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**E-cadherin mediates the aggregation of breast cancer cells
induced by tamoxifen and epidermal growth factor**

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Abstract

In the present study, we evaluated the ability of 4-hydroxytamoxifen (OHT) and epidermal growth factor (EGF) to regulate homotypic adhesion in MCF7 breast cancer cells. Our results demonstrate that OHT and EGF activate the E-cadherin promoter, increase E-cadherin mRNA and protein expression and enhance homotypic aggregation of MCF7 cells. Interestingly, an ER α and EGFR cross-talk is involved in the E-cadherin expression by OHT and EGF as demonstrated by knocking-down either receptor. On the basis of our findings, the well-established cross-talk between ER α and EGFR could be extended to the modulation of E-cadherin expression by OHT and EGF. Thus, the potential ability of tamoxifen to induce cell-cell aggregation may contribute to the biological response of pharmacological intervention in breast cancer patients.

Introduction

Breast cancer is the most common malignancy in women worldwide with over one million cases diagnosed each year [1-3]. Estrogen is required for normal proliferation and differentiation of breast epithelial cells and is implicated in the development and progression of breast cancer [4]. The classical mechanism of estrogen action mainly involves two members of the nuclear receptor superfamily, the estrogen receptor (ER) α and ER β [5]. Steroid receptors, including ER α , have two main transcriptional activation functions named AF1 and AF2 which are associated with the N-terminal and the hormone binding domains, respectively. Agonists of ER α induce a conformational change in the hormone binding pocket that allows the recruitment of coactivators, which in turn maximize the transcriptional response to ligands [6]. Antagonists of ERs bind to the hormone binding pocket, however they block the AF2 in an inactive conformation. In addition, ER α can be activated by a host of extracellular signals, including growth factors, in the absence of the cognate ligand 17 β -estradiol (E₂) [7-9]. Of interest, partial antagonists of ER α , such as 4-hydroxytamoxifen (OHT), may display cell- and promoter-specific agonist effects that are thought to be due, at least in part, to AF1 activity and/or to the ability to recruit cofactors [10-12]. OHT was the first antiestrogen approved for the clinical management of ER-positive breast cancers and has been recommended as first-line treatment for the past three decades [4]. Despite the beneficial effects of OHT for patients at all stages of ER-positive breast cancer [13], the major obstacle to its use is the development of tamoxifen-resistance which either occurs *de novo* or in patients showing initial benefits [14]. Growth factors, including EGF, are involved in breast cancer progression [15,16]. Binding of EGF to the cognate receptor EGFR initiates a transduction cascade with sequential phosphorylation and activation of a series of intracellular signaling molecules. Among the downstream pathways activated by EGF, it is important to mention the cell survival cascade mediated by PI3-kinase (PI3-K) and AKT/PKB [17,18], the cell proliferation pathway mediated by the mitogen activated protein kinases (MAPK) ERK1/2 [19,20] and the stress-

induced pathways mediated by the stress activated protein kinase/JNK and p38 MAPKs [21]. On the other hand, rapid E₂ stimulation through ER α also activates these transduction pathways which synergistically trigger cell survival and proliferation [22,23]. In addition, ER α can be promptly activated in a ligand-independent manner by EGF which mimics E₂ in inducing agonist effects including breast cancer cell growth [24-26].

In the epithelium and in epithelium-derived tumors, cell-cell adhesion and tumor mass mostly rely on functions of E-cadherin, which is an intercellular adhesion molecule generally considered as a tumor suppressor [27,28]. Alteration in the activity of E-cadherin and the cadherin-catenin complex has been implicated in cancer progression [29], invasion [30-32], and metastasis [33,34]. Considering that in our recent study ER α -mediated homotypic tumor cell adhesion was induced by E₂ through an increase of E-cadherin expression [35], in the present investigation we evaluated whether the mixed ER agonist/antagonist OHT and the well-known ER α activator EGF could mimic the aforementioned action of E₂ in MCF7 breast cancer cells.

Materials and Methods

Plasmids. The plasmid containing the human E-cadherin promoter (-p1008/+49) was obtained from Dr. Y. S. Chang (Chang-Gung University, Republic of China) [36].

Cell lines and culture conditions. MCF7 cells were grown in DMEM/F-12 containing 10% FBS supplemented with 1% L-glutamine and 1% penicillin/streptomycin (Sigma, Milan, Italy). Cells were switched to medium without serum 48h before RT-PCR, immunoblots, and evaluation of ERK1/2 phosphorylation.

Transfections and Luciferase Assays. MCF7 cells were plated into 24-well dishes with 500µl regular growth medium/well the day before transfection. The medium was replaced with that lacking serum on the day of transfection, which was performed using FuGene6 reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy) with a mixture containing 0.5µg reporter plasmid and 2ng pRL-thymidine kinase. After 24h, we renewed the serum-free medium containing the indicated treatment, and then cells were incubated for approximately 16h. Luciferase activity was measured with the dual luciferase kit (Promega Italia, Milan, Italy) according to the manufacturer's recommendations. Firefly luciferase values were normalized to the internal transfection control provided by the Renilla luciferase activity. The data are reported as normalized relative light unit (RLU) values.

