

Re-expression of Estrogen Receptor α in Estrogen Receptor α -negative MCF-7 Cells Restores both Estrogen and Insulin-like Growth Factor-mediated Signaling and Growth¹

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ABSTRACT

Estrogen can increase insulin-like growth factor-I receptor (IGF-IR) and insulin receptor substrate-1 (IRS-1) expression, two key components of IGF-I-mediated signaling. The result is sensitization of breast cancer cells to IGF-I and synergistic growth in the presence of estrogen and IGF-I. We hypothesized that loss of estrogen receptor α (ER α) would result in reduced IGF-mediated signaling and growth. To test this hypothesis, we examined IGF-I effects in MCF-7 breast cancer cell sublines that have been selected for loss of ER α (C4 and C4-12 cells are ER α -negative) by long-term estrogen withdrawal. C4 and C4-12 cells had reduced IGF-IR and IRS-1 mRNA and protein expression (compared with MCF-7 cells) that was not inducible by estrogen. Furthermore, C4 and C4-12 cells showed reduced IGF-I signaling and failed to show any growth response to either estrogen or IGF-I. To prove that loss of IGF and estrogen-mediated signaling and growth was a consequence of loss of ER α , we re-expressed ER α in C4-12 cells by stable transfection with HA-tagged ER α . Three independent C4-12 ER α -HA clones expressed a functional ER α that (a) was down-regulated by estrogen, (b) conferred estrogen-induction of cyclin D1 expression, and (c) caused estrogen-mediated increase in the number of cells in S phase. All of the effects were completely blocked by antiestrogens. Interestingly, ER α -HA expression in C4-12 cells did not restore estrogen induction of progesterone receptor expression. However, ER α -positive C4-12 cells now exhibited estrogen-induction of IGF-IR and IRS-1 levels and responded mitotically to both estrogen and IGF-I. These data show that ER α is a critical requirement for IGF signaling, and to our knowledge this is the first report of functional ER α expression that confers estrogen-mediated growth of an ER-negative breast cancer cell line.

INTRODUCTION

The steroid hormone estrogen and the peptide growth factor IGF-I³ are both critical for normal mammary development (1, 2) and act synergistically to promote mammary ductal growth (3). Synergism between IGF-I and estrogen has also been documented in a number of other model systems including normal uterus (4), endometrial cancer (5), and breast cancer (6). Although the signal transduction pathways used by these mitogens are distinct, one involving a nuclear hormone receptor and the other involving a membrane-bound receptor tyrosine kinase, a greater understanding of their respective signaling mechanisms has revealed considerable cross-talk between the pathways (7).

The IGF family consists of two ligands (IGF-I and II), two receptors (IGF-IR and IGF-IIR), six high-affinity binding proteins (IGFBP 1–6), and several IGFBP-related proteins (8). IGF-I and II are circulating peptide hormones that can act in an endocrine, paracrine, or autocrine manner and can affect a wide variety of processes such as cell growth, survival, transformation, and differentiation (9).

Estrogen can affect IGF signaling at multiple levels, altering expression of nearly all of the IGF family members including IGF-I (10), IGF-II (11), IGF-IR (12), IGF-IIR (13), IGFBPs (14), and IRS-1 (6, 15). The result of estrogen action is to increase expression of IGF activators (IGF-I/IGF-II, IGF-IR, and IRS-1) and decrease expression of IGF inhibitors (IGFBP-3, IGF-IIR), resulting in enhanced response to IGF. Furthermore, it has been shown recently (16) that estrogen activation of ER α causes a direct interaction between ER α and IGF-IR and results in activation and phosphorylation of IGF-IR and IGF-IR downstream signals such as ERK1/2. In addition to the estrogen activation of IGF-signaling pathways, IGF-I in turn enhances ER-mediated transactivation (17), possibly via ERK1/2 (18), Akt (19), src/JNK (20), or pp90rsk1 (21).

Supporting the concept of cross-talk between ER and IGF is evidence that specific targeting of either pathway can affect signaling and mitogenesis through the other pathway. Antiestrogens can inhibit IGF signaling by down-regulation (6, 15, 22) or dephosphorylation (23, 24) of signaling molecules such as IGF-IR or IRS-1, thus inhibiting IGF-mediated growth. Anti-IGF strategies, such as overexpression of IGFBP-1 (17, 25) or IGFBP-3 (26), blocking antibody against IGF-IR (27), or down-regulation of IRS-1 (28), not only inhibit IGF-mediated signaling, but also inhibit ER-mediated transactivation and estrogen-mediated growth.

In this study, we describe the effects of ER α expression on IGF-mediated signaling and growth. We show that loss of ER α in MCF-7 cells causes reduced expression of IGF-signaling molecules, diminished IGF signaling, and failure to proliferate in response to estrogen or IGF-I. Re-expression of ER α in the same cell line restores ER α function (signaling and proliferation) but also restores the IGF-responsive phenotype, with re-expression of IGF-signaling molecules and growth in response to IGF. Thus, in MCF-7 cells, ER α is a critical regulator of IGF-mediated signaling and growth.

