Epidermal Growth Factor Induces *WISP-2/CCN5* Expression in Estrogen Receptor-α-Positive Breast Tumor Cells through Multiple Molecular Cross-talks

Snigdha Banerjee, Krishanu Sengupta, Neela K. Saxena, Kakali Dhar, and Sushanta K. Banerjee

Cancer Research Unit, VA Medical Center, Kansas City, Missouri and Division of Hematology and Oncology, Department of Medicine, University of Kansas Medical Center, Kansas City, Kansas

Abstract

Epidermal growth factor (EGF) is a mitogen for estrogen receptor (ER)–positive breast tumor cells, and it has been proven that EGF occasionally mimicked estrogen action and cross-talks with ER-α to exert its activity. Therefore, the present study was undertaken to explore whether EGF is able to modulate the expression of Wnt-1-induced signaling protein-2/connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed 5 (*WISP-2/CCN5*), an estrogen-responsive gene, in normal and transformed cell lines of the human breast and, if so, whether this induction is critical for EGF mitogenesis and what downstream signaling pathways are associated with this event. Here, we show that EGF-induced *WISP-2* expression in ER- and EGF receptor–positive noninvasive MCF-7 breast tumor cells was dose and time dependent and that expression was modulated at transcription level. A synergism was seen in combination with estrogen. Moreover, small interfering RNA–mediated inhibition of WISP-2/CCN5 activity in MCF-7 cells resulted in abrogation of proliferation by EGF. The multiple molecular cross-talks, including the interactions between phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase signaling pathways and two diverse receptors (i.e., ER-α and EGFR), were essential in the event of EGF-induced *WISP-2/CCN5* up-regulation in MCF-7 cells. Moreover, EGF action on WISP-2/CCN5 is restricted to ER- and EGFR-positive noninvasive breast tumor cells, and this effect of EGF cannot be instigated in ER-α-negative and EGFR-positive normal or invasive breast tumor cells by introducing ER-α. Finally, regulation of phosphorylation of ER-α and EGFR may play critical roles in EGF-induced transcriptional activation of *WISP-2* gene in breast tumor cells. (Mol Cancer Res 2005;3(3):151–62)

Introduction

Wnt-1-induced signaling protein-2/connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed 5 (WISP-2/CCN5) is a secreted protein identified as a member of CCN family of growth factors (1-3), a newly identified family of growth factors that shared conserved multimodular domains and has been implicated as having an important role in both angiogenesis and carcinogenesis (2, 4-6). Recent studies from our laboratory and others have suggested that WISP-2/CCN5 has a particular relevance to human breast disease (1, 7, 8). This is because WISP-2/CCN5 mRNA and protein levels were elevated in different human breast tumor-derived cell lines as well as in breast tumor samples while being barely detectable, or undetectable, in normal breast epithelial cells (7-9). Additionally, abnormal expression of the *WISP-2/CCN5* gene was significantly higher in noninvasive carcinoma cells compared with invasive carcinoma cells. These observations are consistent with human studies demonstrating that WISP-2/CCN5 mRNA and protein expression are "biphasic" and markedly higher in noninvasive lesions compared with adjacent invasive tumor cells where expression levels are reduced and only sporadically detected (7). Furthermore, like other members of CCN family, including Cyr61 (10), *WISP-2/CCN5* is a serum- and sex steroid (i.e., estrogen and progesterone)–inducible early responsive gene in human breast tumor cells (7, 11, 12). Silencing of *WISP-2/CCN5* gene functions minimizes serum-induced breast tumor cell proliferation (7). Although these studies are beginning to identify the specific properties of WISP-2/CCN5 in breast cancer cells, the regulation of WISP-2/CCN5 by growth factors and its role in regulation of growth factor–mediated proliferation in breast cancer cells have been elusive.

Malignant disease in the breast is the result of uncontrolled cellular growth and proliferation (13-15). In general, growth of normal mammary epithelial cells is controlled by a delicate balance between growth-promoting and growth-inhibiting factors. The perturbations of these tightly regulated events by modulating the growth-promoting or growth-inhibiting factors

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Requests for reprints: Sushanta K. Banerjee, Cancer Research Unit, Research Division 151, VA Medical Center, 4801 Linwood Boulevard, Kansas City, MO 64128. Phone: 816-861-4700, ext. 57057; Fax: 816-922-3320. E-mail: sbanerjee2@kumc.edu

enhance aberrant cell growth. A key driver for aberrant growth of breast tumor cells is the epidermal growth factor (EGF) and its receptor (the EGFR/erB1/HER-1; ref. 16). EGF is a 6-kDa heat and acid stable polypeptide and a potent mitogen for many normal and malignant cells, including breast tumor cells (17, 18). The biological and pathobiological effects of EGF are mediated through its receptor EGFR, a member of the growth factor receptor family, which also include erB2/HER-2, erB3/HER-3, and erB4/HER-4 (19-21). However, this is not always the case, because estrogen receptor (ER)–negative MDA-MB-231 breast cancer cells possess huge quantity of EGFR in their cell membranes, but these cells do not react in response to EGF (22). Although the specific reasons of this nonresponse to EGF in this cell line are unclear, the various studies have shown that the actions of EGF-EGFR complex occasionally mimicked the effects of estrogen and these effects are most likely ER-dependent but estrogen-independent phenomena (23-26). Moreover, these studies and others suggested a causal link between ER and EGF-EGFR signaling leading to mitogenic activity by EGF in breast tumor cells (23, 27). Despite this link, however, downstream signaling molecule(s) modulated by the convergent action of ER and EGF-EGFR complex has not yet been fully identified.

To address all above issues, the current studies have been designed to determine whether EGF is able to induce WISP-2/CCN5 expression in breast tumor cells and, if so, whether this induction is critical for cellular proliferation by EGF and what signaling pathways are involved in this event. We report that WISP-2/CCN5 expression is elevated by EGF in ER-α/EGFR-positive noninvasive breast tumor cells leading to activation of the proliferative function of EGF through multiple molecular cross-talks. Moreover, hyperphosphorylation of EGFR and ER-α at Ser^{118} residue in the activation function-1 region of the NH_2-terminal domain (28) may also be a necessary step for the induction of WISP-2/CCN5 expression by EGF.

Results

EGF Up-Regulates WISP-2/CCN5 mRNA Expression in a Dose- and Time-Dependent Manner in Noninvasive Breast Carcinoma Cells

The dose-dependent effect of EGF on *WISP-2/CCN5* mRNA expression in ER-α- and EGFR-positive noninvasive MCF-7 breast tumor cells was determined for an identical period. MCF-7 cells (~70% confluent) were grown in a serum-free condition for 3 days. Starved cells were then exposed to different doses (i.e., 1, 10, and 100 ng/mL) of EGF for 24 hours. Total RNA was extracted using the Trizol method followed by Northern blot analysis using a human WISP-2/CCN5-specific nonradioactive probe. As shown in Fig. 1A, the up-regulation of *WISP-2/CCN5* mRNA expression by EGF was identified at 1 ng/mL concentration, which was 5.0-fold higher than the untreated control. The expression levels were elevated correspondingly as the dose of EGF increased.

Because 24-hour EGF (100 ng/mL) exposure reveals maximum induction of WISP-2/CCN5 expression in MCF-7 cells, subsequently, the time-dependent effect of EGF on WISP-2/CCN5 mRNA expression was evaluated. As shown in Fig. 1B, the induction of *WISP-2/CCN5* mRNA level was first detected at 4 hours, which was 2.5-fold higher than the untreated control. The level of expression was improved correspondingly as the time of exposure increased (Fig. 1B). Together, these data showed that EGF transiently and steadily enhanced WISP-2/CCN5 mRNA expression and this regulation is dose and time dependent in MCF-7 noninvasive EGFR- and ER-positive breast carcinoma cells.

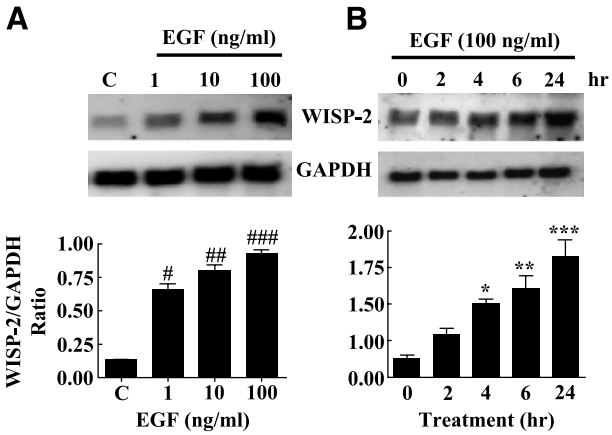

FIGURE 1. Concentration and time dependence of WISP-2/CCN5 up-regulation by EGF in MCF-7 cells. ER- and EGFR-positive noninvasive MCF-7 cells were serum starved for 3 days and exposed to EGF at indicated concentrations for 24 hours (**A**) or EGF (100 ng/mL) for indicated times (**B**). Total RNA was extracted and analyzed by RNA (10 μg) blotting using nonradioactive digoxigenin-labeled, PCR-generated WISP-2/CCN5 probe. GAPDH was used as a control probe to eliminate the loading differences. *X axis,* WISP-2/CCN5 mRNA concentrations. Columns, mean of three different experiments; bars, SD. #, $P < 0.02$; ##, $P < 0.03$; ###, $P < 0.001$; *, $P < 0.01$; **, $P < 0.004$; ***, $P < 0.001$ versus untreated control (Student's *t* test).