RT-PCR. The evaluation of gene expression was performed by semiquantitative RT-PCR as we have previously described [37]. For E-cadherin, pS2 and the acidic ribosomal phosphoprotein P0 (36B4), which was used as a control gene, the primers were, respectively, 5'-GGAATCCAAGCAGAATTGC-3' (E-cadherin forward), and 5'-TATGTGGCAATGCGTTCTCTATCCA-3' (E-cadherin reverse), 5'-

TTCTATCCTAATACCATCGACG-3' (pS2 forward) and 5'-TTTGAGTAGTCAAAGTCAGAGC-3' (pS2 reverse), 5'-CTCAACATCTCCCCCTTCTC-3' (36B4 forward), and 5'-CAAATCCCATATCCTCGTCC-3' (36B4 reverse), to yield products of 285, 210 and 408 bp, respectively, with 20, 15 and 15 PCR cycles.

Western Blotting. Equal amounts of whole protein extract were resolved on a 8-10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (GE Healthcare, Milan, Italy), probed overnight at 4°C with the antibodies against E-cadherin, EGFR, β -actin (Santa Cruz Biotechnology, Milan, Italy), pERK1/2 and total ERK (Cell Signaling Technology and Celbio, Milan, Italy) and then revealed using the enhanced chemiluminescence system (GE Healthcare).

ER α , EGFR and Sp1 silencing experiments. Cells were plated onto 10-cm dishes, maintained in antibiotic-free medium for 24h and then transfected for additional 24h before treatments with a mixture containing Opti-MEM, 8 μ l/well LipofectAMINE 2000 (Invitrogen, Milan, Italy) and 0.5 μ g/well control siRNA or ER α siRNA (Sigma), vector or shEGFR purchased from SuperArray (Bioscience Corporation, Frederick, MD), 200nM/well of control siRNA or Sp1 siRNA (Applied Biosystems, Milan, Italy).

Chromatin immunoprecipitation (ChIP) assay. MCF7 cells were treated with 1 μ M OHT or 100ng/ml EGF for 1h. Thereafter, cells were cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with salmon DNA/protein A agarose (Upstate Biotechnology, Lake Placid, NY) and immunoprecipitated with anti-ER α antibody or with anti-RNA Pol II (Santa Cruz Biotechnology). Pellets were washed as reported [38], eluted with elution buffer and digested with proteinase K. DNA was obtained by phenol/chloroform extraction and precipitated with ethanol; 3 μ l of

each sample were used for PCR with Sp1 primers: 5'-TAGCAACTCCAGGCTAGAGG-3' and 5'-AACTGACTTCCGCAAGCTCACA-3'. The PCR conditions were 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min. The amplification products obtained in 30 cycles were analysed in a 2% agarose gel and visualized by ethidium bromide staining.

Three-dimensional spheroid cultures. MCF7 cells were plated in single-cell suspension in 2%-agar-coated plates and left untreated or treated with 1µM OHT or 100ng/ml EGF for 48h. To block E-cadherin function, the medium was supplemented with E-cadherin antibody (1:100 dilution; Chemicon International, Temecula, CA) or EGTA to a final concentration of 4mM. To generate three-dimensional spheroids, the plates were rotated for 4h at 37°C. The three-dimensional cultures were photographed using a phase-contrast microscope (Olympus, Sun Valley, CA). The extent of aggregation was scored by measuring the spheroids with an ocular micrometer. The spheroids between 25 and 50, 50 and 100, and >100µm (in the smallest cross-section) were counted in 10 different fields under ×10 magnification.

Statistical Analysis. Statistical analysis was performed using ANOVA, followed by Newman-Keuls testing to determine differences in means.

Results

OHT and EGF transactivate the E-cadherin promoter and induce E-cadherin mRNA and protein expression.

On the basis of our previous study demonstrating that E-cadherin expression is induced by E_2 [35], and considering the potential of OHT and EGF to exert agonist activity through $ER\alpha$, we evaluated whether these compounds could regulate E-cadherin expression like E_2 . An expression vector containing the human E-cadherin promoter was transactivated by OHT and EGF in MCF7 cells (Fig. 1A). Treating cells with OHT in combination with EGF the transcriptional response was similar using each compound alone (Fig. 1A). In line with these findings, OHT and EGF induced E-cadherin mRNA expression at the same extent treating cells with both compounds (Fig. 1B, C). Then, we explored whether the agonistic effect displayed by OHT in upregulating E-cadherin expression was also evident in the modulation of the classical estrogen target gene pS2. Hence, MCF7 cells were treated with E_2 , OHT, the combination of both compounds and EGF. The up-regulation of pS2 by E_2 was abolished by OHT (Fig. 2A, B), confirming that OHT can act as a mixed agonist-antagonist depending on the gene response evaluated. Moreover, also EGF was able to upregulate pS2 mRNA expression (Fig. 2A, B). Thereafter, we asked whether OHT and EGF could up-regulate E-cadherin expression at the protein level. Upon exposure to each compound or a combination of both, we observed an increase of E-cadherin protein levels which was prevented by the ER antagonist ICI, the Src and ERK inhibitors PP2 and PD, respectively (Fig. 3A-D). The latter findings showing the involvement of the MAPK pathway was also confirmed by the ability of OHT and EGF to trigger a rapid ERK1/2 phosphorylation similar to that observed upon E_2 exposure (Fig. 4). Taken together these results suggest that both OHT and EGF involve $ER\alpha$ as well as Src and MAPK signaling in the regulation of E-cadherin expression.