MATERIALS AND METHODS

Materials. All of the materials and chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. IGF-I was purchased from GROPEP (Adelaide, Australia). ICI 182780 was a kind gift from Zeneca Pharmaceuticals (Macclesfield, England). All of the tissue culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY) unless otherwise stated.

Cell Lines. MCF-7 cells were routinely maintained in α MEM + 5% fetal bovine serum (Hyclone, Logan, Utah) + 2 mM glutamine + 50 IU/ml penicillin, 50 μ g/ml streptomycin. C4 and C4-12 cells were routinely maintained in α MEM without phenol red + 5% charcoal/dextran-treated fetal bovine serum (Hyclone) + 2 mM glutamine + 50 IU/ml penicillin, 50 μ g/ml streptomycin. SFM has been described previously (6).

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³ The abbreviations used are: IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; IGFBP, IGF-binding protein; IRS-1, insulin receptor substrate-1; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; SFM, serum-free medium; PR, progesterone receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; E2, estradiol; ER α -HA, HA-tagged ER α ; HA, hemagglutinin.

Immunoblotting and Immunoprecipitation. Cells were stimulated with hormones and then lysed in TNESV buffer as described previously (6). Total protein (50 μ g) was separated by 8% SDS-PAGE and electrophoretically transferred to nitrocellulose overnight at 4°C. Immunoblotting was performed using techniques and antibodies described previously (6). In addition, lysates were immunoblotted for cyclin D1 (Santa Cruz Biotechnology; 1:200) and PR (1:1000; Dako, Carpinteria, CA).

RNase Protection Assay. MCF-7 cells were plated at 3×10^6 cells in 15-cm dishes (Becton Dickinson) and allowed to adhere overnight. Cells were harvested by trypsin/EDTA and pelleted in 15-cm tubes. Total RNA was prepared by Qiagen RNeasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and checked for integrity by separation on a 1% agarose gel. RNase protection was performed according to our method published previously (6), and RNA loading was normalized to mRNA of the ribosomal protein 36B4 (29).

Transient and Stable Transfection. Construction of the HA-tagged ER α expression construct has been described previously (30). Stable transfection of C4-12 cells with pCDNA3.1 or pCDNA3.1ER α -HA was performed using lipofectin according to the manufacturer's instructions. Briefly, C4-12 cells were plated at 1×10^6 cells in 10-cm dishes and transfected with 30 μ g of ER α -HA plasmid and 1 μ g of pSVneo. Transfected cells were selected in 800 μ g/ml G418S (Life Technologies, Inc.) and screened by HA immunoblot.

Growth Assay. MTT proliferation assay has been described previously (6). Briefly, 20 μ l of MTT (5 mg/ml in PBS) was added to the medium and incubated for 4 h, after which the medium was aspirated and color developed by the addition of DMSO + 2.5% Improved Modified Eagle Medium. Absorbance was read at 540 nm. All of the time points were performed in quadruplicate.

Flow Cytometry. Flow cytometry and flow data analysis were performed by the Flow Cytometry Core at Baylor College of Medicine using a Beckman Coulter EPICS XL-MCS. Cells were plated at 1×10^6 cells in 6-cm dishes and allowed to adhere overnight. The cells were incubated in SFM for 48 h and then stimulated with E2 (1 nM), ICI 182780 (100 nM), or a combination for 16 h. Cells were trypsinized, washed with PBS, resuspended in 0.2 ml of PBS, and fixed by adding 0.1 ml of ethanol with vortexing. After 30 min at room temperature, the cells were stored at 4°C until analysis. Before analysis, the cells were centrifuged, fixative was removed, and the cells were resuspended in 0.5 ml of PBS. One ml of propidium iodide (50 μ g/ml) in PBS was added, and 30 min before analysis, 100 μ l of RNase A (1 mg/ml) was added.

Statistical Analysis. All of the statistical analysis was performed using Prism 3.0. The changes between individual treatments of cells were analyzed using one-way unpaired *t* tests. To analyze statistical differences among all of the treatment groups within each cell line, we performed one-way ANOVA.

RESULTS

ER-negative MCF-7 Cells Have Reduced IGF-IR and IRS-1 Expression. We and others have shown previously (6, 15, 22, 31) that IGF-IR and IRS-1 expression (protein and mRNA) is inducible by estrogen. In this study, we examined expression of these IGF-signaling molecules in MCF-7 cells that were selected for loss of ER α expression.⁴ C4 and C4-12 cells were derived by clonal selection from MCF-7 cells grown in the absence of estrogen for 9 months. The derivation of C4 and C4-12 cells from MCF-7 was confirmed by DNA fingerprinting. Although the initial passages of C4 and C4-12 cells showed variable or very low ER, the passages used in these studies were stably ER-negative by Western blotting, ligand-binding assay, and RNase protection assay. C4-12 cells were immunoblotted for ER α in every experiment to confirm that they were negative. Additionally, C4 and C4-12 cells did not exhibit estrogen induction of PR mRNA or binding sites.⁴