To explore if WISP-2/CCN5 is up-regulated by EGF at the transcription level or whether the mRNA stability was altered, the effect of a transcriptional inhibitor, actinomycin D (3 μg/mL), on EGF-induced *WISP-2/CCN5* mRNA expression was determined. As shown in Fig. 2, pretreatment of actinomycin D completely inhibited the induced expression of the *WISP-2/CCN5* gene to the basal level, suggesting that EGF-induced *WISP-2/CCN5* mRNA expression is under transcription control.

Next, we investigated whether *de novo* protein synthesis is required for EGF action on WISP-2/CCN5 regulation. To test this, MCF-7 cells were exposed to either EGF alone or in combination with a protein synthesis inhibitor, puromycin (10 μg/mL), for 4 hours, and *WISP-2/CCN5* mRNA expression was evaluated. As shown in Fig. 2, puromycin is able to inhibit the EGF-induced expression of the *WISP-2/CCN5* gene in these cells, thus indicating that *de novo* protein synthesis is required for the EGF response.

Synergistic Effects of Estrogen and EGF on WISP-2/CCN5 mRNA Expression

EGF and estrogen act as mitogens and promote breast cancer cell proliferation through multiple signaling pathways, which are usually intertwined [for details, see the review of Levin (23)]. Interestingly, synergism between EGF and estrogen action has been reported previously (25). These findings persuaded us to resolve whether estrogen modulates EGF action on *WISP-2/CCN5* gene expression in MCF-7 cells. To test this, serum-starved cells were exposed to 100 ng/mL EGF, 10 nmol/L

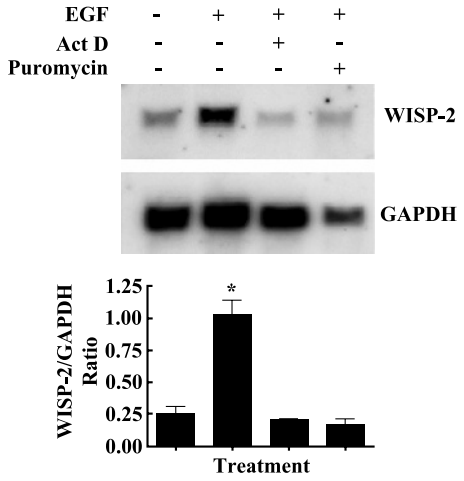

FIGURE 2. Effects of transcription inhibitor or protein synthesis inhibitor on EGF-induced WISP-2/CCN5 mRNA expression. Exponentially growing MCF-7 cells were serum starved for 3 days, and cells were either exposed to EGF (100 ng/mL) alone for 4 hours, pretreated for 1 hour with 3 μg/mL actinomycin D (*Act D*; transcription inhibitor), or pretreated for 30 minutes with 10 μg/mL puromycin (protein synthesis inhibitor) before exposure to EGF (100 ng/mL) for 4 hours. Total RNA was extracted and analyzed by Northern blot using digoxigenin-labeled, PCR-generated probes for WISP-2/CCN5 and GAPDH. Columns, mean of three different experiments; bars, SD. *, $P < 0.01$ versus untreated control (Student's *t* test).

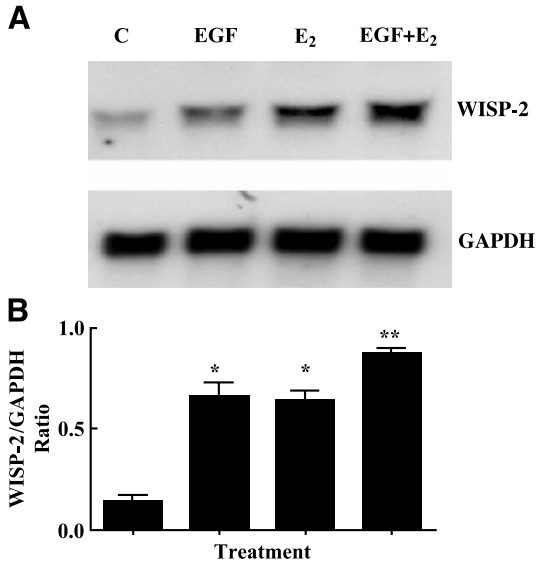

FIGURE 3. Synergistic effects of estrogen and EGF on WISP-2/CCN5 mRNA in MCF-7 cells. Serum-starved MCF-7 cells were exposed to E_2 (10 nmol/L) or EGF (100 ng/mL) alone or in combination with E_2 and EGF for 24 hours. Total RNA was extracted and analyzed by RNA blotting using nonradioactive digoxigenin-labeled, PCR-generated probe for WISP-2/CCN5. *Y axis,* WISP-2/CCN5 to GAPDH ratio. Columns, mean of three different experiments; bars, SD. *, $P < 0.01$; **, $P < 0.001$ versus untreated control (Student's *t* test).

17β-estradiol (E_2), or combination of EGF and E_2 for 24 hours. Total RNA was extracted and *WISP-2/CCN5* mRNA levels were evaluated. As shown in Fig. 3, *WISP-2/CCN5* mRNA expression enhances significantly when cells were exposed to E_2 and EGF together compared with E_2- or EGF-treated cells. This study, therefore, suggests a synergistic effect of E_2 and EGF on *WISP-2/CCN5* mRNA expression in MCF-7 cells.

Knockdown of WISP-2/CCN5 by Small Interfering RNA Inhibits EGF-Induced MCF-7 Cell Proliferation

To ascertain that WISP-2/CCN5 signaling is necessary for EGF mitogenesis, MCF-7 cells were transiently transfected with pSilencer vectors containing small interfering RNA (siRNA)-WISP-2 or vector alone for 48 hours, and WISP-2/CCN5 protein levels were detected by immuno-Western blotting the lysates of transfected cells. As expected from our previous study (7), the amount of WISP-2/CCN5 protein level was significantly decreased in siRNA (WISP-2/CCN5#1)–transfected cells compared with vector-transfected control (Fig. 4, *top*). After the validation of the inhibitory effect of both siRNAs, MCF-7 cells (15,000 per well) were transfected with siRNA-WISP-2 (WISP-2/CCN5#1) containing pSilencer vector or vector alone and were grown in serum-free DMEM with or without EGF (100 ng/mL) in 96-well plate for 24 hours and assayed for cell proliferation using bromodeoxyuridine ELISA kit. As shown in Fig. 4 (*bottom*), siRNA-mediated inhibition of WISP-2/CCN5 expression in MCF-7 cells resulted in significant inhibition of EGF-induced cell proliferation. Inhibition of WISP-2/CCN5 expression by siRNA partially blocks the basal proliferation. Thus, siRNA experiments indicate that mitogenic ability of EGF is mediated by WISP-2/CCN5 signaling.

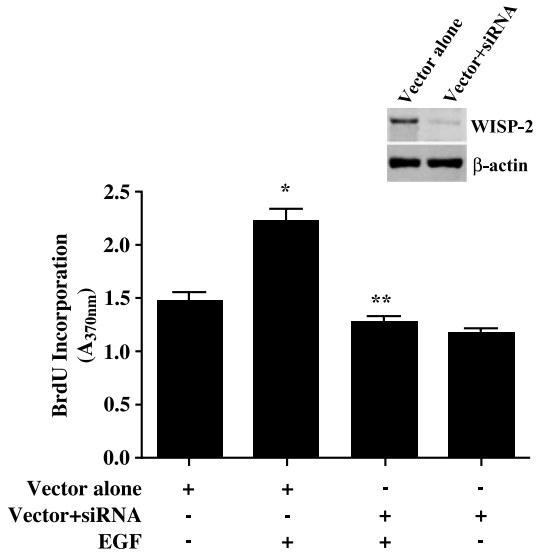

FIGURE 4. WISP-2/CCN5 silencing by siRNA inhibits EGF-induced MCF-7 cell proliferation. Semiconfluent (50-60%) MCF-7 cells were transiently transfected with vector alone or WISP-2/CCN5 siRNA (WISP-2/CCN5#1) containing vectors in serum-free Opti-MEM I as indicated in Materials and Methods. WISP-2/CCN5 protein level was examined by immuno-Western blot analysis with WISP-2-specific polyclonal antibody generated in our laboratory (8) or β-actin monoclonal antibody (*top*). The ability of WISP-2/CCN5 siRNA to inhibit EGF-induced proliferation at 48 hours after transfection was determined in MCF-7 cells using bromodeoxyuridine (*BrdU*) ELISA (*bottom*). Columns, mean of eight different experiments; bars, SD. *, $P < 0.0004$ versus vector-transfected cells; **, $P < 0001$ versus EGF-treated cells (Student's *t* test).

Silencing of EGFR by siRNA Blocks EGF-Induced Up-Regulation of WISP-2/CCN5 mRNA Expression

To test whether up-regulation of WISP-2/CCN5 mRNA expression by EGF is mediated through the EGF-specific receptor, we determined whether silencing of EGFR could modulate the levels of WISP-2/CCN5 expression. For these experiments, MCF-7 cells were transfected with pSilencer vectors containing siRNA-EGFR or vector alone for 48 hours, and EGFR levels were resolved by immuno-Western blotting the lysates of transfected cells. In vector control, the amount of EGFR was significantly higher compared with siRNA-EGFR-transfected cells where siRNA-EGFR completely blocks EGFR protein synthesis (Fig. 5). Inhibitory effects of the other two siRNAs (i.e., siRNA2 and siRNA3) specific for EGFR were also confirmed by transfecting cells with siRNA2 or siRNA3 followed by determining the level of EGFR (data not shown). After the corroboration of the inhibitory effect of siRNA, MCF-7 cells (5×10^4) were transfected with siRNA-EGFR containing pSilencer vector or vector alone and were grown in serum-free DMEM with or without EGF (100 ng/mL) for 24 hours in 25 cm^2 flasks. Total RNA was extracted using Trizol method followed by Northern blot analysis using human WISP-2/CCN5-specific nonradioactive probe. As shown in Fig. 6, prolonged exposure of EGF significantly enhances *WISP-2/CCN5* mRNA expression in MCF-7 cells. This up-regulation of WISP-2/CCN5 mRNA can be blocked by inhibiting EGFR expression using EGFR-specific siRNA. This indicates that EGF-induced up-regulation of WISP-2/CCN5 is mediated through EGFR in MCF-7 cells.

