ERBB4 m80 generates an un tethered cytosolic 4ICD (16–18). We have recently shown that 4ICD harbors an intrinsic nuclear localization signal and 4ICD functions as a nuclear chaperone for the STAT5A transcription factor (19). Furthermore, subsequent binding of 4ICD/STAT5A complexes to STAT5A target promoters results in transactivation of genes involved in lactation (19). In vivo models have revealed a critical role for coupled ERBB4/4ICD and STAT5A signaling during both mammary epithelial differentiation and lactation (20, 21). Alternatively, 4ICD induces cellular apoptosis by localizing to mitochondria (12, 18) and activating a cell death cascade involving proapoptotic members of the BCL-2 family (12). Significantly, cytosolic immunostaining of 4ICD in primary breast tumors was associated with tumor apoptosis (12). Interestingly, the transcriptional coregulation and apoptotic functions of ERBB4 require γ-secretase processing to release 4ICD (18). Thus, a physiologic
function for the membrane-associated ERBB4 holoreceptor remains to be described.

The strong association between ERBB4 and ER expression in breast cancer and the multiple functions of 4ICD led us to investigate the molecular mechanisms underlying ERBB4/ER coexpression and the effect of coupled 4ICD/ER signaling on breast cancer cells. Here we present evidence that 4ICD functions as an ER transcriptional coregulator, selectively binding with ER to gene promoters containing estrogen response elements (ERE). Furthermore, we show that ERBB4 is an estrogen-inducible gene thereby completing a functionally important ERBB4/ER autocrine signaling loop in breast cancer that regulates gene expression and promotes tumor cell proliferation.

Materials and Methods

Cell lines. The T47D human breast cancer cell line was purchased from the American Type Culture Collection (Manassas, VA) and maintained according to the recommendations of the manufacturer. The MCF-7/B cell line stably overexpressing human BCL-2 and resistant to ERBB4 apoptotic activity (12) has been described elsewhere (22).

Plasmid constructs. The ERE luciferase reporter gene (ERE-luc) was a kind gift of Rakesh Kumar (M.D. Anderson, Houston, TX). The ERBB4, ERBB4-EFGP, ERBB4immNLS-EFGP (19), ERBB4V673I-EFGP (18), and 4ICD-Flag (12) expression vectors have been described elsewhere. The glutathione S-transferase (GST) vectors fused to ER domains A/B, C, D, E, and F (23) have been described elsewhere.

Luciferase reporter assay. MCF-7/B cells were seeded at 2 x 10^5 per well in a six-well plate and cultured for 24 hours in phenol red-free medium with 5% charcoal-stripped fetal bovine serum (FBS). Transfections and luciferase transcription assays were performed as described elsewhere (19) using 200 ng or 1 μg of ERE-luc as the reporter. At 24 hours posttransfection, indicated samples were incubated with 100 pmol/L 17β-estradiol (Sigma, St. Louis, MO) and/or 100 ng/mL ICI 182780 (Tocris, Ellisville, MO) for an additional 72 hours. The cells were incubated with 100 pmol/L 17β-estradiol for 1 hour exactly as described elsewhere (19).

In vitro transcription/translation and GST pulldown assay. In vitro transcription/translation was done with linearized pBl4ICD-Flag using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI) supplemented with 20 μCi of Redivue 1-14C methionine (Amersham, Piscataway, NJ) exactly as described by the manufacturer. The GST pulldown assay was done using equal amounts of GST alone or GST fused to the independent ER domains A/B, C, D, E, and F as described elsewhere (25). In some experiments, 10 pmol/L 17β-estradiol was added to the pulldown assay.

Immunoprecipitation and Western blot analysis. Immunoprecipitations from cell lysates prepared from T47D breast cancer cells cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS for 48 hours and stimulated with 100 pmol/L 17β-estradiol for 1 hour were done using rabbit anti-ERBB4 (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody with Alexafluor 488-conjugated goat anti-rabbit immunoglobulin G (IgG; Molecular Probes, Carlsbad, CA) secondary antibody and mouse anti-ER Ab-1 (NeoMarkers) primary antibody with Alexafluor 568-conjugated goat anti-mouse IgG (Molecular Probes) secondary antibody. Deconvolution microscopy was done on cells fixed in 4% paraformaldehyde exactly as described elsewhere (19).