MCF-7, C4, and C4-12 cells were incubated overnight in SFM and then stimulated for 48 h with E2 (1 nM). As a control, we immunoblotted the cells for ER α to confirm that our C4 and C4-12 cells were ER α -negative (Fig. 1A). Treatment of MCF-7 cells with E2 (1 nM)

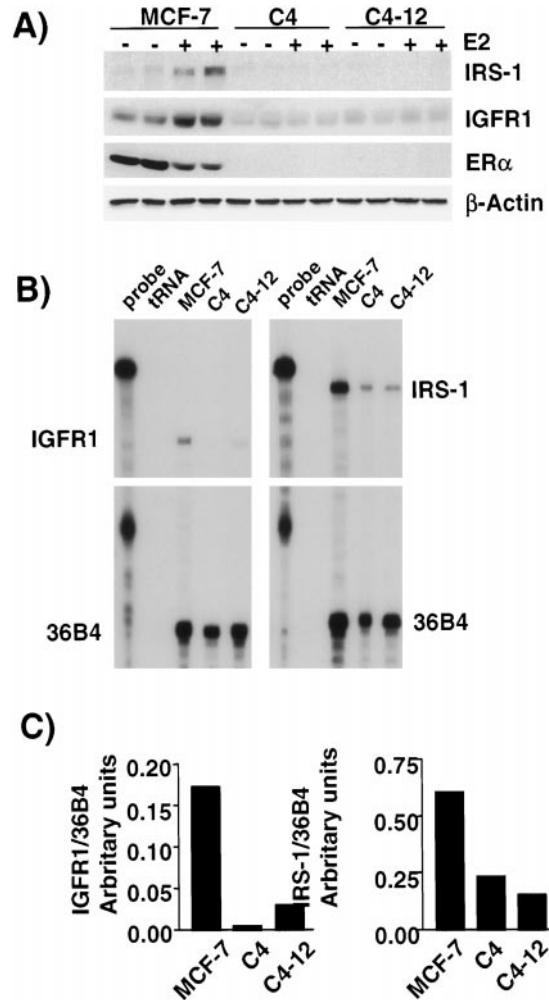


Fig. 1. ER α -negative MCF-7 cells have decreased IGF-IR and IRS-1 protein and mRNA expression. A, duplicate plates of MCF-7, C4, and C4-12 cells were incubated in SFM for 24 h and then stimulated with or without E2 (1 nM) for 24 h. Cells were lysed and immunoblotted for IGF-IR, IRS-1, ER α , and β -actin. Results are representative of three individual experiments. B, MCF-7, C4, and C4-12 cells were grown in maintenance medium and harvested, and total RNA was prepared. RNA (20 μ g) was analyzed for IGF-IR and IRS-1 mRNA by RNase protection assay. 36B4 was used as a loading control. C, graphical representation of data from B using a phosphorimager and correcting for the loading control 36B4. Results are representative of three individual experiments.

resulted in a decrease in ER α expression. C4 and C4-12 cells had undetectable ER α expression. As expected, E2-stimulation of MCF-7 cells resulted in increased protein expression of both IRS-1 and IGF-IR. C4 and C4-12 cells showed lower basal IRS-1 expression and no induction by E2. Similarly, IGF-IR basal expression was lower in C4 and C4-12 cells compared with MCF-7 cells in the absence of E2, and again there was no increase in IGF-IR expression when cells were stimulated with E2.

We next examined whether the decrease in IRS-1 and IGF-IR protein expression was also associated with a decrease in mRNA expression. MCF-7 cells had high levels of both IGF-IR and IRS-1 mRNA, whereas both C4 and C4-12 cells had low IGF-IR and IRS-1 mRNA levels (Fig. 1B). The expression is represented graphically after correcting for the loading control (Fig. 1C). Therefore, loss of ER α is associated with loss of IGF-IR and IRS-1 protein and mRNA expression.

ER-negative MCF-7 Cells Have Diminished IGF Signaling. Because of the decrease in both IGF-IR and IRS-1 expression in the absence of ER α , we examined the ability of IGF to signal through the ERK1/2 and phosphatidylinositol-3 kinase pathways (32). MCF-7,

⁴ E. M. Curran *et al.*, submitted for publication.

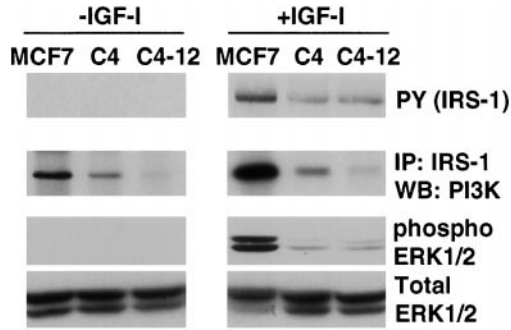


Fig. 2. ER α -negative MCF-7 cells have decreased IGF signaling. MCF-7, C4, and C4-12 cells were incubated in SFM for 24 h and then stimulated with (right) or without (left) IGF-I (5 nM) for 15 min. Cells were lysed, and 500 μ g of cell lysate were immunoprecipitated with antibodies to IRS-1 and immunoblotted for phosphotyrosine (top panel) or p85 (second panel). Additionally, 50 μ g of lysate were immunoblotted for phosphoERK1/2 (third panel). Total ERK1/2 levels were used as a loading control (bottom panel). Results are representative of three individual experiments.