Inhibition of EGF-Induced Up-Regulation of WISP-2/CCN5 mRNA Expression by Anti-EGFR

To confirm the above results that EGF-induced up-regulation of WISP-2/CCN5 mRNA is mediated through EGFR, the effect of Tyrphostin AG30 (100 μmol/L), a selective EGFR inhibitor,

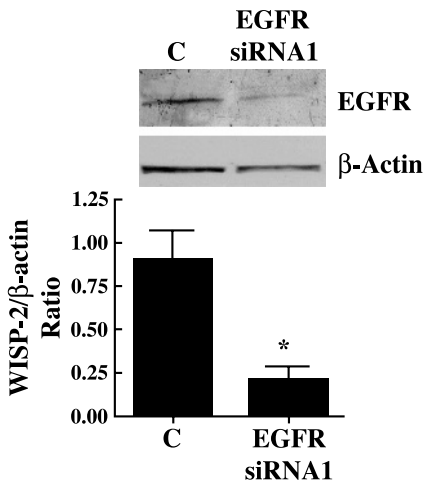

FIGURE 5. Effects of EGFR siRNA on EGFR protein level in MCF-7 cells. Cells were allowed to grow to 50% to 60% confluence and transfected with EGFR siRNA1 or vector alone (*C*) in serum-free Opti-MEM I as indicated in Materials and Methods, and EGFR protein levels were analyzed by Western blot analysis using EGFR-specific monoclonal antibody or antibody specific for β-actin. Columns, mean of three different experiments; bars, SD. *, $P < 0.001$ versus vector-transfected cells (Student's *t* test).

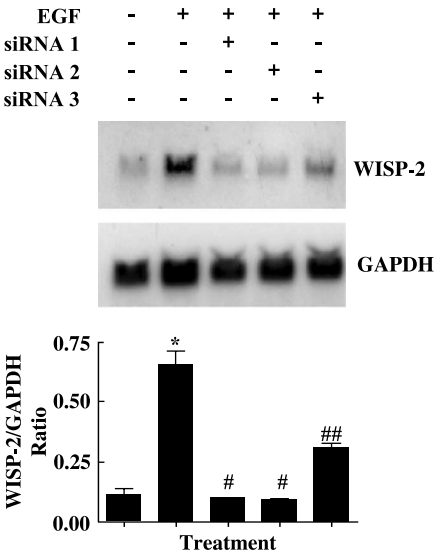

FIGURE 6. Effects of EGFR siRNA on EGF-induced WISP-2/CCN5 mRNA in MCF-7 cells. Exponentially growing MCF-7 cells were transfected with three separate EGFR siRNA (siRNA1, siRNA2, and siRNA3) or vector alone in serum-free Opti-MEM I for 24 hours. After transfection, cells were allowed to grow for additional 24 hours in serum-free conditions, and cells were exposed to EGF (100 ng/mL) for 24 hours. Total RNA was extracted and assessed for nonradioactive Northern blotting analysis using digoxigenin-labeled, PCR-generated probes for WISP-2/CCN5 and GAPDH. *Y axis,* WISP-2/CCN5 to GAPDH ratio. Columns, mean of three separate experiments; bars, SD. #, $P < 0.001$; ##, $P < 0.005$ versus EGF-treated cells; *, $P < 0.01$ versus untreated control (Student's *t* test).

on EGF action was explored. EGF alone increased *WISP-2/CCN5* mRNA levels in MCF-7 cells by ≥4.6-fold compared with the control (Fig. 7). Although cells were exposed to EGF along with AG30, the effect of EGF reduces significantly (Fig. 7). Although inhibition of EGFR by siRNA was more effective at decreasing the levels of EGF-induced WISP-2/CCN5 expression compared with AG30, this study established EGFR-mediated up-regulation of *WISP-2/CCN5* gene expression in MCF-7 cells.

Involvement of Signal Transduction Pathways in EGF-Induced WISP-2/CCN5 Up-Regulation

Multiple signal transduction pathways, including Src, Ras, Raf, mitogen-activated protein kinase (MAPK) kinase, c-Jun NH_2-terminal kinase, and protein kinase C pathways, are involved in EGF-EGFR-induced transcriptional activations of various genes during physiologic and pathophysiologic needs (23, 29-32). Moreover, estrogen, through its membrane receptor, rapidly activates several downstream signaling cascades that may eventually trigger the cross-talk between ER and EGFR, which in turn leads to activation of several signal transduction pathways and genes required to alter the cell biology of breast cancer (23, 33). This information tempted us to assume that similar signaling pathways may be involved in regulation of WISP-2/CCN5 expression by EGF in MCF-7 cells. Therefore, we exposed cells to wortmannin (100 nmol/L), a phosphatidylinositol 3-kinase (PI3K) inhibitor, or U0126 (10 μmol/L), a MAPK kinase/extracellular signal-regulated kinase inhibitor, for

30 minutes before EGF exposure. As shown in Fig. 8, both wortmannin and U0126 were able to inhibit significantly EGF-induced up-regulation of WISP-2/CCN5 expression. However, U0126 alone also blocks constitutive expression of WISP-2/CCN5 in MCF-7 cells, whereas wortmannin only perturbs the EGF action. This study suggests that both PI3K and MAPK signaling pathways are involved in EGF action on the transcriptional regulation of the *WISP-2/CCN5* gene.

Inhibition of EGF-Induced Up-Regulation of WISP-2/CCN5 mRNA Expression by a Pure Anti-Estrogen

The involvement of the ER in estrogen-like actions of EGF in rat uterus and breast tumor cells has been established (24, 25, 27, 34-37). The data described in the preceding section led us to investigate if the ER is involved in regulation of WISP-2/CCN5 expression by EGF in MCF-7 cells. To examine this, starved cells were exposed to EGF (100 ng/mL) alone or in combination with a pure ER antagonist, ICI 182,780 (1 μmol/L), for 24 hours, and *WISP-2/CCN5* mRNA levels were determined using Northern blot analysis. As shown in Fig. 9, EGF alone stimulates WISP-2/CCN5 mRNA expression in MCF-7 cells by 3.5-fold compared with the control, and this induction can be reduced to the basal level by ICI 182,780. This indicates that the effect of EGF on WISP-2/CCN5 mRNA expression in MCF-7 cells can occur dependently of ER-mediated events.

Characterization of WISP-2/CCN5 Expression Levels in Normal and Invasive Human Breast Tumor Cells

To determine whether EGF is able to up-regulate WISP-2/CCN5 expression in EGFR-positive and ER-negative normal human mammary epithelial (HME) cells or MDA-MB-231 human breast invasive carcinoma cell line, both cell lines were treated with EGF (100 ng/mL) for various times (i.e., 4, 24, or 48 hours) and *WISP-2/CCN5* mRNA levels were evaluated using Northern blot analyses. As shown in Fig. 10F to H, EGF (acute or prolonged exposures) is unable to up-regulate WISP-2/CCN5 expression in these cells. These studies, therefore, led us to explore if ER-α is required to potentiate the action of EGF. Both cell lines were transfected with ER-α and the effect of EGF on WISP-2/CCN5 mRNA expression was determined. Transfection of ER-α in these cells was confirmed by Western blot analysis using polyclonal antibody for ER-α that recognizes ER-α and two other variants (Fig. 11A) as shown in our previous study (38) and confocal microscopy (Fig. 10A-D). Expression of ER-α in MDA-MB-231 cells was detected both in the nucleus and in the cytoplasm (Fig. 10D, *inset*), whereas the expression HME cells was only seen in the nuclei (Fig. 10B, *inset*). The functional activity of transfected ER-α was established by confirming the overexpression of *pS2* gene by chronic exposure (24 hours) of E_2 in MDA-MB-231 cells (Fig. 10E). *pS2* is a well-recognized, estrogen-inducible gene in ER-positive breast tumor cells (39, 40) and is known to up-regulate estrogen in ER-transfected breast tumor cells (41). Because phosphorylation at Ser^{118} is critically associated with the activation of ER-α (42), the activity of ER-α was further confirmed by determining the phosphorylation of Ser^{118} in ER-transfected cells by Western blot analysis using phospho-ER-α (Ser^{118})–specific antibody (Fig. 11A). Phosphorylation

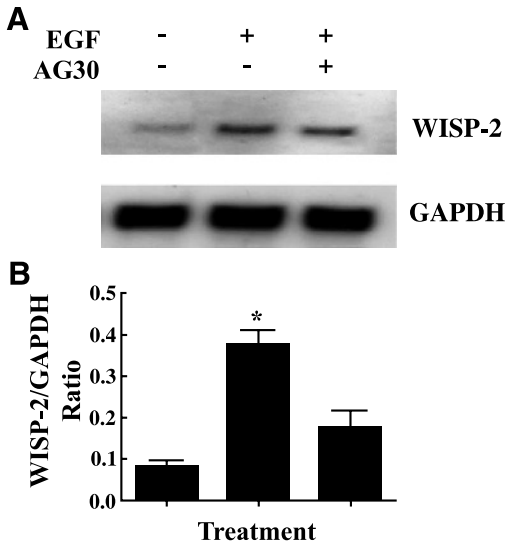

FIGURE 7. Effect of EGFR antagonist AG30 on EGF-induced WISP-2/CCN5 mRNA expression in MCF-7 cells. Serum-starved cells were exposed to EGF (100 ng/mL) alone or in combination of EGF and AG30 (100 μmol/L) for 24 hours. Total RNA was extracted, blotted, and hybridized with digoxigenin-labeled, PCR-generated WISP-2/CCN5 and GAPDH probes. *Y axis,* WISP-2/CCN5 to GAPDH ratio. Columns, mean of three separate experiments; bars, SD. *, $P < 0.01$ versus untreated control (Student's *t* test).