Isolation of nuclear and cytosolic/membrane subcellular fractions. Nuclear and cytosolic subcellular fractions were isolated from T47D breast cancer cells cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS for 48 hours and stimulated with 100 pmol/L 17β-estradiol for 1 hour. Differences in gene expression were determined using paired Student’s t test.

Suppression of ERBB4 and ER expression. To suppress expression of endogenous ERBB4 or ER, T47D cells were transfected with erbB-4/HER4 siRNA SMARTpool or ESR1 siRNA SMARTpool, respectively, using siIMPORTER transfection reagent (Upstate Biotechnology, Charlottesville, VA) according to the instructions of the manufacturer.

Reverse transcription-PCR. T47D breast cancer cells were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS for 48 hours, treated with 100 pmol/L 17β-estradiol for 16 hours, and total RNA was extracted using a RNeasy Mini Kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. First-strand cDNA was synthesized from 5 μg of total RNA in a volume of 40 μl using the Superscript First-Strand Synthesis System for reverse transcription-PCR (RT-PCR; Invitrogen; Carlsbad, CA) and 1 μl was amplified with 35 cycles of PCR using PCR SuperMix (Invitrogen) according to the instructions of the manufacturer. Oligonucleotide primers for progesterone receptor (PgR) PCR were forward 5'-CCATTGGCGGATCCACAAGGT and reverse 5'-TGCGGAATTCACATCTGCGG; for stromal cell-derived factor 1 (SDF-1), forward 5'-GGCCAGACACATGGAATCT and reverse 5'-GCCGAAATGTCCAAAACAGGCC; for trefoil factor 1 precursor (pS2) PCR, forward 5'-GGCCTCTGTCCTCTGCTTCC and reverse 5'-GAAACCACTCCTGTCTTCC; for ERBB4 PCR, forward 5'-GAGAGAGTCTGCTGGAAGAGAGGAC and reverse 5'-GGATGATGACCTATGTCAT; and for ER 5'-CCAAACAGACGACCTTGC.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation was done as previously described (19) with the following modifications. Chromatin was prepared from T47D breast cancer cells cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS for 48 hours and stimulated with 100 pmol/L 17β-estradiol for 1 hour. Fragmented chromatin was immunoprecipitated using antibodies directed against ERBB4 (Upstate) or ER Ab-1 (NeoMarkers, Fremont, CA) and amplified with 35 to 45 cycles of PCR using PCR SuperMix (Invitrogen). Oligonucleotide primers for the PgR promoter were forward 5'-TCTCGTGGCTCGTACACTCGG and reverse 5'-GGCTTTGGCAGGGCCTCC; for the SDF-1 promoter region harboring an ERE half-site and associated SP-1 site, forward 5'-GAGCGTGAGAGCTCAAAG (nt –216 to –197; ref. 24) and reverse 5'-GGCGTTAGAGGGAGGAC (nt –17 to –35; ref. 24); for the pS2 promoter, forward 5'-GGTGCTGCGCAGGCTTTT and reverse 5'-GGAGCTTAGAACAATTCG; for ERBB4 ERE half-site I, forward 5'-GCTTTGATGAGAGGTTCG and reverse 5'-CGTCTCATGGAGCCCTGT; for ERBB4 ERE half-site II, forward 5'-CATCACAGGACAGAGCGTAC and reverse 5'-GCTTGAATATTCCATAAGATC; and for ERBB4 ERE half-site III, forward 5'-GAGAGAGGAGAGGAGAAGAGCAGCAGCAG and reverse 5'-CCAACACGACGACCTTGC.
in triplicate and the data represent the mean and SE of at least three independent experiments. Statistically significant differences between data sets were determined using paired Student’s *t* test.