C4, and C4-12 cells were incubated in SFM overnight and then stimulated with IGF-I (5 nM) for 10 min (Fig. 2). IRS-1 immunoprecipitation followed by antiphosphotyrosine immunoblotting revealed that IGF-I stimulation of MCF-7 cells caused tyrosine phosphorylation of IRS-1, but that both C4 and C4-12 cells had reduced IGF-I-mediated tyrosine phosphorylation of IRS-1.

Immunoblotting IRS-1 immunoprecipitates for the p85 α regulatory subunit of phosphatidylinositol-3 kinase revealed enhanced association between IRS-1 and P85 α in MCF-7 cells treated with IGF-I. In C4 and C4-12 cells, less p85 α was associated with IRS-1, and little or no increase in association was seen after IGF treatment. Immunoblotting of total cell lysates for ERK1/2 activation (Fig. 2) showed that IGF-I caused rapid and strong activation of ERK1/2 in MCF-7 cells. In stark contrast, activation of ERK1/2 was either reduced or totally absent in C4 and C4-12 cells. Total ERK1/2 levels are shown as a loading control. Therefore, C4 and C4-12 cells exhibit substantially decreased IGF signaling compared with MCF-7 cells.

ER-negative MCF-7 Cells Have Increased Basal Cell Number but No Increase in Response to E2 or IGF-I. It is well established that E2 and IGF-I are potent mitogens for ER-positive breast cancer cells and that, in most instances, addition of both mitogens results in additive or synergistic growth (6). MCF-7, C4, and C4-12 cells were incubated in SFM with E2 (1 nM), IGF-I (5 nM), or a combination, and cell number was assessed by MTT assay (Fig. 3). C4 and C4-12 cells increased in cell number under basal conditions (SFM) faster than the parental MCF-7 cells. MCF-7 cell number was increased in cell number by both IGF-I and E2 (Fig. 3, top graph; growth curves overlap), and the combination of mitogens (E2 + IGF-I) caused an increase that was greater than either ligand alone. C4 and C4-12 cells failed to respond to E2 as a result of their lack of ER α , but both cell lines also failed to respond to IGF-I.

Stable Transfection of C4-12 Cells with ER α . To prove that the loss of IGF signaling and growth was a result of loss of ER α expression and not simply a simultaneous event, we reintroduced ER α into C4-12 cells by stable transfection of ER α -HA. The initial screen of clones revealed a very high number of positive ER α -expressing clones (35 positive of 75 clones tested), suggesting that expression of ER α in C4-12 cells is not growth inhibitory or toxic. Indeed, the positive clones expressed ER α at levels that were both below and significantly above that of MCF-7 cells (data not shown). For further analysis, we chose transfectants that expressed levels of ER α similar to MCF-7 cells. As can be seen in Fig. 4, C4-12 and C4-12pCDNA clones were ER α -negative. C4-12ER α -HA clones (#4, #17, and #29) expressed ER α (bottom) that was also HA immunoreactive (top).

C4-12ER α -HA Clones Have Estrogen-inducible Cyclin D1 Expression but Not PR Expression. Previous studies (33–41) that have expressed ER α in ER-negative cell lines have resulted in ER α that either is nonfunctional, and thus does not increase expression of E2-responsive genes or alter growth, or actually inhibits proliferation. This phenomenon is not seen when ER α is expressed in ER α -positive cells (42). It has been found recently (43) that estrogen does not

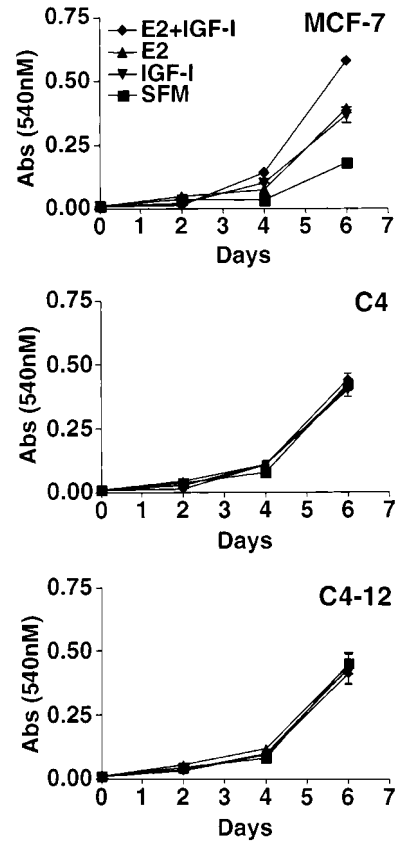


Fig. 3. ER α -negative MCF-7 cells have increased basal cell number but no increase in response to E2 or IGF-I. MCF-7, C4, and C4-12 cells were plated at 15,000 cells/well in a 24-well plate and incubated in SFM for 24 h. Triplicate wells were then stimulated with no ligand, E2 (1 nM), IGF-I (5 nM), or a combination, and proliferation was assessed by MTT assay at the indicated days. All of the points represent the average of triplicate wells \pm SE. Results are representative of four individual experiments.