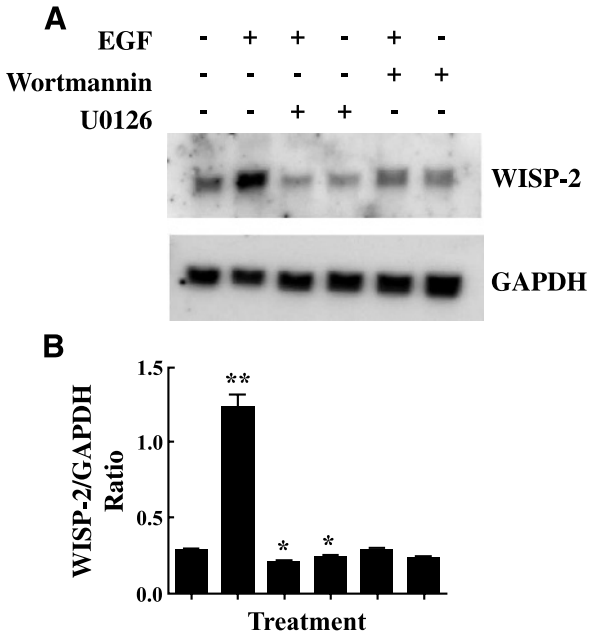

FIGURE 8. Effects of PI3K and extracellular signal-regulated kinase inhibition on EGF-induced up-regulation of WISP-2/CCN5 mRNA in MCF-7 cells. Starved cells were incubated with or without 100 nmol/L wortmannin (PI3K inhibitor) and 10 μmol/L U0126 (extracellular signal-regulated kinase inhibitor) for 30 minutes before incubation for 24 hours with or without EGF (100 ng/mL). The blot was probed with digoxigenin-labeled, PCR-generated WISP-2/CCN5 and GAPDH. *Y axis,* WISP-2/CCN5 to GAPDH ratio. Columns, mean of three different experiments; bars, SD. *, $P < 0.05$ versus EGF-treated cells; **, $P < 0.001$ versus untreated control (Student's *t* test).

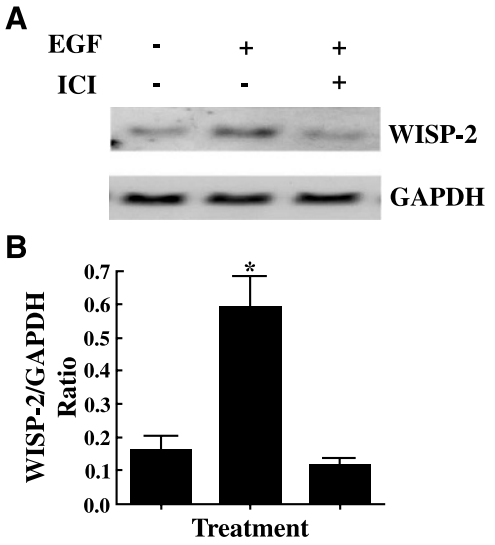

FIGURE 9. WISP-2/CCN5 up-regulation by EGF is mediated through ER-α in MCF-7 breast cancer cell line. Cells (~70% confluent) were serum starved for 3 days and then exposed to EGF (100 ng/mL) or in combination of EGF and a pure anti-estrogen ICI 182,780 (1 μmol/L) for 24 hours. Total RNA was extracted and analyzed by RNA blotting using nonradioactive digoxigenin-labeled, PCR-generated probes for WISP-2/CCN5 and GAPDH. *Y axis,* WISP-2/CCN5 to GAPDH ratio. Columns, mean of three separate experiments; bars, SD. *, $P < 0.001$ versus untreated control (Student's *t* test).

of ER-α was detected in both ER-α-transfected HME cells and MDA-MB-231 cells (Fig. 11A). However, the level of phosphorylation of ER-α was a little less in ER-α-transfected HME cells and MDA-MB-231 cells compared with MCF-7 cells (Fig. 11A). Once the transfection of ER-α and its activity were confirmed, the transfected cells were exposed to EGF and WISP-2/CCN5 expression was determined. The study shows that, despite the introduction of active ER-α, EGF is unable to enhance the *WISP-2/CCN5* mRNA expression in these cells (Fig. 10G and H), therefore suggesting that other multiple molecular events are associated with EGF-induced up-regulation of WISP-2/CCN5 expression, which are lacking in HME or MDA-MB-231 cells.

EGF Elicits Contrast Effects on ER-α Phosphorylation in Breast Tumor Cells

Phosphorylations on various serine residues (i.e., Ser^{104}, Ser^{106}, Ser^{118}, Ser^{167}, and Ser^{236}) of ER-α can be potentiated by estradiol as well as through the action of second messenger signaling pathways (28, 43, 44). Ser^{118} is the major site that is phosphorylated by estradiol and EGF through the MAPK signaling pathways and plays a critical role in ER-α function (28). In this study, the effect of EGF on phosphorylation at Ser^{118} was determined in MCF-7 and ER-α-transfected MDA-MB-231 breast tumor cell lines. The results presented in Fig. 11B show that EGF (100 ng/mL) caused an induction of phosphorylation of Ser^{118} in serum-starved MCF-7 cells after 24 hours of exposure. In contrast, EGF reduces phosphorylation of Ser^{118} in ER-α by 3.2-fold in transfected MDA-MB-231 breast tumor cells compared with untreated cells. Although this study does not show directly that the involvement of phosphorylation of Ser^{118} in EGF-induced WISP-2/CCN5 expression, the onset of Ser^{118} phosphorylation paralleled to the onset of up-regulation of WISP-2/CCN5 mRNA by EGF suggests a possible link between these two events.

Transfection of ER-α Reduces EGFR Levels and Activities in MDA-MB-231 Cells

The data presented in the preceding sections indicate that the cross-talk between ER-α and EGFR is crucial in EGF-induced up-regulation of WISP-2/CCN5 expression in MCF-7 cells. However, similar interaction is probably not potentiated in ER-α-transfected MDA-MB-231 EGFR-positive cells, as the WISP-2/CCN5 expression was unaltered by EGF. Therefore, we determined if transfection of ER-α reacts differently and changes the status and activity of EGFR in MDA-MB-231 cells. To test this, EGFR mRNA and protein levels and phospho-EGFR levels were evaluated in ER-α-transfected and nontransfected MDA-MB-231 cells. EGFR mRNA level was significantly elevated in ER-α-transfected cells compared with nontransfected parent cells (Fig. 12A). This result is consistent with the previous study (41). A single protein band of EGFR was detected by immuno-Western blot analyses in MDA-MB-231 cells. However, ER-α transfection to MDA-MB-231 cells causes the EGFR to migrate as a doublet on SDS-PAGE and can be detected by immuno-Western blot analysis. This electrophoretic shift may be due to the phosphorylation and dephosphorylation of this protein, because phosphorylations of specific sites have been shown to reduce the rate of migration of several proteins on SDS-PAGE (45). The intensity of the upper band, which can be considered as phospho-EGFR, was significantly reduced in transfected cells compared with the corresponding single protein band of parental cells (Fig. 12B). The combined intensity of upper and lower bands (doublet) exhibits no significant difference in comparison with non-transfected parent cells (Fig. 12B). The down-regulation of phosphorylation of EGFR was further confirmed by immuno-Western blot detection of phosphorylation of EGFR using anti-phospho-EGFR antibody and their ratios (Fig. 12C). Together, these studies suggest that negative regulation of EGFR by ER-α in MDA-MB-231 cells may block the EGF action in induction of WISP-2/CCN5 expression rather than creating an interaction of ER-α and EGFR to facilitate the EGF to exert its action.