**Results**

**ERBB4 functions as an ER coregulator.** We have previously shown that ERBB4/4ICD functions as a STAT5A transcriptional coactivator (18, 19, 27) and others have shown that ERBB4 potentiates expression of an ERE reporter gene fusion (6). To determine if ERBB4 contributes to 17-β-estradiol (estrogen)-stimulated ER transactivation, we cotransfected the ER(+) MCF-7/B breast cancer cell line with an ERBB4 expression vector and an ERE-luciferase (ERE-luc) fusion vector. Luciferase expression in the presence or absence of estradiol was determined. As expected, we observed a robust 10-fold increase in luciferase activity when ERE-luc–transfected cells were stimulated with estrogen (Fig. 1A). The estrogen-induced expression was abolished in the presence of the pure antiestrogen ICI-182780, indicating that estrogen-stimulated ERE-luc activity in these cells requires a functional ER (Fig. 1A). ERBB4 expression potentiated ERE-luc response to estrogen by 30-fold and this activity was ablated in the presence of ICI-182780 (Fig. 1A). These results suggest that ERBB4 is an estrogen-regulated ER coactivator.

**ERBB4 is a selective ER coregulator recruited to gene promoters harboring EREs.** To confirm coactivation of estrogen-regulated gene expression by endogenous ERBB4, we treated T47D breast cancer cells with estrogen and determined the expression levels of the three estrogen-regulated genes PgR, SDF-1, and pS2. The T47D breast cancer cell line expresses both ER and a cleavable form of ERBB4. As expected, estrogen stimulated expression of PgR, SDF-1, and pS2 in this experimental system (Fig. 1B). When ERBB4 expression in the presence of estrogen was suppressed through transfection of an interfering RNA directed against ERBB4 (ERBB4 RNAi), we found that estrogen-stimulated PgR and SDF-1 expression returned to basal levels (Fig. 1B). These results suggest that ERBB4 is required for ER transactivation of PgR and SDF-1 but not the pS2 gene.

We have previously shown that the 4ICD nuclear protein is recruited to gene promoters recognized by STAT5A (19). To determine if ERBB4 is recruited to promoter regions harboring EREs, we did a chromatin immunoprecipitation assay using chromatin prepared from estrogen-stimulated T47D cells and antibodies directed against ERBB4 or ER. Estrogen stimulated recruitment of both ERBB4 and ER to the PgR and SDF-1 promoters (Fig. 1C), genes for which estrogen-regulated expression requires ERBB4 (Fig. 1B). Although estrogen recruited ER to the pS2 promoter, an increase in ERBB4 recruitment was not observed (Fig. 1C). Consistent with this observation, estrogen stimulates pS2 expression independent of ERBB4 (Fig. 1B). Taken together, our results show that ERBB4 is an estrogen-stimulated ER coregulator that selectively binds to and activates gene promoters harboring EREs.

**Estrogen stimulates nuclear accumulation of the 4ICD.** Our results suggest that ERBB4 regulates ER transactivation, in part, through estrogen-stimulated recruitment of ERBB4 and ER to gene promoters. Because physiologic signals that activate ERBB4 promote proteolytic cleavage of ERBB4 releasing 4ICD, we hypothesized that 4ICD may play a role in coregulating ER transactivation functions. To elucidate the molecular mechanism underlying ERBB4 coregulation of ER at gene promoters harboring EREs, we first determined the effect of estrogen stimulation of T47D cells on 4ICD nuclear translocation. In mock-stimulated T47D cells, some basal nuclear accumulation of both ER and ERBB4/4ICD was observed (Fig. 2A). Following estrogen stimulation, the majority of both proteins were also detected in the perinuclear region (Fig. 2A, middle, compare asterisks between top and middle). Nuclear translocation of 4ICD in response to estrogen was abolished in cells where ER expression was suppressed by RNAi (Fig. 2A, bottom), suggesting that ligand-bound ER functions as a 4ICD nuclear chaperone. Western blot analysis of cytosolic/membrane extracts and nuclear extracts shows that estrogen stimulated nuclear accumulation of both ER and 4ICD (Fig. 2B). Consistent with our previous results, the ERBB4 holoreceptor was excluded from the nuclear compartment (ref. 19; Fig. 2B).