ER α and EGFR silencing abrogates the E-cadherin up-regulation by OHT and EGF.

The results obtained prompted us to define the role exerted by ER α and EGFR in mediating E-cadherin stimulation by both OHT and EGF. Interestingly, after knocking-down ER α expression neither OHT nor EGF was able to up-regulate E-cadherin expression (Fig. 5A, B) confirming the data obtained using the ER antagonist ICI. Moreover, EGFR silencing also resulted in the abrogation of the E-cadherin increase upon exposure to each compound (Fig. 5C, D). The role of EGFR signaling was further ascertained observing an upregulation of EGFR protein expression particularly treating MCF7 cells with OHT (Fig. 5E). These results suggest that a functional cross-talk between ER α and EGFR triggers E-cadherin expression by the agents used.

OHT and EGF induce the recruitment of ER α to the Sp1 site located within the E-cadherin promoter sequence.

The interaction of ER α with the E-cadherin promoter sequence was investigated by ChIP assay. To this end, the MCF7 chromatin was immunoprecipitated with anti-ER α or anti-RNA-Pol II antibodies. PCR was used to determine the recruitment of ER α to the E-cadherin region containing the Sp1 site (-144/-132nt). Interestingly, the weak ER α recruitment to the E-cadherin promoter observed in cells treated with vehicle increased upon OHT and EGF treatment (Fig. 6A). In addition, both compounds induced the recruitment of RNA-Pol II indicating the ability to stimulate E-cadherin transcription (Fig. 6A). Next, the E-cadherin protein increase stimulated by OHT and EGF was no longer evident silencing Sp1 in MCF7 cells (Fig. 6B). A similar result was obtained using mithramycin, an inhibitor of Sp1-DNA interactions (Fig. 6C). These data confirm the role exerted by the Sp1 site in mediating the up-regulation of E-cadherin upon OHT and EGF treatments.

OHT and EGF induce MCF7 cell-cell adhesion in three-dimensional cultures.

On the basis of our previous study showing that the increase of E-cadherin expression by E₂ is responsible for enhanced MCF7 cell aggregation [35], we evaluated whether OHT and EGF could induce the same effect. Considering that multicellular spheroid cultures can closely reproduce some biological features of tumors and improve the relevance of results obtained by *in vitro* studies [39-41], we used three-dimensional MCF7 cultures as an experimental model. MCF7 cell-cell adhesion was enhanced following a 48h exposure to OHT and EGF (Fig. 7), which both induced the formation of spheroids prevalently with a diameter greater than 50µm (Table 1). Moreover, supplementing the culture medium with function-blocking E-cadherin antibody or the calcium-chelating agent EGTA we verified the direct involvement of E-cadherin in the enhanced cell-cell adhesion upon OHT and EGF treatments. In the presence of the E-cadherin antibody, the MCF7 cells showed the formation of small aggregates and limited intercellular contact, whereas in the presence of EGTA MCF7 cells remained rounded and separately suspended (Fig. 7).

Discussion

Estrogens play a major role in the development of breast cancer. Nearly 70% of breast tumors express the ER α which allows for the use of antiestrogen therapy as a reliable therapeutic approach for all stages of the disease [42]. The ER antagonist tamoxifen has been shown to improve survival in early breast cancer [43] as well as the quality of life in patients with metastatic disease [13]. Despite the documented benefits of ER-targeted therapy in breast cancer, approximately 50% of patients with advanced disease do not respond to first-line treatment with tamoxifen due to *de novo* or intrinsic resistance. Furthermore, almost all patients with metastatic disease and many who receive tamoxifen as adjuvant therapy eventually experience tumor relapse (acquired resistance). Thus, tamoxifen resistance occurs frequently in breast cancer patients and seriously limits treatment efficacy [42,44]. Multiple mechanisms have been proposed to contribute to the failure of tamoxifen therapy, including changes in the uptake or metabolism of tamoxifen, presence of ER mutants and ligand-independent ER activation, loss of expression of ER and/or cofactors and modification of the estrogen response element [42,45]. A large body of clinical and experimental studies has suggested that alteration of ER function by growth factor pathways may be involved in tamoxifen resistance [14, 42,46-48]. In this context, it has been reported that phosphorylation of ER α by EGFR-mediated signaling enhances the estrogen-induced transcriptional activity [49,50]. An increased recruitment of coactivators, such as AIB1 [51], and conformational changes [52] after receptor phosphorylation also contribute to enhanced transcriptional activity of ER α in tamoxifen-resistant breast cancer cells. In addition, an inverse relationship between the expression of ER α and EGFR or c-erbB2 was observed in primary breast cancer samples and was associated with decreased sensitivity to endocrine therapy and a poorer prognosis [47,53]. Hormone-dependent breast cancer cells engineered to overexpress EGFR and c-erbB2 resulted in stimulated hormone-independent cell growth [54,55].

Our present data demonstrate that EGF enhances E-cadherin expression and promotes homotypic aggregation of breast cancer cells maintained as three-dimensional cultures.