Discussion

Despite the functional disparity of WISP-2/CCN5 in various cell types (7, 46, 47), this signaling molecule is becoming an increasingly important focus in breast cancer research for multiple reasons. For example, WISP-2/CCN5 was overexpressed in several breast tumor cell lines (9), and this expression is a requirement for ER-α-positive human breast tumor-derived MCF-7 epithelial tumor cell proliferation (7). This protein is overexpressed in ~70% of breast tumor samples but particularly so in atypical hyperplasia and ductal carcinoma *in situ* (7), a carcinoma in ducts that has not progressed beyond the basement membrane barrier (48). Moreover, *WISP-2/CCN5* is an estrogen- and progesterone-responsive gene and its expression in breast tumor samples is closely associated with ER-α positivity (7, 11). The present study further amplified the

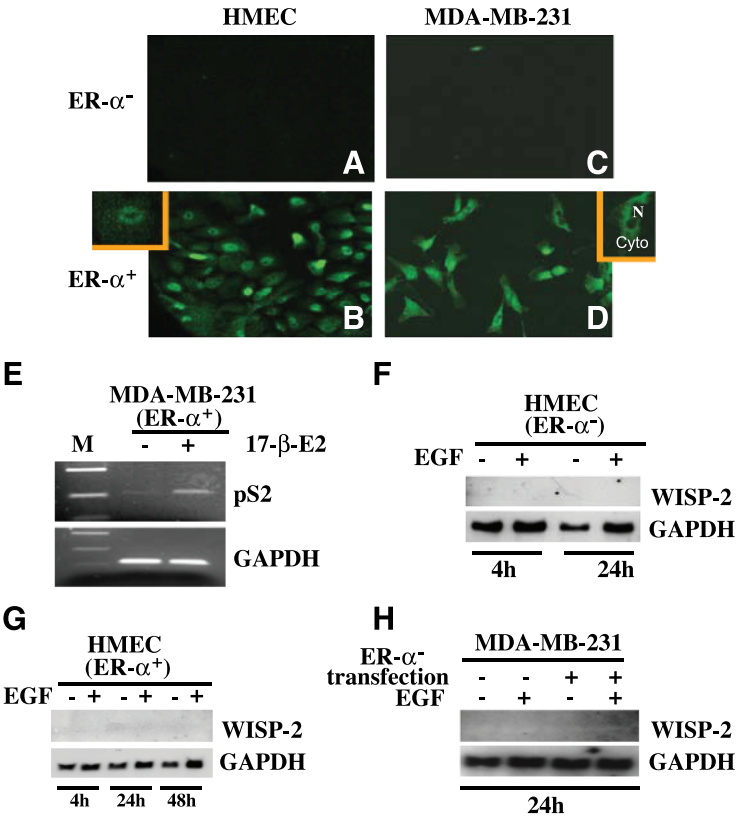

FIGURE 10. Effects of EGF on WISP-2/CCN5 expression in ER-α nontransfected and transfected normal and transformed human breast tumor cell lines. **A-D.** Cells (~70% confluent) were grown in chamber slides and transfected with human ER-α. After 48 hours of transfection, cells were fixed with paraformaldehyde and stained with anti-ER-α-specific polyclonal antibody and anti-rabbit IgG conjugated with FITC (Alexa Fluor 488) as the secondary antibody. **A** and **C.** Nontransfected ER-α-negative HME cells and MDA-MB-231 tumor cells. **B** and **D.** ER-α-transfected HMEC and MDA-MB-231 cells. Selected portions of **B** and **D** are shown in higher magnification. *N,* nucleus; *Cyto,* cytoplasm. **E.** Reverse transcription-PCR analysis of E_2-induced pS2 mRNA expression in nontransfected and ERα-transfected MDA-MB-231 cells. GAPDH mRNA expression ensures the quality of RNA and equal loading on agarose gel. The amplified product sizes of pS2 and GAPDH are 210 and 114 bp, respectively. *M,* DNA standards. **F.** ER-α-negative and EGFR-positive HME cells were grown in phenol red–free medium for 24 hours and exposed to EGF (100 ng/mL) for 4 or 24 hours followed by a nonradioactive Northern blot analysis for the detection of WISP-2/CCN5 expression. **G.** ER-α-transfected or vector alone-transfected HME cells show the WISP-2/CCN5 status following EGF exposure for 4, 24, or 48 hours. **H.** MDA-MB-231 cells were transfected with human ER-α expression vector (hER-pSG5) or vector alone and then exposed to EGF for 24 hours. WISP-2/CCN5 mRNA expression was determined in ER-α-transfected or vector-transfected cells.

significance of the *WISP-2/CCN5* gene in the carcinogenesis of human breast, because the results of this study reveal that WISP-2/CCN5 expression can be up-regulated in noninvasive MCF-7 human breast tumor cells by EGF, a growth factor that is believed to be linked to poor prognosis of breast cancer and an important modulator of cell proliferation, survival, adhesion, migration, and differentiation of breast epithelial cells (16, 49, 50). EGF dose-dependently induced WISP-2/CCN5 mRNA levels in a time-dependent manner (Fig. 1). Both low and high doses of EGF increase WISP-2/CCN5 mRNA expression in MCF-7 cells, and a dramatic synergism was seen in combination with estrogen (Fig. 3). Moreover, inhibition of WISP-2/CCN5 expression with siRNA abrogated MCF-7 cell proliferation induced by EGF, signifying that WISP-2/CCN5 signaling is essential for mitogenic ability of EGF. This study, therefore, further increased the importance of WISP-2/CCN5 signaling and sustains a positive role of WISP-2/CCN5 in the growth of noninvasive breast cancer cells.

There is evidence that a significant number of growth factors, including EGF-responsive genes, are regulated via the control of transcriptional activation and/or mRNA stabilization (51-53) through direct and/or indirect protein synthesis-dependent pathways (54). Inhibition of EGF-induced up-regulation of *WISP-2/CCN5* mRNA expression by either a transcription inhibitor actinomycin D or a protein synthesis inhibitor puromycin (Fig. 2) suggests that, for the *WISP-2/CCN5* gene, EGF may regulate the expression at the transcription level rather than mRNA stability, and intermediate proteins are required for this event.

Because EGF exerts its physiologic and pathophysiologic effects through its tyrosine kinase receptor, EGFR/HER-1, by activating multiple signal transduction pathways (16, 17, 30, 55, 56), we used several standard approaches to explore whether analogous signaling pathways were involved in the regulation of WISP-2/CCN5 expression by EGF. The studies have shown that EGFR/HER-1 mediates the action of EGF because up-regulation of *WISP-2/CCN5* mRNA in MCF-7 cells by EGF could be inhibited by either EGFR-specific siRNA or EGFR-specific tyrosine kinase inhibitor AG30 (Figs. 5, 6, and 7). Moreover, this study implicated both PI3K/Akt and MAPK signaling pathways in this event as inhibition of PI3K or MAPK activities using specific inhibitors to block EGF-induced WISP-2/CCN5 expression in MCF-7 cells. Consistent with the previous report (53), this study suggests a cross-talk of intracellular signals involved in EGF-EGFR/HER-1-mediated activation of a gene associated with breast tumor cell proliferation. Although this is a unique mechanism for amplitude modulation of a gene, it is unclear how these two signaling pathways are involved in enhancing the expression of the *WISP-2/CCN5* gene in MCF-7 cells. Probably, the activation of MAPK/extracellular signal-regulated kinase requires the participation of PI3K/Akt signaling in induction of WISP-2/CCN5 by EGF (57-59). Therefore, further studies are warranted.

Regardless of this inconsistency, it has been well established that EGF-EGFR/HER-1 signaling imitates the estrogenic action *in vitro* and *in vivo* through a classic ER (ER-α; refs. 23, 26, 27, 60). Accordingly, EGF actions can be blocked by

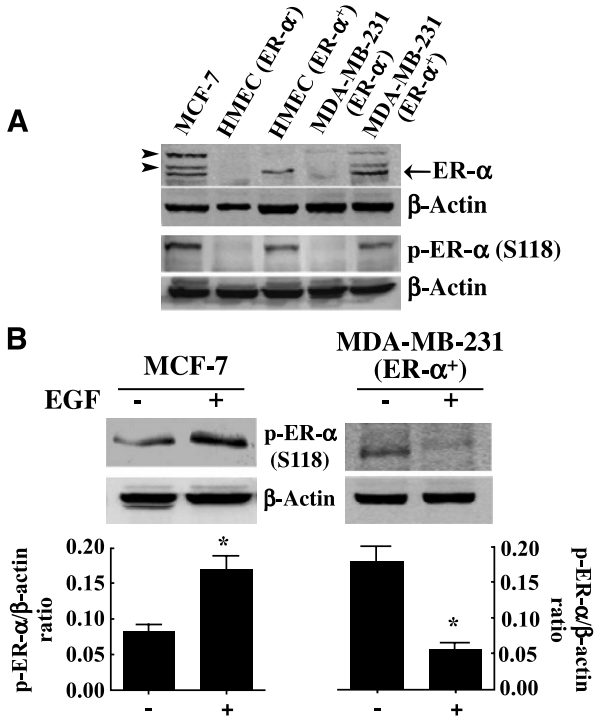

FIGURE 11. Differential regulations of phospho-ER-α in MCF-7 and MDA-MB-231 breast tumor cells. **A.** Expressions of phospho-ER-α and ER-α proteins in different ER-α-transfected and nontransfected breast tumor cells. *Arrowheads,* the two ER-α variants (38). Percentage of ER-α phosphorylated in different cells as indicated. Percentage of p-ER-α is calculated by the formula: intensity of p-ER-α/total ER-α × 100. **B.** Single representative immunoblot exhibits differential effects of EGF on phospho-ER-α levels in MCF-7 and ER-α-transfected MDA-MB-231 cells. Columns, mean of three separate experiments; bars, SD. *, $P < 0.01$ versus untreated control (Student's *t* test).

anti-estrogen (60-62). To test whether amplitude modulation of WISP-2/CCN5 expression by EGF-EGFR signaling is mediated through ER-α, we carried out multiple experiments to investigate (*a*) if pure anti-estrogen, ICI 182,780, attenuates EGF action on *WISP-2/CCN5* mRNA expression, (*b*) what would be the effect of EGF on WISP-2/CCN5 mRNA levels in ER-α-negative but EGFR-positive MDA-MB-231 breast tumor cells as well as in normal breast epithelial cells, and (*c*) whether transfection of ER-α in EGFR-positive cells altered the action of EGF. In ER- and EGFR-positive MCF-7 breast tumor cells, EGF enhances *WISP-2/CCN5* mRNA expression levels, and this can be reduced to the basal level by exposing cells to ICI 182,780 (Fig. 9). In contrast, EGF was unable to modulate WISP-2/CCN5 mRNA expression in ER-negative breast tumor cells or normal mammary epithelial cells, which express high levels of EGFR (63, 64). Together, these data suggest that a molecular cross-talk between ER-α and EGFR signaling pathways is essential for induction of WISP-2/CCN5 expression by EGF. Nevertheless, this phenomenon cannot be generalized because ER-α transfections in these cells were not able to enhance the EGF action (Fig. 10) and so leave open the possibility that interaction between these two receptors, EGFR/HER-1 and ER-α, occurs under specific circumstances, which are probably lacking in MDA-MB-231 invasive carcinoma cells.