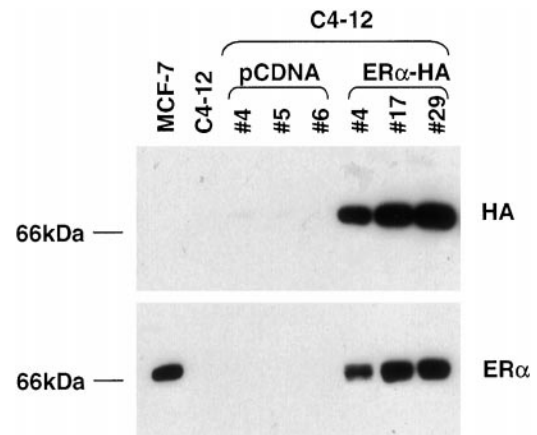


Fig. 4. Stable transfection of C4-12 cells with ER α -HA. C4-12 cells were stably transfected with control plasmid (pCDNA3.1) or ER α -HA (pCDNA3.1ER-HA). After selection in 800 μ g/ml G418S, individual clones were screened by HA (top) or ER α (bottom) immunoblot. Clones, which expressed ER α at levels equal to MCF-7 and were used for further study, are shown (C4-12ER α -HA #4, #17, and #29).

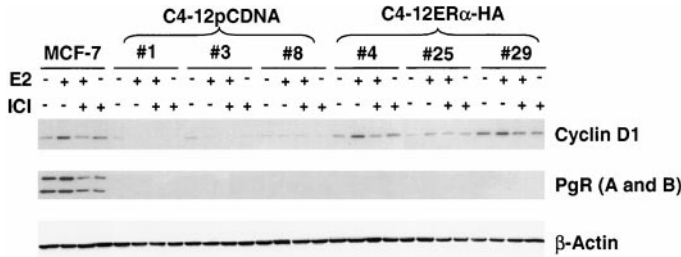


Fig. 5. C4-12ER α -HA cells have estrogen-inducible cyclin D1 but not estrogen-inducible PR expression. MCF-7, C4-12pCDNA clones (#1, #3, and #8), and C4-12ERHA clones (#4, #25, and #29) were incubated in SFM for 24 h, stimulated with E2 (1 nM), ICI (100 nM), or a combination of E2 (1 nM) and ICI (100 nM) for 6 h, lysed, and immunoblotted for cyclin D1 (top), PR (middle), and β -actin (bottom) expression. Results are representative of two individual experiments.

increase cyclin D1 expression in ER α -negative cells (MDA-321 and HELA) that are stably transfected with ER α . Therefore, we examined MCF-7, C4-12, and C4-12ER α -HA clones for ER function by analyzing two estrogen-regulated genes, PR and cyclin D1. MCF-7, C4-12pCDNA, and C4-12ER α -HA clones were incubated overnight in SFM, stimulated with E2 for 6 h, and then immunoblotted for ER α (data not shown), cyclin D1 (Fig. 5, top), and PR (Fig. 5, bottom). ER α levels in C4-12ER α -HA cells were down-regulated in response to E2 (data not shown; and Fig. 7), suggesting that ER α -HA was functional and responded in a similar manner to wild-type ER (Fig. 2). MCF-7 cells in SFM expressed low levels of cyclin D1. E2 stimulation caused an increase in cyclin D1 expression that was blocked by ICI (Fig. 5, top). C4-12pCDNA clones all expressed low or undetectable cyclin D1 levels. A long exposure revealed that cyclin D1 expression was not altered by E2 or ICI in these cells (data not shown). C4-12ER α -HA clones all had higher basal expression of cyclin D1 compared with C4-12pCDNA clones, with cyclin D1 expression being similar to that of MCF-7. Additionally, C4-12ER α -HA clones all exhibited estrogen-induction of cyclin D1 that was inhibited by ICI. Thus, C4-12ER α -HA clones express a functional ER that can regulate expression of cyclin D1. Immunoblotting for PR expression, using an antibody that recognizes both the A and B isoforms, we found that MCF-7 cells had estrogen-induction of PR expression, which was blocked by ICI (the relatively minor induction of PR levels is attributable to the short time of estrogen stimulation). As expected, C4-12 cells did not express PR; however, C4-12ER α -HA cells also failed to express any PR. Therefore, re-expression of ER α in C4-12 cells restores estrogen responsiveness to cyclin D1 but not to PR.