E-cadherin is an intercellular adhesion molecule generally implicated as tumor suppressor in several types of epithelial tumors, based on findings that the expression of this homotypic adhesion molecule is frequently lost in human epithelial cancers [29-31]. However, it has well been shown in ovarian epithelial tumors that E-cadherin expression is much more elevated than normal ovaries, suggesting that E-cadherin can play a role in the development of ovarian carcinomas [56]. For instance, it is worth to mention that E-cadherin may serve not only as an intercellular adhesion molecule, but it may also trigger intracellular activation of proliferation and survival signals [57, 35]. Our recent findings provided evidence that E₂ may be involved in mammary tumorigenesis on the basis of its stimulatory effects on primary tumor mass either *in vivo* using MCF7 cell tumor xenograft or *in vitro* using MCF7 three-dimensional cultures [35]. These actions were elicited by E₂ through homotypic tumor cell adhesion consequent to an increase of E-cadherin expression [35].

A number of studies have evaluated the usefulness of E-cadherin expression with respect to survival of breast cancer patients. Several studies indicate that reduced E-cadherin expression is an independent indicator of poor survival, particularly in node-negative patients [58-61]. However, other studies have found E-cadherin to be of little use in predicting clinical behavior of breast cancer patients [62]. Indeed, one study even noted that strong E-cadherin immunostaining correlated with poor survival [63]. In short, there currently are conflicting opinions about the prognostic value of altered E-cadherin expression. Thus, the usefulness of E-cadherin as a predictor of clinical behavior for breast cancer patients is still under debate.

In the present study, we showed that a cross-talk between ER α and EGFR was involved in E-cadherin expression by both OHT and EGF as demonstrated using the pure ER antagonist ICI, the Src and ERK inhibitors PP2 and PD, respectively, and most importantly silencing either ER α or EGFR expression.

Moreover, OHT and EGF stimulated ER α recruitment to the Sp1 site within the E-cadherin promoter region, whereas blocking this site, either by silencing Sp1 or using mithramycin, ER α binding to the E-cadherin promoter region was no longer evident.

Thus, in conclusion in this study, we provide further insights into the molecular mechanisms involved in the agonist activity exerted by tamoxifen in breast cancer, through the evidence that OHT *per se* is able to activate the promoter of E-cadherin, increasing E-cadherin expression at the mRNA and protein levels and thus may enhance homotypic aggregation of MCF7 breast cancer cells.

In addition, we have demonstrated that E-cadherin is a target of a cross-talk between EGF and OHT/ER signaling, since the upregulatory effect induced by OHT on E-cadherin expression was abrogated in the presence of either ER α or EGFR silencing. It is worth to note that in our experimental conditions tamoxifen induced an enhanced expression of EGFR protein levels, which may contribute to the failure of adjuvant endocrine therapy in breast cancer, as suggested by a growing body of data. The latter observations highlights how the agonistic effect of tamoxifen and EGF may sustains tumor mass growth through cell-cell aggregation.

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Legend for figures

Figure 1. OHT and EGF transactivate the E-cadherin promoter and increase E-cadherin mRNA expression.

A. MCF7 cells were transiently transfected with a luciferase reporter plasmid containing the full-length human E-cadherin promoter p-1008/+49 and treated with 1 μ M OHT, 100ng/ml EGF or a combination of both compounds. The luciferase activities were normalized to the internal transfection control. Each data point represents the mean \pm S.D. of three independent experiments performed in triplicate. **B.** The expression of E-cadherin was evaluated by semiquantitative RT-PCR in MCF7 cells treated with 1 μ M OHT, 100ng/ml EGF or a combination of both compounds for 16h; 36B4 levels were determined as a control. **C.** The quantitative representation of data (mean \pm S.D.) of three independent experiments. * indicates $p < 0.05$ for cells receiving vehicle (-) versus treatments.

Figure 2. OHT acts as an agonist for E-cadherin mRNA expression but as an antagonist for pS2 mRNA expression.

A. The expression of E-cadherin and pS2 was evaluated by semiquantitative RT-PCR in MCF7 cells treated with 1 μ M OHT, 100nM E₂, a combination of both compounds and 100ng/ml EGF for 16h; 36B4 levels were determined as a control. **B.** The quantitative representation of data (mean \pm S.D.) of three independent experiments. * indicates $p < 0.05$ for cells receiving vehicle (-) versus treatments.

Figure 3. Immunoblots of E-cadherin from MCF7 cells.

MCF7 cells were exposed for 16h to 1 μ M OHT, 100ng/ml EGF or a combination of both compounds (**A**). MCF7 cells were exposed for 16h to 1 μ M OHT and 100ng/ml EGF alone or in combination with 10 μ M ICI 182,780 (**B**), Src family tyrosine kinase inhibitor PP2 (**C**), MEK inhibitor PD 98059 (**D**).

β -actin serves as loading control. The quantitative representation of data (mean \pm S.D.) of three independent experiments after densitometry. * indicates $p < 0.05$ for cells receiving vehicle (-) versus treatments.

Figure 4. ERK1/2 phosphorylation in MCF7 cells.