ER-α is a phosphoprotein, and its biological functions through the transcriptional activations of various genes can be modulated by phosphorylations on various serine residues of this protein (36, 65). A well-studied phosphorylation site in ER-α is Ser^{118} (28). Both estrogen and EGF are able to enhance phosphorylation of ER-α at Ser^{118} residue in various cell lines (28, 35, 43, 45, 66). EGF-induced acute phosphorylation of ER-α may be associated with the molecular cross-talk between ER and EGFR in MCF-7 cells (35, 36). Consistent with previous studies, our findings indicate that EGF enhances phosphorylation of ER-α at Ser^{118} in MCF-7 cells parallel with WISP-2/CCN5 up-regulation (Fig. 11). However, EGF action on phosphorylation of ER-α was reversed in ER-α-transfected MDA-MB-231 invasive carcinoma cells (Fig. 11). Together, these studies suggest that phosphorylation of ER-α may be an essential intermediate event to enhance WISP-2/CCN5 expression in noninvasive MCF-7 breast cancer cells by EGF. Further studies are warranted.

EGFR/HER-1 has an extracellular domain, a transmembrane domain, and a cytoplasmic domain, which has tyrosine kinase enzyme activity (17). On stimulation of EGFR/HER-1 signaling capacity by its ligand EGF, autophosphorylation on various residues within cytoplasmic domains and phosphorylation of intracellular substrates were achieved and increased (17, 67, 68). The signaling capacity of EGFR can be reduced through the inhibition of autophosphorylation by treating cells with tyrosine kinase inhibitors (69, 70). In this study, to test whether EGFR signaling capacity, measured by autophosphorylation levels, was related to ER-α concentration in breast tumor cells, the *ER-α* gene was introduced into the ER-α-negative MDA-MB-231 breast tumor cells and phosphorylation of Tyr^{1173} was determined. The results of this study showed that the constitutive autophosphorylation levels of EGFR in MCF-7 and MDA-MB-231 proliferative cells were identical (Fig. 12). However, on transfection of ER-α in MDA-MB-231 cells, the phosphorylation levels were significantly reduced from the basal values (Fig. 12), and this reduction cannot be recovered by exogenous EGF treatment (data not shown). Because the autophosphorylation of EGFR for kinase activity and cross-talk with other receptors for mitogenic signaling are allied and linear events (68), our studies suggest that inhibition of phosphorylation of EGFR by ER-α transfection may hamper the cross-talk between these two receptors, which eventually blocks the EGF action on regulation of WISP-2/CCN5 signaling in invasive breast carcinoma cells. However, it is uncertain why ER-α transfection reduces autophosphorylation of EGFR in invasive carcinoma cells.

In summary, the data presented here reveal that WISP-2/CCN5 is an EGF-induced signaling molecule in noninvasive ER-positive MCF-7 breast tumor cells but not in invasive carcinoma cells, and this induction is regulated at transcriptional levels. Multiple levels of complex molecular cross-talks are involved in this regulatory event. Phosphorylations of ER-α and/or EGFR may need to regulate the expression of *WISP-2/CCN5* gene by EGF in breast tumor cells. However, further studies are warranted to confirm the premise. Finally, the accumulated data suggest that WISP-2/CCN5, like other members (Cyr61/CCN1) of CCN family (10, 71), could be a well-suited signaling molecule to study possible mechanisms

of differential action of EGF on breast tumor cell proliferation. Moreover, this work provides a novel insight into the mechanism underlying differential sensitivity of noninvasive and invasive breast cancer cells.

Materials and Methods

Reagents

E_2, EGF, wortmannin, puromycin, actinomycin D, and anti-actin monoclonal antibody were obtained from Sigma Chemical Co. (St. Louis, MO). U0126 was purchased from Promega Corp. (Madison, WI). EGFR inhibitor, Tyrphostin AG30, was obtained from Calbiochem (San Diego, CA). Anti-estrogen (ICI 182,780) was purchased from Tocris (Ellisville, MO). Mouse anti-EGFR, anti-phospho-EGFR (Tyr^{1173}) monoclonal antibodies, rabbit anti-ER-α, and phospho-ER-α polyclonal antibodies were purchased from BD Biosciences (San Jose, CA), Upstate (Lake Placid, NY), Santa Cruz (Santa Cruz, CA), and Cell Signaling (Beverly, MA), respectively. The digoxigenin high prime DNA labeling and detection kit was obtained from Roche Diagnostics GmbH (Indianapolis, MD). WISP-2/CCN5 rabbit polyclonal antibody was generated previously in our laboratory (8).

Cell Lines, Culture Conditions, and Treatment

MCF-7 (noninvasive) and MDA-MB-231 (invasive) human breast carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The nontransformed HME cells were obtained from Clonetics (San Diego, CA). ER-α-transfected HMEC was a gift from Dr. Deborah Zajchowski (Berlex Biosciences, Richmond, CA). The human carcinoma cell lines were grown in DMEM (Life Technologies, Grand Island, NY) supplemented with 1 mmol/L sodium pyruvate (Life Technologies), 50 units/mL penicillin associated with 50 μg/mL streptomycin (Life Technologies), and 10% fetal bovine serum (Life Technologies) at 37°C in humidified tissue culture incubator containing 5% CO_2 and 95% air. HME cells were grown in mammary epithelial basal medium (Clonetics) supplemented with mammary epithelial growth medium single quots (Clonetics) and ER-α-transfected cell line was grown in DFCI-1 medium with 1% fetal bovine serum (72). Cells (~70% confluent) were serum deprived for 3 days to reach an estrogen- and growth factor–free environment. The deprived cells were then treated with E_2 (10 nmol/L) and EGF (1-100 ng/mL) for different times indicated in the figure legends. All the inhibitors, except ICI 182,780, were administered into the culture medium for 30 minutes or 1 hour before the addition of E_2 or EGF depending on the experimental protocol.

siRNA Synthesis and Cloning in Cellular Expression System

To design WISP-2/CCN5- and EGFR-specific double-stranded siRNAs 21 to 22 nucleotides long, which initiate a sequence-specific, post-transcriptional gene silencing and known as RNA interference, two different siRNAs complementary to the target sites of *WISP-2/CCN5* gene and three different target sites of *EGFR* gene were selected and chemically synthesized from the Midland Certified Reagent Co. (Midland, TX). This strategy for choosing siRNA target sites is based on

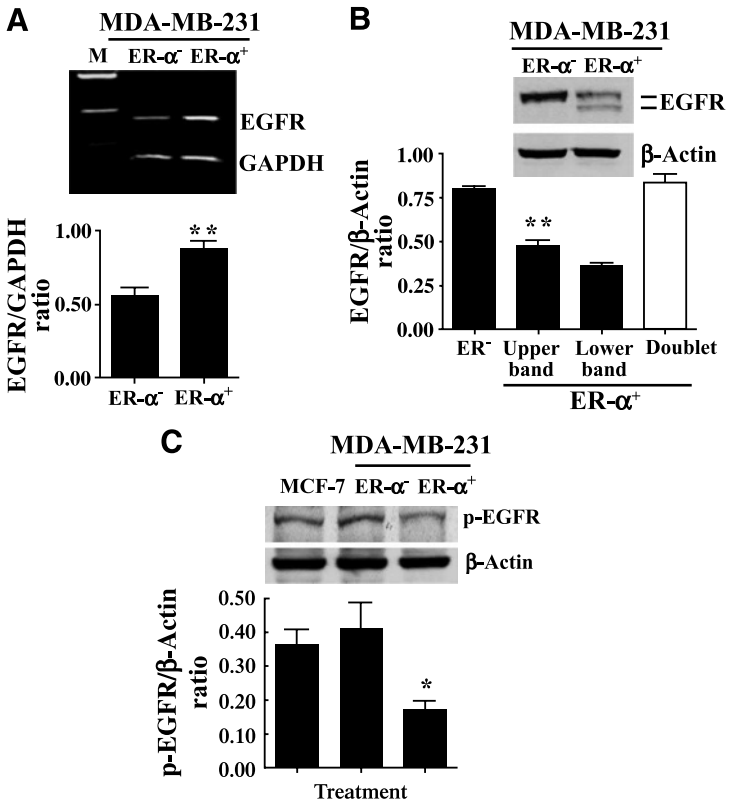

FIGURE 12. Detection of EGFR mRNA, total protein, and phospho-EGFR in MCF-7, MDA-MB-231, and ER-transfected MDA-MB-231 breast tumor cells using reverse transcription-PCR and Western blot analysis. **A.** Reverse transcription-PCR analysis of EGFR mRNA expression in nontransfected and ERα-transfected MDA-MB-231 cells. GAPDH mRNA expression ensures the quality of RNA and equal loading on agarose gel. The amplified product sizes of EGFR and GAPDH are 341 and 114 bp, respectively. Ratio between EGFR and GAPDH mRNA ER-α-transfected and nontransfected cells. **B.** Proteins were extracted from semiconfluent cells and proteins (30 μg) were loaded for Western immunoblotting. EGFR was detected by chemiluminescent immunoblotting using monoclonal anti-mouse EGFR antibody. **C.** Phospho-EGFR was detected using phospho-EGFR-specific monoclonal antibody. Columns, mean of three separate experiments; bars, SD. *, $P < 0.01$; **, $P < 0.005$ versus nontransfected control (Student's t test).

previous observation of Elbashir et al. (73). The siRNA antisense sequences of WISP-2/CCN5 and EGFR are WISP-2/CCN5#1, 5′-CACAGCUGGGUACGCACC tt; WISP-2/CCN5#2, 5′-GAAGGCCAGGAGGUGGGU Ctt; EGFR1, 5′-UGCCUUGG-CAAACUUUCU Utt; EGFR2, 5′-AGUGCCCAACUGCGU-GAG Ctt; and EGFR3, 5′-GAAUUUUCGUAGUACAUA Utt. cRNA strands (2 μg) were annealed by heating at 90°C for 3 minutes followed by incubation at 37°C for 1 hour. The ligated product was inserted into the expression vector (pSilencer 1.0-U6 siRNA expression vector) and then transformed into DH5α *Escherichia coli* according to the manufacturer's instruction (Ambion, Austin, TX). The positive clones were confirmed by PCR, amplifying the insert portion of the clones with a gene-specific forward primer and the T3 promoter primer.