**Estrogen-stimulated ERBB4 coregulation of ER and nuclear translocation of 4ICD require ERBB4 proteolytic processing but an intact 4ICD nuclear localization signal is dispensable.** We have previously shown that ERBB4 coregulation of STAT5A transactivation required both proteolytic processing of ERBB4 at the cell membrane (18) and an intact 4ICD nuclear localization signal (19). These results identified 4ICD as a nuclear chaperone for the STAT5A transcription factor (19). To determine if a similar mechanism mediates nuclear cotranslocation of 4ICD and ER, the nuclear receptor STAT5A transactivates gene expression.
we determined the effect of independent ERBB4 mutations that abolish γ-secretase processing (referred to as ERBB4V673I; ref. 18) or abrogate nuclear accumulation of 4ICD (referred to as ERBB4muNLS; ref. 19) on ERBB4 transcriptional coregulation of ER. Interestingly, the ERBB4 nuclear localization signal was dispensable for estrogen-stimulated ERBB4 coactivation of ERE-luc; however, γ-secretase processing of ERBB4 to release a soluble 4ICD was required for ERBB4 coactivation of ERE-luc (Fig. 3A).

Nuclear accumulation of 4ICD following ligand activation of the ERBB4 holoreceptor requires an intact 4ICD nuclear localization signal (19); however, stimulation of ERE-luc by an ERBB4 receptor with a mutated nuclear localization signal raises the possibility that ligand-bound ER mediates 4ICD nuclear translocation independent of an intrinsic 4ICD nuclear localization signal. To test this possibility, we transfected MCF-7/B cells with ERBB4, ERBB4muNLS, or ERBB4V673I, each fused to enhanced green fluorescent protein (EGFP), and determined the effect of estrogen treatment on nuclear translocation of each receptor. Consistent with our previous results (Fig. 2A and B), estrogen stimulated nuclear accumulation of 4ICD (Fig. 3B, ERBB4/Estrogen). In concordance with our transcriptional coactivation data (Fig. 3A), estrogen stimulated nuclear accumulation of 4ICD harboring a mutated nuclear localization signal (ERBB4muNLS) but failed to mobilize the unprocessed ERBB4V673I mutant to the nucleus (Fig. 3B, ERBB4V673I/Estrogen). These results imply that estrogen-stimulated ER facilitates nuclear translocation of 4ICD and, by extension, ER can function as a nuclear chaperone for 4ICD lacking an intact nuclear localization signal.

**Estrogen stimulates a direct interaction between 4ICD and ER.** Our results raise the possibility of a estrogen-induced cytosolic 4ICD and ER complex that cotranslocates to the nucleus and regulates gene expression. To support this contention, we used an in vitro GST pulldown assay and a coimmunoprecipitation assay with endogenous protein to characterize the putative 4ICD and ER interaction. In an in vitro pulldown assay, each ER functional domain was independently fused to GST (Fig. 4A), affinity purified, and incubated with 35S-labeled 4ICD (residues 673-1309). Our results indicate that 4ICD directly interacts with ER in a domain-dependent manner and binds to ER via a ligand-independent activation function (AF-1) and AF-2 (Fig. 4B).
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We further show that 4ICD interaction with ER domain E/AF-2 requires estrogen, indicating a ligand-dependent interaction with this region (Fig. 4C).

To support our in vitro assay, we stimulated T47D cells with estrogen and determined if ER exists in a complex with ERBB4/4ICD. Indeed, estrogen stimulation of T47D cells resulted in recruitment of ER to the ERBB4/4ICD immunoprecipitated complex (Fig. 4D). Taken together, our results suggest a novel signaling mechanism with estrogen stimulating an association between ER and 4ICD. Subsequent nucleotranslocation of the complex results in selective ER/4ICD recruitment to gene promoters containing EREs and activation of gene expression.

**ERBB4 is an estrogen-inducible gene recruited with ER to the ERBB4 promoter.** Clinically, ERBB4 expression in breast cancer is significantly associated with ER expression (5–8), raising the possibility that ERBB4 is an estrogen-inducible gene. We therefore determined the effect of estrogen treatment of T47D cells on ERBB4 expression by RT-PCR. Our results show that estrogen stimulation of T47D cells resulted in the recruitment of ER to an ERBB4 immunoprecipitated complex (Fig. 4D). Taken together, our results suggest a novel signaling mechanism with estrogen stimulating an association between ER and 4ICD. Subsequent nucleotranslocation of the complex results in selective ER/4ICD recruitment to gene promoters containing EREs and activation of gene expression.