MCF7 cells were treated for 10 min with 100nM E₂, 1 μ M OHT or 100ng/ml EGF. Total ERK proteins were used to normalize ERK1/2 expression. The quantitative representation of data (mean \pm S.D.) of three independent experiments after densitometry. * indicates $p < 0.05$ for cells receiving vehicle (-) versus treatments.

Figure 5. ER α and EGFR silencing abrogates the up-regulation of E-cadherin by OHT and EGF.

E-cadherin protein expression in MCF7 transfected with 0.5 μ g/well of ER α siRNA (A) or control siRNA (B), shEGFR (C) or vector (D). After 24h of transfection cells were treated with 1 μ M OHT or 100ng/ml EGF for 16h. EGFR protein expression in MCF7 treated for 16h with 1 μ M OHT or 100ng/ml EGF (E). β -actin serves as loading control. This experiment was repeated three times with similar results. Most representative experiment is shown.

Figure 6. OHT and EGF induce the recruitment of ER α on the Sp1 site within E-cadherin promoter sequence.

A. MCF7 cells were treated for 1h with 1 μ M OHT or 100ng/ml EGF or left untreated then crosslinked with formaldehyde and lysed. The soluble chromatin was immunoprecipitated with the anti-ER α or the anti-RNA Pol II Abs. The E-cadherin promoter sequence containing Sp1 was detected by PCR with specific primers, as described in Materials and Methods. To determine input DNA, the E-cadherin promoter was amplified from 5 μ l of initial preparations of soluble chromatin (before

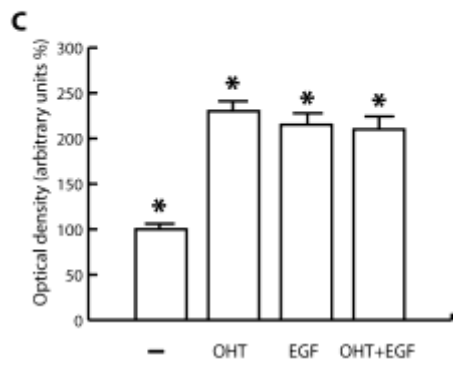
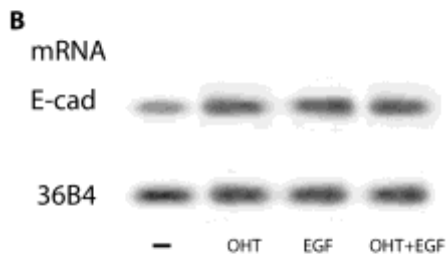
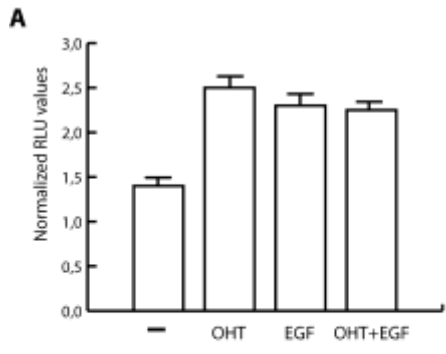
immunoprecipitations). N, negative control provided by PCR amplification without DNA sample. This experiment was repeated three times with similar results. Most representative experiment is shown. E-cadherin protein expression evaluated by immunoblot in MCF7 cells treated for approximately 16h and exposed to 1 μ M OHT or 100ng/ml EGF after knocking-down Sp1 (**B**) or preincubation with 200 nM mithramycin (**C**). β -actin serves as loading control. The *side panels* show the quantitative representation of data (mean \pm S.D.) of three independent experiments including that of **A**, **B** and **C**. * and ● indicate $p < 0.05$ for cells received vehicle (-) versus treatments.