Transfection of Cells with siRNA or ER-α

Transfection of expression vectors alone or containing either siRNA or ER-α were carried out in breast tumor cell lines by using the Lipofectin reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. MCF-7 or MDA-MB-231 cells were seeded in 25 cm^2 flasks and six-well culture plates in DMEM supplemented with 10% fetal bovine serum to achieve 50% to 60% confluence. The cells were then fed with Opti-MEM I (Invitrogen) for 1 to 2 days. The siRNA constructs (10 μg) or ER-α constructs (10 μg; a gift of Pierre Chambon, Université Louis Pasteur, France) were transfected in MCF-7 cells or MDA-MB-321 cells, respectively, with 1 mL Opti-MEM I containing 20 μg/mL Lipofectin for 24 hours at 37°C in a humidified tissue culture incubator containing 5% CO_2. The transfection medium was replaced by Opti-MEM I containing no Lipofectin and treated with EGF (100 ng/mL) for additional 24 hours. Cells were harvested to quantitate the RNA level of WISP-2/CCN5 and protein level of EGFR or ER-α. Vector alone-transfected cells were considered as negative controls.

RNA Extraction, cDNA Synthesis, and Probe Preparation

Total cellular RNA was extracted from different cell lines using the Trizol-mediated extraction method. cDNA synthesis and probe preparation were done according to the method described by Banerjee et al. (74).

Northern Blot Analysis

Total RNA (10 μg) of each sample was separated by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane (Schleicher & Schuell, Keene, NH). The membranes were hybridized with nonradioactive digoxigenin-labeled, PCR-generated WISP-2/CCN5 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Relative expressions of *WISP-2/CCN5* mRNA were calculated by densitometric analyses using One-dimensional Image Analysis Software version 3.6 (Eastman Kodak Co., Rochester, NY).

Reverse Transcription-PCR Analysis

Total RNAs from different samples were reverse transcribed into cDNAs and PCR amplified with pS2, EGFR, and GAPDH specific primers following the method described earlier (8). The sequences of primers are pS2, 5′-ATGGCCACCATGGAGAA-CAA-3′ (forward) and 5′-ATAGAAGCACCAGGGGACCC-3′ (reverse); EGFR, 5′-GAGTCGGGCTCTGGAGGAAA-3′ (forward) and 5′-GCTCCTTCAGTCCGGTTTTA-3′ (reverse); and GAPDH, 5′-ATGAGAAGTATGACAACAGCC-3′ (forward) and 5′-TGAGTCCTTCCACGATACC-3′ (reverse). The annealing temperature was 55°C for pS2 and 60°C for EGFR and GAPDH. Each PCR amplification was run for 30 cycles. A 100-bp ladder DNA molecular weight marker (Life Technologies, Gaithersburg, MD) was used to determine the sizes of the amplified products.

Subcellular Fractionation and Immuno-Western Blot

The subcellular fractions and immuno-Western blot analysis were the same as described previously (7). Briefly, the protein (30 μg) of each sample was separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with Superblock (Pierce, Rockford, IL) for 1 hour at room temperature followed by incubation with appropriate primary antibodies overnight at 4°C. Membranes were then washed and incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour. Membranes were washed extensively and developed with an enhanced chemiluminescence kit (Amersham Pharmacia, Piscataway, NJ). Measurement of band intensities and densitometric analyses were done by using One-dimensional Image Analysis Software version 3.6.

Confocal Immunofluorescence Microscopy

After 48 hours of transfection, cells were fixed with 2% paraformaldehyde/PBS for 20 minutes and then permeabilized with 0.1% Triton X-100/PBS for 5 minutes. Samples were preblocked with a ready-to-use blocking solution (Histostain kit, Zymed Laboratories, South San Francisco, CA) and incubated with rabbit anti-ER-α antibody overnight at 4°C. Cells were then stained with anti-rabbit IgG fluorescent conjugate (Alexa Flour 488, Molecular Probes, Eugene, OR), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Cells were mounted in PBS-glycerin and examined by confocal fluorescence microscopy (Bio-Rad, Hercules, CA).

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References

1. Pennica D, Swanson TA, Welsh JW, et al. WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proc Natl Acad Sci U S A 1998;95:14717–22.

2. Brigstock DR. The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. Endocr Rev 1999;20:189–206.

3. Brigstock DR, Goldschmeding R, Katsube KI, et al. Proposal for a unified CCN nomenclature. Mol Pathol 2003;56:127–8.

4. Moussad EE, Brigstock DR. Connective tissue growth factor: what's in a name? Mol Genet Metab 2000;71:276–92.

5. Kireeva ML, Mo FE, Yang GP, Lau LF. Cyr61, a product of a growth factor-inducible immediate-early gene, promotes cell proliferation, migration, and adhesion. Mol Cell Biol 1996;16:1326–34.

6. Lin C, Leu SJ, Chen N, et al. CCN3 (NOV) is a novel angiogenic regulator of the CCN protein family. J Biol Chem 2003;278:24200–8.

7. Banerjee S, Saxena N, Sengupta K, Tawfik O, Mayo MS, Banerjee SK. WISP-2 gene in human breast cancer: estrogen and progesterone inducible expression and regulation of tumor cell proliferation. Neoplasia 2003;5:63–73.

8. Zoubine MN, Banerjee S, Saxena NK, Campbell DR, Banerjee SK. WISP-2: a serum-inducible gene differentially expressed in human normal breast epithelial cells and in MCF-7 breast tumor cells. Biochem Biophys Res Commun 2001;282:421–5.

9. Saxena N, Banerjee S, Sengupta K, Zoubine MN, Banerjee SK. Differential expression of WISP-1 and WISP-2 genes in normal and transformed human breast cell lines. Mol Cell Biochem 2001;228:99–104.

10. Sampath D, Winneker RC, Zhang Z. Cyr61, a member of the CCN family, is required for MCF-7 cell proliferation: regulation by 17β-estradiol and overexpression in human breast cancer. Endocrinology 2001;142:2540–8.

11. Inadera H, Hashimoto S, Dong HY, et al. WISP-2 as a novel estrogen-responsive gene in human breast cancer cells. Biochem Biophys Res Commun 2000;275:108–14.

12. Inadera H, Dong HY, Matsushima K. WISP-2 is a secreted protein and can be a marker of estrogen exposure in MCF-7 cells. Biochem Biophys Res Commun 2002;294:602–8.

13. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.

14. Weinberg RA. The molecular basis of carcinogenesis: understanding the cell cycle clock. Cytokines Mol Ther 1996;2:105–10.

15. Weinberg RA. How cancer arises. Sci Am 1996;275:62–70.

16. Dickson RB, Lippman J. Oncogenes, suppressor genes, and signal transduction. In: Harris AL, Lippman J, Morroow M, Osborne CK, editors. Disease of the breast. 2nd ed. New York: LW&W; 2000. p. 281–317.

17. Carpenter G, Cohen S. Epidermal growth factor. J Biol Chem 1990;265:7709–12.

18. Cao H, Lei ZM, Rao CV. Transcriptional and posttranscriptional mechanisms in epidermal growth factor regulation of human chorionic gonadotropin (hCG) subunits and hCG receptor gene expression in human choriocarcinoma cells. Endocrinology 1994;135:962–70.

19. Earp HS, Dawson TL, Li X, Yu H. Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. Breast Cancer Res Treat 1995;35:115–32.

20. Riese DJ, Komurasaki T, Plowman GD, Stern DF. Activation of ErbB4 by the bifunctional epidermal growth factor family hormone epiregulin is regulated by ErbB2. J Biol Chem 1998;273:11288–94.

21. Riese DJ, Stern DF. Specificity within the EGF family/ErbB receptor family signaling network. Bioessays 1998;20:41–8.

22. Verber N, Prevost G, Planchon P, Starzec A. Evidence for growth effect of epidermal growth factor on MDA-MB-231 breast cancer cells. Eur J Cancer 1994;30A:1352–9.

23. Levin ER. Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol 2003;17:309–17.

24. Vignon F, Bouton MM, Rochefort H. Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens. Biochem Biophys Res Commun 1987;146:1502–8.

25. Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA. Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. Mol Endocrinol 1993;7:992–8.

26. Apostolakis EM, Garai J, Lohmann JE, Clark JH, O'Malley BW. Epidermal growth factor activates reproductive behavior independent of ovarian steroids in female rodents. Mol Endocrinol 2000;14:1086–98.

27. Ignar-Trowbridge DM, Nelson KG, Bidwell MC, et al. Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. Proc Natl Acad Sci U S A 1992;89:4658–62.