C4-12ER-HA Clones Increase DNA Synthesis in Response to Estrogen. Next, we examined whether the increase in cyclin D1 was associated with increased DNA synthesis measured by the number of cells in S phase. MCF-7, C4-12pCDNA, and C4-12ER α -HA clones were incubated in SFM for 24 h, stimulated with estrogen or antiestrogen for 16 h, and then analyzed by fluorescence-activated cell sorter (Fig. 6). MCF-7 cells incubated in SFM were growth-arrested, with the majority of cells (67%) being in G₀/G₁ (data not shown). E2 stimulation caused a 98% increase in the percentage of cells in S phase ($P < 0.01$), and this increase was completely blocked by ICI ($P < 0.001$). As expected, the E2-mediated increase in S phase was associated with a decrease in cells in G₀/G₁, whereas the ICI-mediated decrease in S phase was associated with an increase in the number of cells in G₀/G₁ (data not shown). C4-12pCDNA #1 and #3 were unaffected by E2 or ICI ($P = 0.27$ and $P = 0.32$). In contrast, C4-12ER α -HA clones showed a 38.0% (#4) and 31.9% (#29) increase in S phase in response to E2. This response was completely blocked by ICI (#4, $P < 0.05$; and #29, $P < 0.001$). Therefore, C4-12ER α -HA cells contain a functional ER α that (a) is down-regulated by E2 (data

not shown; and Fig. 7), (b) confers E2 induction of cyclin D1 but not PR expression (Fig. 5), and (c) causes an increase in S phase in response to E2 (Fig. 6). Therefore, we next tested whether re-expression of ER α in these cells affected IGF signaling and growth.

C4-12ER α HA Clones Exhibit Estrogen Induction of IGF-IR and IRS-1. MCF-7, C4-12, C4-12pCDNA, and C4-12ER α -HA clones were incubated in SFM overnight and then stimulated with E2 (1 nM) for 24 h. MCF-7 cells had detectable ER α that was down-regulated by E2 (Fig. 7). C4-12 and the C4-12pCDNA clones had no detectable ER α . In contrast, C4-12ER α -HA clones expressed ER α , and it was down-regulated by E2 as in MCF-7 cells. As expected, MCF-7 cells had E2-inducible IRS-1 expression levels. C4-12 and C4-12pCDNA clones all had low or undetectable IRS-1 levels. C4-12ER α -HA clones all had elevated basal IRS-1 expression that was increased by E2. E2 induction of IRS-1 expression was seen in all of the clones tested and mirrored that seen in MCF-7 cells. Exactly the same results were seen for IGF-IR. C4-12ER α -HA clones had higher basal expression of IGF-IR than C4-12 and C4-12pCDNA, and E2 induced IGF-IR expression in the C4-12ER α -HA clones. These data clearly show that ER α is a critical requirement for estrogen induction of IGF-IR and IRS-1, and the fact that the pattern of IGF-IR and IRS-1 up-regulation in ER α -HA-expressing clones mirrored that of cyclin D1 confirms that these cells do indeed express a functional ER.

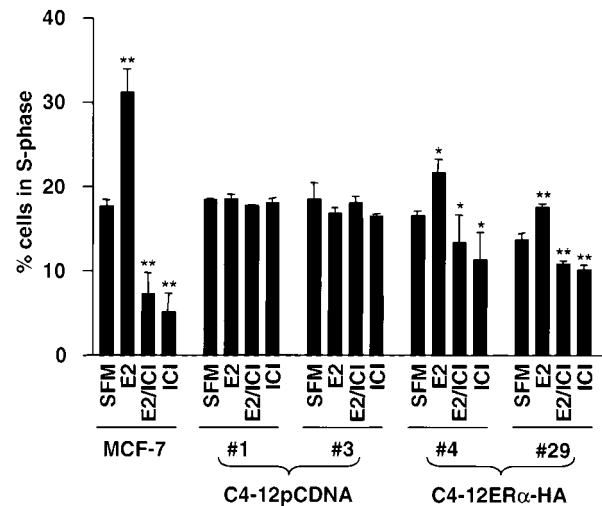


Fig. 6. E2 increases S-phase fraction of C4-12ER α -HA cells. MCF-7, C4-12pCDNA clones (#1 and #3), and C4-12ER-HA clones (#4 and #29) were incubated in SFM for 24 h and then stimulated with E2 (1 nM), ICI (100 nM), or E2 (1 nM) + ICI (100 nM) for 16 h. After this time, cells were trypsinized, labeled with propidium iodide, and analyzed by flow cytometry. Results are presented as the percentage of cells in S phase with each bar representing the average \pm SD of two independent experiments. Statistical significance was tested between two treatment groups using a Student t test and overall within cell lines by ANOVA (*, $P < 0.05$; **, $P < 0.001$).

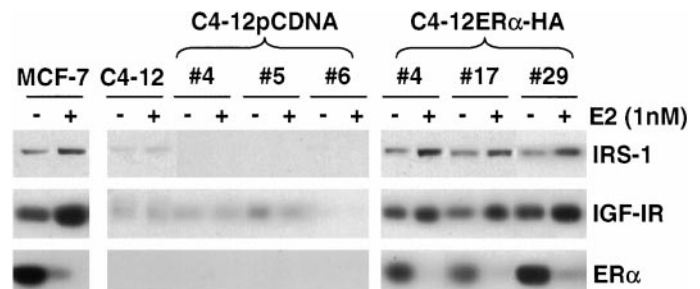


Fig. 7. E2 induces IGF-IR and IRS-1 expression in C4-12ER α -HA cells. MCF-7, C4-12, C4-12pCDNA clones (#4, #5, and #6), and C4-12ERHA clones (#4, #17, and #29) were incubated in SFM for 24 h and then stimulated with or without E2 (1 nM) for 24 h. Cells were lysed and immunoblotted for IGF-IR, IRS-1, and ER α . Results are representative of four independent experiments.