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**ERBB4 contributes to the estrogen response of breast cancer cells.** We next determined the effect of ERBB4 expression on estrogen-stimulated proliferation of the T47D breast cancer cell line. T47D cells were stimulated with estrogen in the presence or absence of RNAi directed against ERBB4. Cell proliferation was determined by MTT assay after 72 hours of estrogen treatment. As expected, estrogen stimulated a significant increase in cellular MTT conversion, an indication of cellular proliferation (Fig. 5D). A significant decrease in MTT conversion was observed, however, when ERBB4 expression was suppressed in estrogen-treated T47D

Figure 4. Estrogen stimulates an interaction between 4ICD and ER. A, schematic of ER functional domains fused to GST with residues indicated. B, mapping of 4ICD interaction domains in ER. Independent ER domain GST fusions were incubated with 35S-labeled 4ICD (ERBB4 residues 673-1309) and 4ICD binding was analyzed by GST pull-down assay. Input of each GST fusion is shown at the bottom. Interaction between 4ICD and ER domain A/B and E harboring AF-1 and AF-2, respectively, was observed. C, 4ICD interaction with AF-2 is ligand dependent. Binding of 4ICD to ER domain E harboring AF-2 was analyzed by GST pull-down assay in the presence or absence of 10 mmol/L 17-β-estradiol. D, estrogen stimulates endogenous ER and ERBB4/4ICD complex formation. T47D cells were incubated in growth medium supplemented with 5% charcoal-stripped FBS for 48 hours and incubated in the presence or absence of 100 pmol/L 17-β-estradiol for 1 hour. Control rabbit IgG and ERBB4 immunoprecipitations were done on cell lysates and analyzed by Western blot for ERBB4/4ICD and ER protein.

Figure 5. ERBB4 is an estrogen-regulated gene contributing to the estrogen response of T47D breast cancer cells. A, estrogen stimulates ERBB4 expression. T47D cells were incubated in growth medium supplemented with 5% charcoal-stripped FBS for 48 hours and incubated in the presence or absence of 100 pmol/L 17-β-estradiol for 16 hours. Total RNA was extracted and analyzed for ERBB4 expression by RT-PCR. β-Actin RNA was amplified as a control for RNA quantitation. B, schematic of ERBB4 promoter. Gray boxes, three potential ERE half-sites identified by Genomatix/MatInspector software; double arrows, regions of chromatin amplified by oligonucleotide primers flanking each ERE half-site. C, estrogen stimulates recruitment of ERBB4 and ER to the ERBB4 promoter. T47D cells were incubated in growth medium supplemented with 5% charcoal-stripped FBS for 48 hours and incubated in the presence or absence of 100 pmol/L 17-β-estradiol for 1 hour. Fragmented chromatin was immunoprecipitated with antibodies directed against ERBB4 or ER and amplified by PCR using oligonucleotide primers flanking ERBB4 promoter ERE half-site I, II, or III. D, ERBB4 contributes to estrogen-induced proliferation of T47D cells. T47D cells were incubated in the presence or absence of ERBB4 RNAI in growth medium supplemented with 5% charcoal-stripped FBS for 48 hours and stimulated with 100 pmol/L 17-β-estradiol for an additional 72 hours. An MTT assay was done. Columns, mean of at least three independent experiments; bars, SE. Asterisks, estrogen alone was significantly greater than control or estrogen with ERBB4 RNAI.
cells (Fig. 5D). These results indicate that the potent estrogen-stimulated proliferative response in T47D breast cancer cells requires ERBB4 expression.

