Keratinocyte Growth Factor (KGF) Induces Tamoxifen (Tam) Resistance in Human Breast Cancer MCF-7 Cells

HSIANG-LIN CHANG[1], YASURO SUGIMOTO[1,2], SULING LIU[3], WEIPING YE[1], LI-SHU WANG[1], YI-WEN HUANG[1] and YOUNG C. LIN[1,2]

[1]*Laboratory of Reproductive and Molecular Endocrinology, College of Veterinary Medicine and*
[2]*The OSU Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210;*
[3]*University of Michigan Comprehensive Cancer Center, Ann Arbor, MI 48105, U.S.A.*

Abstract. *Background: Both estrogen receptor-α (ER-α) and progesterone receptor (PR) are good prognostic factors and indicators of benefit from endocrine therapy in breast cancer patients. The relationship of the ER-α and PR status and the difference in clinical benefit from endocrine therapy in breast cancer is currently unclear. It has been suggested that keratinocyte growth factors (KGFs) are important regulatory factors in breast cancer. Our laboratory has demonstrated that KGF can act as an estromedin for the stimulation of breast cancer cell growth. Also, KGF stimulates aromatase activity in primary cultured human breast cells. This enzyme is a key to the conversion of androgens to estrogens. In the present study, ER-α, two estrogen-regulated genes, PR and PTPγ, KGF and their relationship with endocrine resistance in human breast cancer cells were investigated. Materials and Methods: MCF-7 cells were treated with KGF (1, 5, 10, 20 ng/ml), KGF-13 (0.1, 1, 10 μM) or vehicles as control for 24 hours. KGF-13 is a potential receptor-binding pentapeptide constructed using the KGF peptide residues 101-105 (RTVAV) as a template, located within the beta 4 - beta 5 loop. Total RNA were isolated and real-time PCR was employed to identify ER-α, PR and PTPγ gene expressions in response to KGF and KGF-13. Western blot analysis was used to verify the levels of ER-α and PR protein, whereas immunohistochemical staining was used to detect PTPγ expression in MCF-7 cells. To determine the response of MCF-7 cells to endocrine therapy, MCF-7 was treated with either 20 ng/ml KGF or 10 μM KGF-13 combined with 1, 3 and 5 μM of 4-hydroxytamoxifen (4OH-Tam). A non-radioactive cell proliferation assay was applied to determine the growth rate of MCF-7 cells. The results of real-time PCR and the cell proliferation assay were analyzed by Student's t-test and p-values of less than 0.05 were considered statistically significant. Results: Our data showed that KGF significantly suppressed ER-α, PR and PTPγ expression in MCF-7 cells. KGF suppressed ER-α, PR and PTPγ mRNA to a maximal inhibition at 20 ng/ml by 88%, 57% and 61%, respectively. Western blot analysis and immunohistochemical staining confirmed the down-regulation of ER-α, PR and PTPγ by KGF in protein levels. Ten μM KGF-13 also decreased ER-α expression. Ten μM KGF-13 significantly decreased ER-α, PR and PTPγ mRNA expressions by 51%, 57% and 67%, respectively. These KGF-13-mediated mRNA down-regulations were also observed in protein levels. In a cell proliferation assay, 4OH-Tam (3, 5 μM) induced MCF-7 cell death. KGF and KGF-13 alone did not stimulate MCF-7 cell growth. KGF significantly disrupted 4OH-Tam cell killing effects by 1.2- and 1.3-fold at 4OH-Tam concentrations of 3 μM and 5 μM, respectively. KGF-13 significantly disrupted 4OH-Tam cell killing effects by 1.2- and 1.7-fold at 4OH-Tam concentrations of 3 μM and 5 μM, respectively. Conclusion: Our results suggested that not only ER-α and PR but also PTPγ could be potential bio-makers for growth factor-induced endocrine resistant in breast cancer. KGF might increase the endocrine resistance via decreasing ER-α, PR and PTPγ as well. Moreover, the functional analysis of KGF-13 implied possible applications of using short receptor-binding peptides derived from intact KGF as breast cancer therapeutic agents. Thus, our experimental data provided evidence of KGF-induced anti-hormone resistance in human breast cancer and suggested novel strategies for breast cancer via interference with KGF signaling.*