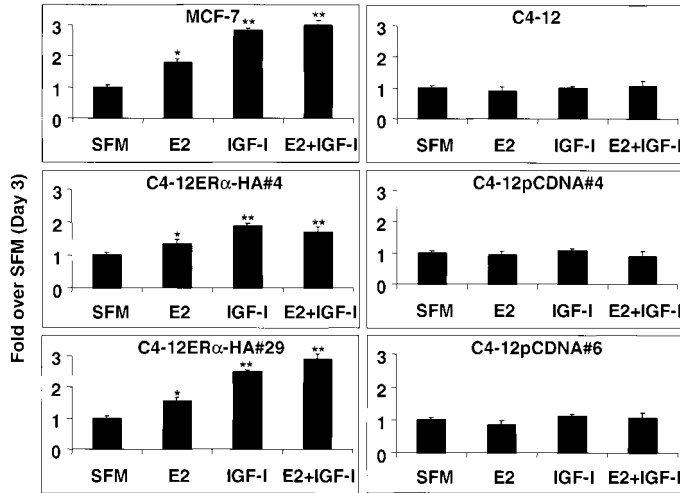


Fig. 8. E2 and IGF-I stimulate proliferation of C4-12ER-HA cells. MCF-7, C4-12, C4-12pCDNA clones (#4 and #6), and C4-12ER-HA clones (#4 and #29) were plated at 15,000 cells/well in a 24-well plate. Cells were incubated for 24 h in SFM and then stimulated with IGF-I (5 nM), E2 (1 nM), or IGF-I (5 nM) + E2 (1 nM). After 3 days, cell proliferation was assessed by MTT assay. Bars represent the average of triplicate wells \pm SE as fold over control (SFM alone at 3 days). Results are representative of three independent experiments. Statistical significance was tested between two treatment groups using Student's *t* test and overall within cell lines by ANOVA (*, $P < 0.05$; **, $P < 0.001$).

C4-12 ER α HA Clones Proliferate in Response to both IGF-I and E2.

The previous characterization of C4-12ER α -HA cells indicated that they contained a functional ER α and expressed E2-inducible IGF-IR and IRS-1. Next, we tested whether this conferred mitogenic responsiveness to IGF on these cells. Cells were stimulated with E2 (1 nM), IGF-I (5 nM), or a combination for 3 days, and cell numbers were assessed by MTT assay (Fig. 8). For simplicity and because of the fact that cells had varying basal proliferation rates, data are presented as fold over control (SFM) after 3 days. However, for direct comparison of basal differences, some of the absorbance values were MCF-7 (0.294), C4-12 (0.512), C4-12pCDNA #6 (0.531), and C4-12ERHA #29 (0.367). As can be seen, C4-12 and its derivatives all had higher basal cell numbers than MCF-7 (similar to Fig. 3). In MCF-7 cells, E2 caused an 82% increase in cell number ($P < 0.05$). IGF-I was more potent, causing a 195% increase in cell number ($P < 0.01$). C4-12 cell proliferation was not affected by either E2 or IGF-I (as also shown previously in Fig. 3). Control transfectants (C4-12pCDNA #4 and #6) also showed no change in proliferation in response to E2 or IGF-I, substantiating the cell-cycle analysis in Fig. 6. In contrast, C4-12ER α -HA clones proliferated in response to E2 with a 56% increase (C4-12ER α -HA #29, $P < 0.05$) and a 26% increase (C4-12ER α -HA #4, $P < 0.01$) in cell number over control (cells in SFM). Most importantly, reintroduction of HA-ER α now allowed these cells to respond to IGF-I, with a 126% (C4-12ER α -HA #29, $P < 0.01$) and 87% (C4-12ER α -HA #4, $P < 0.01$) increase in cell number. These data and data from Fig. 6 show that reintroduction of ER α into C4-12 cells confers responsiveness to both estrogen and IGF. In both S-phase and cell number analysis, we consistently observed that the response of C4-12ER α -HA cells to estrogen was approximately one-half that of wild-type MCF-7 cells.

DISCUSSION

The desire to obtain estrogen-independent or antiestrogen-resistant MCF-7 cells has led many investigators to maintain MCF-7 cells long-term either without estrogen (44–47) or in the presence of antiestrogen (48, 49). In all of these cases, MCF-7 cells were gener-

ated that still contained ER. Indeed, a recent report (50) has described estrogen-independent MCF-7 cells that still contain ER α and are actually hypersensitive to low concentrations of E2. C4 and C4-12 are unique in that they are ER α -negative and estrogen-unresponsive. C4 and C4-12 cells were derived by clonal selection from MCF-7 cells grown in the absence of estrogen for 9 months. The derivation of C4 and C4-12 cells from MCF-7 was confirmed by DNA fingerprinting. Although the initial passages of C4 and C4-12 cells showed variable or very low ER, the passages used in these studies were stably ER-negative by Western blotting, ligand-binding assay, and RNase protection assay. C4-12 cells were immunoblotted for ER α in every experiment to confirm that they were negative. Additionally, C4 and C4-12 cells did not exhibit estrogen induction of PR mRNA or binding sites.⁴