Discussion

Crosstalk between cell-surface and nuclear receptors and their contribution to the development and progression of endocrine regulated cancers have been areas of intense investigation. In breast cancer, these studies have identified signaling crosstalk between the EGFR-family and ER involving activation of parallel or overlapping signaling pathways (28, 29). Here we show for the first time direct coupling of transmembrane and nuclear receptor signaling, forming an autocrine feedback loop that regulates breast cancer cell gene expression and contributes to tumor cell proliferation. Our results support a model of ERBB4/ER coupled signaling in breast cancer cells, which involves an estrogen-stimulated interaction between extranuclear ER and ERBB4/4ICD. Subsequent proteolytic processing of ERBB4 by TNFα-converting enzyme followed by γ-secretase results in release of an independently signaling 4ICD. Estrogen promotes nuclear cotranslocation of ER and 4ICD and ultimately recruitment of ER and selective recruitment of 4ICD to estrogen responsive gene promoters. Nuclear 4ICD functions as an ER coactivator when bound with ER to gene promoters including PgR and SDF-1, and possibly ERBB4 itself. Estrogen activation of ERBB4 expression establishes a novel transmembrane and nuclear receptor autocrine signaling loop that selectively potentiates expression of estrogen-regulated genes and affects breast tumor cell proliferation (Fig. 6).

Our results support a novel ER signaling pathway that directly integrates the extranuclear and genomic actions of estrogen. Classic or genomic estrogen signaling involves binding of estrogen to the ER followed by activation or repression of gene expression regulated by direct association of the estrogen/ER complex with gene promoters harboring EREs. Extranuclear actions of estrogen are thought to be mediated through a membrane-associated ER, and estrogen activation of this nonclassic receptor pathway results in lateral stimulation of receptor tyrosine kinases and G-protein coupled receptors (30) or in activation of secondary messengers including intracellular calcium (31) and cyclic AMP (32, 33). Here we show that extranuclear ER associates with ERBB4 in response to estrogen stimulation and estrogen promotes nuclear cotranslocation of ER and the ERBB4 proteolytic product, 4ICD. This ER/4ICD complex directly regulates estrogen genomic activity by binding to and activating expression of estrogen-regulated genes. Our results support this model over a model where the 4ICD nuclear protein independently translocates and interacts with nuclear ER residing at target promoters. Indeed, we show that 4ICD lacking a functional nuclear localization signal translocates to the nucleus in response to estrogen, implying that extranuclear ER functions as a nuclear chaperone for 4ICD. Furthermore, estrogen-induced nuclear translocation of 4ICD is abolished when estrogen expression is suppressed by RNAi, further supporting a critical role for ER during estrogen-induced 4ICD nuclear translocation. Although estrogen stimulates association between extranuclear ER and other cell-surface receptors, including the receptor tyrosine kinases ERBB2 (34) and insulin-like growth factor (35), crosstalk between ER and these receptors results in activation of non-genomic estrogen signaling pathways, including mitogen-activated protein kinase and phosphatidylinositol 3-kinase (28, 35), which may indirectly affect ER transactivation (30). In this context, the ERBB4/ER signaling axis is a unique cell-surface and nuclear receptor signaling pathway that directly couples extranuclear and nuclear estrogen actions.

The exact molecular mechanisms regulating the transcriptional coactivator function of 4ICD remain to be determined. Similar to the EGFR-family members EGFR (36) and ERBB2 (37), 4ICD harbors independent transcriptional activity (17, 38). However, artificial transcription assays and an independently expressed 4ICD protein fusion were employed in these studies, thus limiting the significance of these observations. A physiologically activated and proteolytically processed ERBB4 holoreceptor with independent transactivation activity remains to be confirmed. Alternatively, ERBB4 may regulate transcription indirectly by facilitating transcription factor association with target promoters. For example, we have previously shown that the STAT5A transcription factor interacts with 4ICD, and 4ICD mediates nuclear translocation and subsequent DNA binding of STAT5A at STAT5A target promoters (19). Similarly, estrogen stimulates 4ICD/ER complex binding to the PgR, SDF-1, and ERBB4 promoters, and transcriptional activation of the PgR and SDF-1 genes, and possibly ERBB4 itself, requires ERBB4 expression. In contrast, 4ICD fails to associate with ER at the pS2 promoter. One interpretation of these observations is that 4ICD sequesters and enhances recruitment of transcription factors, including ER, to selectively activate a subset of ER target promoters. In the absence of ERBB4/4ICD, ER and other unbound transcriptional complexes may now be recruited to transactivate gene promoters indirectly modulated by 4ICD. We are currently doing a global analysis of transcription factors recruited with 4ICD to estrogen-regulated genes to substantiate this molecular model of 4ICD transcriptional regulation.