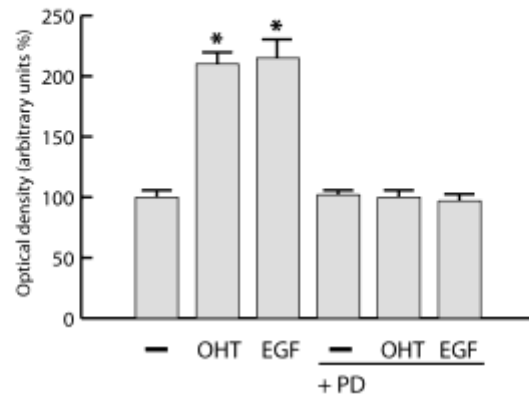
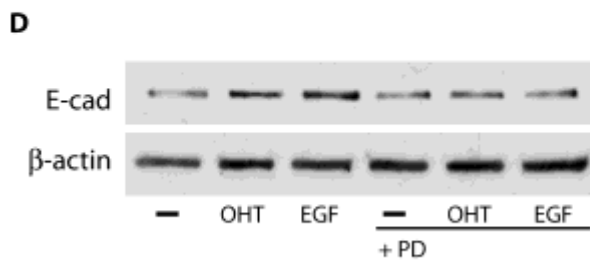
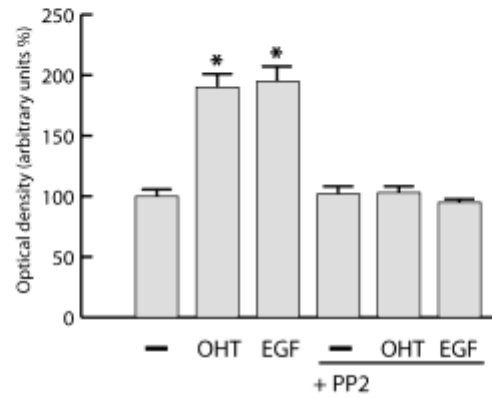
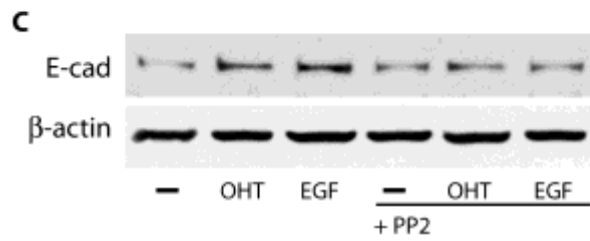
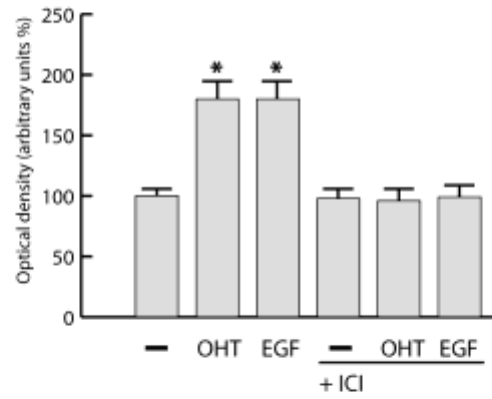
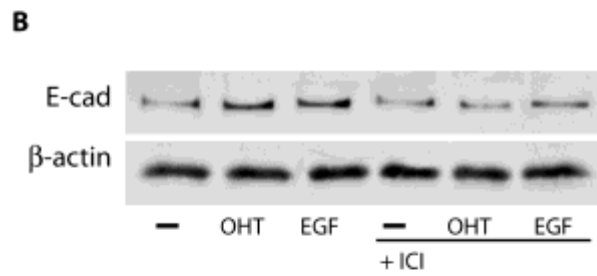
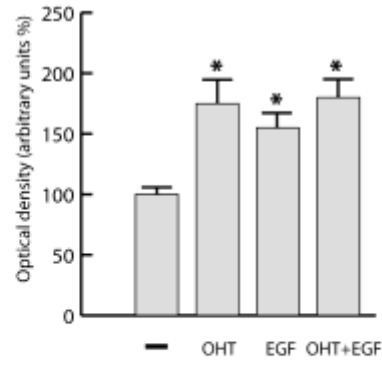
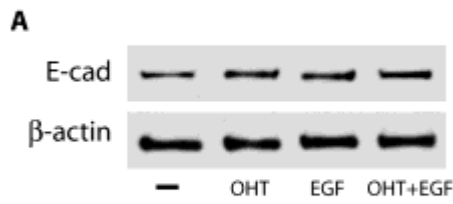
Figure 7. OHT and EGF induce cell-cell adhesion in three-dimensional cultures.

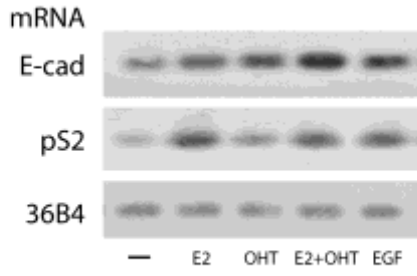
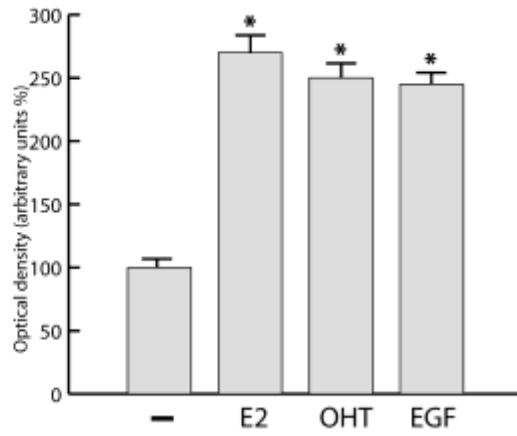
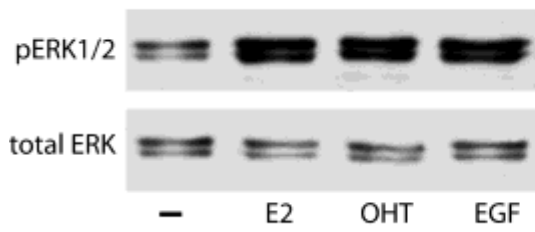
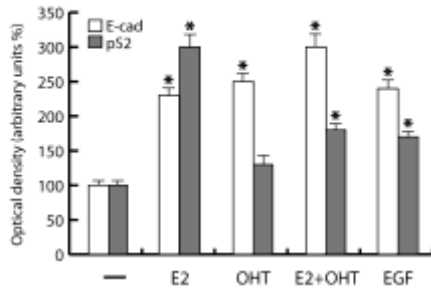
E-cadherin-positive MCF7 cells were seeded in 2% agar-coated plates and cultured as three dimensional spheroids. To block E-cadherin function, the medium was supplemented with E-cadherin antibody (1:100 dilution; a-E-cad) or EGTA (4 mM). Cells were untreated or treated with 1 μ M OHT or 100ng/ml EGF for 48h and then photographed under phase-contrast microscopy. Bar, 50 μ m.