We have shown here that loss of ER α in MCF-7 cells is associated with reduced expression of critical IGF-signaling components (IGF-IR and IRS-1) and that this is associated with an inability to respond mitogenically to IGF-I. This result mimics the effect of antiestrogens in IGF action both *in vitro* and *in vivo* (6) and confirms that ER is a critical regulator of IGF signaling and growth in MCF-7 cells. Importantly, this is not a single cell-line phenomenon, because Dougherty *et al.* (51) have recently reported similar findings using the T47D cell line. Thus, in two different cell lines, loss of ER α results in changes in expression of critical IGF-signaling molecules. Can these cell-line models be used to explain why the majority of ER-negative cells do not proliferate in response to IGFs, whereas all of the ER-positive cells are IGF-responsive? ER-negative cell lines tend to have decreased IGF-IR and IRS-1 expression compared with ER-positive breast cancer cells. However, simple re-expression of IRS-1 (52) or IGF-IR (53) does not restore IGF-mediated growth, suggesting that these cells have further disruption of their IGF-signaling pathways. Despite ER-negative cell lines not proliferating in response to IGFs, these cells seem to depend upon IGFs for their invasive and metastatic potential because several strategies that inhibit IGF signaling, *e.g.*, expression of dominant-negative IGF-IR, are most successful at inhibiting in ER-negative cell growth and metastasis *in vivo* (54). Although evidence suggests that IGFs are important in ER-positive breast cancer, our data does not imply that IGFs have no role in ER-negative cancer. IGF-IR (55) and IRS-1 (6) are higher in ER-positive patients than in ER-negative patients, correlating with expression seen in our ER α -negative MCF-7 model. However, although our data relate to a single cell-line model, there are presumably many pathways for a tumor to become ER-negative *in vivo*; *e.g.*, it has been shown that IGF-IR is expressed in a subset of ER-negative tumors and that high expression is correlated with a worse prognosis (56, 57). Furthermore, as stated previously, most anti-IGF strategies thus far have been successful in ER-negative cell lines, suggesting that IGFs may have a role in ER-negative breast cancer.

The main purpose of the present study was to characterize the loss of IGF signaling in these ER-negative cells and not how these cells overcame the need for ER signals, but data from our studies can rule out certain possibilities for this transition; *e.g.*, although C4 and C4-12 cells have increased cell number in SFM, they actually have decreased cyclin D1 expression (Fig. 5). Additionally, these cells clearly do not have increased basal ERK1/2 activity (Fig. 2), suggesting that signaling through ERK1/2 is not responsible for the increased cell number.

How have the C4-12 cells adapted to grow in the absence of E2? Clearly, C4-12 cells show increased cell numbers in SFM compared with MCF-7 cells (Fig. 3). However, this increase in basal cell number was not associated with a change in distribution of cells in the cell cycle, suggesting that the cells are not proliferating greater in SFM. Indeed, we believe that these cells may have adapted to survive estrogen withdrawal by increasing cell survival pathways and, thus,

lowering their rates of apoptosis. We are currently testing this hypothesis in C4-12 cells. We cannot, however, rule out the possibility that C4-12 cells have increased proliferation in the absence of increased S phase. It is possible that these cells have an overall shortened cell cycle that results in altered proliferative rate without changes in specific phases of the cell cycle. This has been noted previously (58) in IGF-IR null fibroblasts.

Several groups have introduced ER α into ER-negative cells (33–41). However, in all of the previous cases, this has resulted in ER α expression either having no effect or actually being growth inhibitory (33, 59). It has been found recently (43) that estrogen does not increase cyclin D1 expression in ER α -negative cells (MDA-321 and HELA) that are stably transfected with ER α . The lack of cyclin D1 induction has been suggested as a reason why these cells do not proliferate in response to E2. In contrast to previous studies, C4-12 cells expressing ER α do exhibit estrogen induction of cyclin D1 and do proliferate in response to estrogen (increase in S phase and cell number). This suggests that C4-12 cells have retained the appropriate cofactors for normal ER function that are not found in other ER-negative cell lines. This result probably reflects the fact that these cells have only recently lost ER α expression and are genetically similar to their MCF-7 counterparts, whereas other ER-negative cell lines may have lost ER expression and critical ER cofactors many passages ago. Despite C4-12ER α -HA cells having estrogen induction of cyclin D1 expression, these cells do not show estrogen induction of PR. Thus, ER cannot induce expression of all of the estrogen-regulated genes, and comparison of MCF-7 with C4-12ER α cells will be a unique model for elucidating the ER cofactors necessary for estrogen induction of genes such as PR.

In summary, we provide evidence that stable expression of ER α , in an ER α -negative cell line (C4-12), can confer an estrogen-responsive phenotype with estrogen induction of gene expression and proliferation. Furthermore, we have shown in this model system that ER α is a critical requirement for IGF signaling and growth, with changes in ER α expression absolutely controlling responses to IGFs.

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