One unexpected finding from these studies was that ERBB4 itself is an estrogen-inducible gene with both ER and 4ICD recruited to the ERBB4 promoter in response to estrogen. Estrogen stimulated binding of ER and 4ICD to a region of the ERBB4 promoter between −2275 and −1989 nucleotides upstream of the translational start. This region contained a consensus ERE half-site (GGTCA) at −2097 in tandem with an imperfect half-site (CGTCA).
at −2086. Although ER dimers bind inefficiently to ER half-sites (39–41), binding is enhanced by juxtaposed SP-1 or activator protein 1 (AP-1) sequence elements (42–46). Interestingly, the bottom strand of each ERBB4 ERE overlaps with sequences homologous to the AP-1 core binding site (TGAC). A combined combination of an ERE half-site with overlapping AP-1 sites regulates estrogen response of the c-fos promoter (46). Although the exact mechanism of ER recruitment to ERE half-sites remains unresolved, one prevailing model suggests that dimeric ER binding to an ERE half-site is stabilized by interactions with AP-1 and SP-1 transcriptional complexes recruited to juxtaposed response elements (40, 42, 47, 48). Currently, the exact molecular mechanism regulating the selective recruitment of 4ICD to EREs remains to be determined. It is interesting, however, that 4ICD binds to the PgR, SDF-1, and ERBB4 promoters harboring ERE half-sites with associated SP-1/AP-1 sequence elements, but not to the PS2 promoter with its near consensus ERE. These observations require confirmation by examining 4ICD recruitment to other estrogen-regulated genes, but one intriguing possibility is that sequences surrounding target ERE sites determine 4ICD recruitment to estrogen-regulated promoters.

Our findings have led to the molecular characterization of a novel ER/ERBB4 autocrine signaling loop in breast cancer cells; however, does this unique signaling pathway affect cell growth? Similar to earlier reports (13, 14), we show that suppression of ERBB4 expression in the T47D cell line results in a significant decrease in estrogen-induced cell proliferation. In concordance with these findings, others have shown that ectopic overexpression of ERBB4 enhances estrogen-stimulated growth of the ER(+) MCF-7 breast cancer cell line (6). Our results suggest that the 4ICD transcriptional coactivator may contribute to estrogen-induced cellular responses by promoting selective expression of estrogen-regulated genes. Thus, 4ICD may coactivate growth-promoting genes while suppressing expression of growth inhibitory genes. In support of this contention, we show that 4ICD coactivates expression of PgR, which acts in combination with ER to promote breast epithelial proliferation (49–51). Likewise, SDF-1, another 4ICD transcriptional target, contributes to estrogen-induced breast tumor cell proliferation (52) and is associated with poor prognosis in breast cancer patients (53). Identification of additional candidate tumor-promoting estrogen response genes directly regulated by 4ICD is an area of ongoing research in our laboratory.

In concordance with our experimental findings, clinical evidence supports estrogen stimulation of a functional ER/ERBB4 autocrine signaling loop regulating PgR expression in breast cancer. Indeed, ERBB4 expression in breast cancer is significantly associated with ER expression (5–8). Furthermore, loss of PgR expression, an ER/ERBB4 expression in breast cancer is significantly associated with estrogen stimulation of a functional ER/autocrine model would account for the improved clinical response to endocrine therapy observed in patients coexpressing ER and ERBB4 when compared to patients with tumors expressing ER alone (10). Nevertheless, clinical observations and our recent descriptions of multiple 4ICD activities imply that the ER and 4ICD signaling axis has a significant effect on estrogen action and therapeutic response in breast cancer patients. Therefore, the potentially complex influence of ER and ERBB4 autocrine signaling should be considered when interpreting therapeutic responses of patients with ER-positive breast tumors.

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This work is dedicated to June Allison, caring mother, wife, and friend, who after 14 disease-free years must stage another battle against breast cancer.

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Yun Zhu, Lacey L. Sullivan, Sujit S. Nair, et al.


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