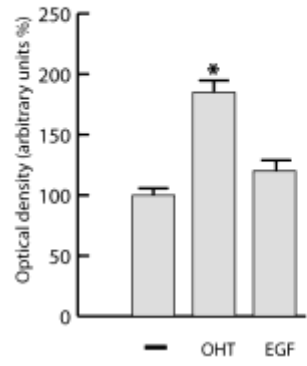
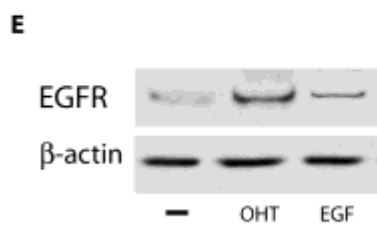
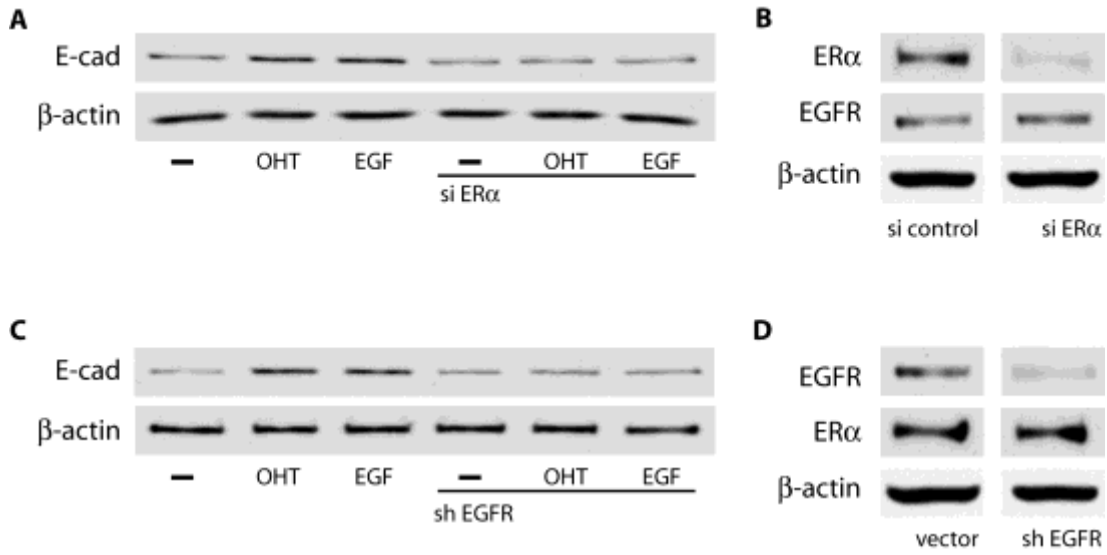
Table 1. Effect of OHT and EGF on cell aggregation in MCF7 breast cancer cells.