28. Lannigan DA. Estrogen receptor phosphorylation. Steroids 2003;68:1–9.

29. Mahabeleshwar GH, Das R, Kundu GC. Tyrosine kinase, p56lck-induced cell motility, and urokinase-type plasminogen activator secretion involve activation of epidermal growth factor receptor/extracellular signal regulated kinase pathways. J Biol Chem 2004;279:9733–42.

30. Olszewska-Pazdrak B, Ives KL, Park J, Townsend CM Jr, Hellmich MR. Epidermal growth factor potentiates cholecystokinin/gastrin receptor-mediated Ca^{2+} release by activation of mitogen-activated protein kinases. J Biol Chem 2004;279:1853–60.

31. Sasaki E, Tominaga K, Watanabe T, et al. COX-2 is essential for EGF induction of cell proliferation in gastric RGM1 cells. Dig Dis Sci 2003;48:2257–62.

32. Iwabu A, Smith K, Allen FD, Lauffenburger DA, Wells A. Epidermal growth factor induces fibroblast contractility and motility via a protein kinase C δ-dependent pathway. J Biol Chem 2004;279:14551–60.

33. Schiff R, Massarweh SA, Shou J, Bharwani L, Mohsin SK, Osborne CK. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. Clin Cancer Res 2004;10:331–6S.

34. Katzenellenbogen BS, Norman MJ. Multihormonal regulation of the progesterone receptor in MCF-7 human breast cancer cells: interrelationships among insulin/insulin-like growth factor-I, serum, and estrogen. Endocrinology 1990;126:891–8.

35. Marquez DC, Lee J, Lin T, Pietras RJ. Epidermal growth factor receptor and tyrosine phosphorylation of estrogen receptor. Endocrine 2001;16:73–81.

36. Pietras RJ. Interactions between estrogen and growth factor receptors in human breast cancers and the tumor-associated vasculature. Breast J 2003;9:361–73.

37. Lee AV, Cui X, Oesterreich S. Cross-talk among estrogen receptor, epidermal growth factor, and insulin-like growth factor signaling in breast cancer. Clin Cancer Res 2001;7:4429–35s.

38. Sengupta K, Banerjee S, Saxena NK, Jonathan NB, Campbell DR, Banerjee SK. Differential expression of VEGF-A mRNA by 17β-estradiol in breast tumor cells lacking classical ER-α may be mediated through a variant form of ER-α. Mol Cell Biochem 2004;262:215–24.

39. Luqmani YA, Ricketts D, Ryall G, Turnbull L, Law M, Coombes RC. Prediction of response to endocrine therapy in breast cancer using immunocytochemical assays for pS2, oestrogen receptor and progesterone receptor. Int J Cancer 1993;54:619–23.

40. Luqmani YA, Campbell T, Soomro S, Shousha S, Rio MC, Coombes RC. Immunohistochemical localisation of pS2 protein in ductal carcinoma *in situ* and benign lesions of the breast. Br J Cancer 1993;67:749–53.

41. Sheikh MS, Shao ZM, Chen JC, Li XS, Hussain A, Fontana JA. Expression of estrogen receptors in estrogen receptor-negative human breast carcinoma cells: modulation of epidermal growth factor-receptor (EGF-R) and transforming growth factor α (TGF α) gene expression. J Cell Biochem 1994;54:289–98.

42. Chen D, Riedl T, Washbrook E, et al. Activation of estrogen receptor α by S118 phosphorylation involves a ligand-dependent interaction with TFIIH and participation of CDK7. Mol Cell 2000;6:127–37.

43. Le Goff P, Montano MM, Schodin DJ, Katzenellenbogen BS. Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. J Biol Chem 1994;269:4458–66.

44. Denton RR, Koszewski NJ, Notides AC. Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. J Biol Chem 1992;267:7263–8.

45. Joel PB, Traish AM, Lannigan DA. Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase. J Biol Chem 1998;273:13317–23.

46. Mason HR, Lake AC, Wubben JE, Nowak RA, Castellot JJ Jr. The growth arrest-specific gene CCN5 is deficient in human leiomyomas and inhibits the proliferation and motility of cultured human uterine smooth muscle cells. Mol Hum Reprod 2004;10:181–7.

47. Lake AC, Bialik A, Walsh K, Castellot JJ Jr. CCN5 is a growth arrest-specific gene that regulates smooth muscle cell proliferation and motility. Am J Pathol 2003;162:219–31.

48. Tavassoli FA. Pathology of breast. 2nd ed. New York: McGraw-Hill; 1999.

49. Dickson RB, Lippman ME. Growth factors in breast cancer. Endocr Rev 1995;16:559–89.

50. Tovey SM, Witton CJ, Bartlett JM, Stanton PD, Reeves JR, Cooke TG. Outcome and human epidermal growth factor receptor (HER) 1-4 status in invasive breast carcinomas with proliferation indices evaluated by bromodeoxyuridine labelling. Breast Cancer Res 2004;6:R246–51.

51. Wilusz CJ, Wormington M, Peltz SW. The cap-to-tail guide to mRNA turnover. Nat Rev Mol Cell Biol 2001;2:237–46.

52. Okamoto M, Ono M, Uchiumi T, et al. Up-regulation of thrombospondin-1 gene by epidermal growth factor and transforming growth factor β in human cancer cells-transcriptional activation and messenger RNA stabilization. Biochim Biophys Acta 2002;1574:24–34.

53. Zhu Y, Wang M, Lin H, Huang C, Shi X, Luo J. Epidermal growth factor upregulates the transcription of mouse lon homology ATP-dependent protease through extracellular signal-regulated protein kinase- and phosphatidylinositol-3-kinase-dependent pathways. Exp Cell Res 2002;280:97–106.

54. Gilchrist CA, Shull JD. Epidermal growth factor induces prolactin mRNA in

GH4C1 cells via a protein synthesis-dependent pathway. Mol Cell Endocrinol 1993;92:201–6.

55. Chang M, Liu H, Jordan VC. Hormonal and growth factors. In: Donegan WL, Spratt JS, editors. Cancer of the breast. 5th ed. New York: Saunders; 2002. p. 417–41.

56. Bost F, McKay R, Dean N, Mercola D. The JUN kinase/stress-activated protein kinase pathway is required for epidermal growth factor stimulation of growth of human A549 lung carcinoma cells. J Biol Chem 1997;272:33422–9.

57. Kitamura T, Kimura K, Jung BD, et al. Proinsulin C-peptide rapidly stimulates mitogen-activated protein kinases in Swiss 3T3 fibroblasts: requirement of protein kinase C, phosphoinositide 3-kinase and pertussis toxin-sensitive G-protein. Biochem J 2001;355:123–9.

58. Yart A, Laffargue M, Mayeux P, et al. A critical role for phosphoinositide 3-kinase upstream of Gab1 and SHP2 in the activation of ras and mitogen-activated protein kinases by epidermal growth factor. J Biol Chem 2001;276:8856–64.

59. Lopez-Ilasaca M. Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. Biochem Pharmacol 1998;56:269–77.

60. Tong GM, Rajah TT, Zang X, Pento JT. Antiestrogen inhibition of EGF-mediated invasiveness of human breast cancer cells. In Vitro Cell Dev Biol Anim 2001;37:578–80.

61. Chalbos D, Philips A, Galtier F, Rochefort H. Synthetic antiestrogens modulate induction of pS2 and cathepsin-D messenger ribonucleic acid by growth factors and adenosine 3′,5′-monophosphate in MCF7 cells. Endocrinology 1993;133:571–6.

62. Yarden RI, Lauber AH, el Ashry D, Chrysogelos SA. Bimodal regulation of epidermal growth factor receptor by estrogen in breast cancer cells. Endocrinology 1996;137:2739–47.

63. Davidson NE, Gelmann EP, Lippman ME, Dickson RB. Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. Mol Endocrinol 1987;1:216–23.

64. Ahmad T, Farnie G, Bundred NJ, Anderson NG. The mitogenic action of insulin-like growth factor I in normal human mammary epithelial cells requires the epidermal growth factor receptor tyrosine kinase. J Biol Chem 2004;279:1713–9.

65. Ali S, Coombes RC. Estrogen receptor α in human breast cancer: occurrence and significance. J Mammary Gland Biol Neoplasia 2000;5:271–81.

66. Chen D, Washbrook E, Sarwar N, et al. Phosphorylation of human estrogen receptor α at serine 118 by two distinct signal transduction pathways revealed by phosphorylation-specific antisera. Oncogene 2002;21:4921–31.

67. Sako Y, Minoghchi S, Yanagida T. Single-molecule imaging of EGFR signalling on the surface of living cells. Nat Cell Biol 2000;2:168–72.

68. Prenzel N, Zwick E, Leserer M, Ullrich A. Tyrosine kinase signalling in breast cancer. Epidermal growth factor receptor: convergence point for signal integration and diversification. Breast Cancer Res 2000;2:184–90.

69. Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 2000;14:1649–60.

70. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.

71. Sampath D, Winneker RC, Zhang Z. The angiogenic factor Cyr61 is induced by the progestin R5020 and is necessary for mammary adenocarcinoma cell growth. Endocrine 2002;18:147–59.

72. Zajchowski DA, Sager R, Webster L. Estrogen inhibits the growth of estrogen receptor-negative, but not estrogen receptor-positive, human mammary epithelial cells expressing a recombinant estrogen receptor. Cancer Res 1993;53:5004–11.

73. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J 2001;20:6877–88.

74. Banerjee S, Saxena N, Sengupta K, Banerjee SK. 17α-Estradiol-induced VEGF-A expression in rat pituitary tumor cells is mediated through ER independent but PI3K-Akt dependent signaling pathway. Biochem Biophys Res Commun 2003;300:209–15.

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