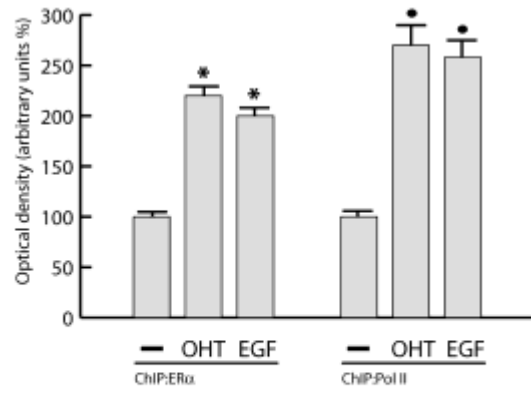
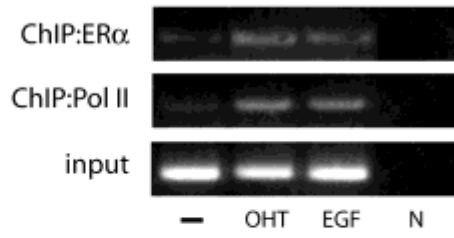
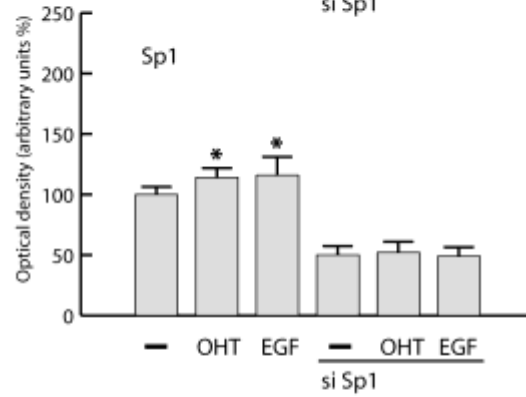
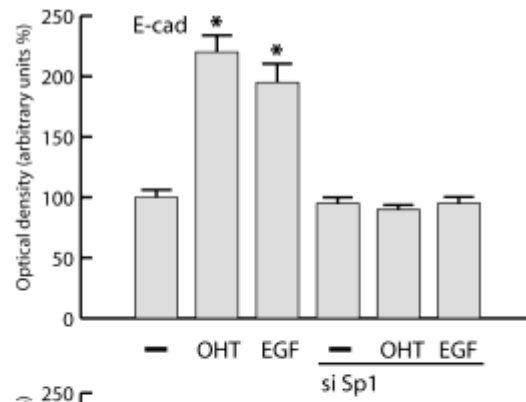
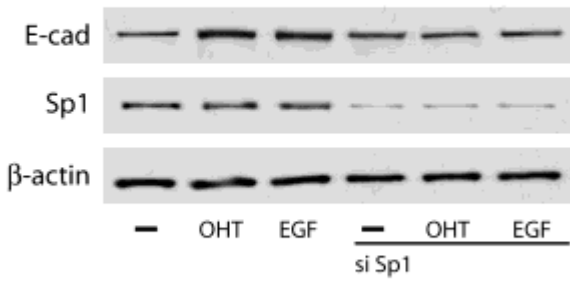
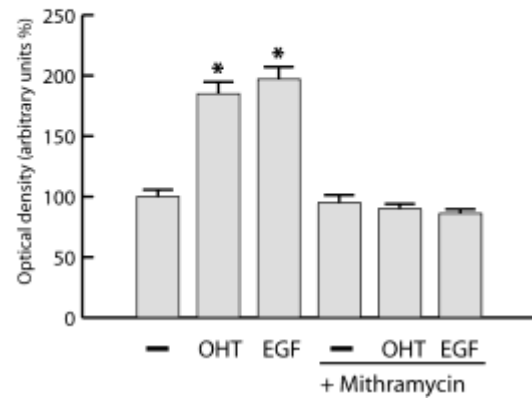
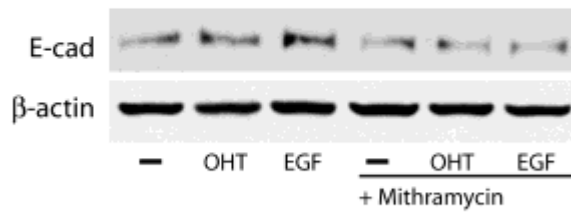
MCF7 cells were cultured as three-dimensional spheroids in medium without serum and treated as reported. The extent of aggregation was scored by measuring the spheroid diameters with an ocular micrometer. The values represent a sum of spheroids in 10 optical fields under x10 magnification. The results are mean mean \pm S.D. from at least three experiments. Representative three-dimensional cultures are shown in Fig. 7.

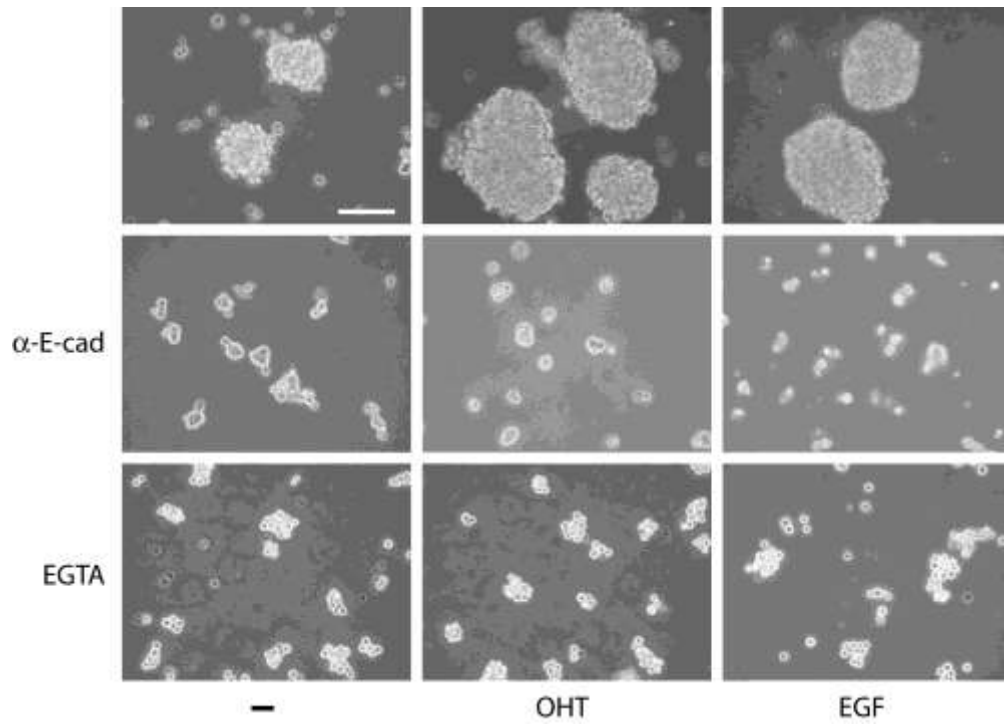




A**B**



A**B****C**



Spheroids

	25 ≤ 50 μm	50 ≤ 100 μm	> 100 μm
Control	<i>20 ± 1.3</i>	<i>0.4 ± 0.1</i>	<i>0.0 ± 0.0</i>
OHT	<i>3 ± 0.9</i>	<i>15 ± 1.5</i>	<i>6 ± 0.8</i>
EGF	<i>7 ± 0.8</i>	<i>21 ± 1.9</i>	<i>4 ± 0.6</i>