Correspondence to: Young C. Lin, D.V.M., Ph.D., Laboratory of Reproductive and Molecular Endocrinology, College of Veterinary Medicine, The Ohio State University, 1900 Coffey Road, Columbus, OH 43210-1092, U.S.A. e-mail: lin.15@osu.edu

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The steroid hormone estrogen is an essential factor for the normal human breast and for the development and progression of human breast cancer (1, 2). The effects of estrogens are mediated primarily through interaction with the

estrogen receptor-α (ER-α). Upon estrogen binding, the ER-α becomes hyper-phosphorylated at several serine and tyrosine residues, resulting in dissociation of heat-shock proteins, receptor dimerization and nuclear localization (3). These facilitate ER-α association with estrogen response elements (EREs) or with proteins bound to other responsive elements within promoters of target genes (4). Functional analysis of ER-α has shown it to be a modular protein, with a DNA-binding domain (DBD) flanked by two transcriptional activator functions (AF), AF-1 and AF-2 (5, 6). The activity of AF-2 appears to be largely ligand-enabled, showing relatively strict specificity for estrogens, while AF-1 activity is ligand-independent. Anti-estrogens primarily compete for ER-α and are widely used for the management of estrogen-responsive human breast cancers. Tamoxifen (Tam) is the most frequently prescribed anti-estrogenic agent and is also used in prevention for women at high risk of developing breast cancer (7, 8). Despite the relative safety and the beneficial effects of Tam, many breast cancer patients who initially respond frequently acquire Tam resistance (9).

The progesterone receptor (PR) is an ER-α-regulated gene which requires estrogen and ER-α. PR is synthesized in normal and cancer cells and mediates progesterone's effects in the development of the mammary gland and breast cancer (10). PR is expressed in two isoforms (PR-α and PR-β) from a single gene. Like ER, PR contains a DBD and multiple AFs (11). PR in human breast cancers has recently been reported to be associated with resistance to Tam and the increased risk of breast cancer (12, 13). The increase in breast cancer incidence in women taking both estrogen and progesterone, compared with estrogen alone, emphasizes the importance of progesterone and the PR in breast cancer (14). Both ER-α and PR are good prognostic factors and indicators of benefit from endocrine therapy in breast cancer patients (15). The etiology of the change of ER-α and PR status and the difference in clinical benefit from endocrine therapy in breast cancer is currently unclear. Hypotheses propose that these regulatory mechanisms involve molecules such as ER, PR, growth factor-receptor, and their cross-talk (16-20).

Keratinocyte growth factor (KGF) fibroblast growth factor 7 (FGF7) is one of the FGF family, that comprises over 22 members, such as FGF1 (acidic), FGF2 (basic), FGF3 (int2), FGF4 (hst), FGF5, FGF6, FGF8 (AIGF) and FGF9 (GAF) (21-23). KGF has a stromal origin and appears to act specifically on epithelial cells and is, therefore, exclusively a paracrine growth factor in humans (24, 25). KGF has been detected in normal and cancerous human breast tissues (26-28). Reports have documented the importance of KGF in controlling the growth of mammary epithelium. These include the finding KGF induced the growth of murine mammary epithelial cells in a collagen gel matrix in a heparin-independent manner (29), and that systemic administration of KGF to rodents resulted in mammary epithelial hyperplasia (30, 31). Our laboratory has shown that breast malignant transformation may be associated with a positive feedback stimulation, whereby KGF mRNA expression was elevated by estradiol-17β (E_2), KGF stimulated aromatase mRNA expression and its enzyme activity, which increased the conversion of androgens to estrogens and raised the E_2 level to produce more KGF.

FGFR2IIIb, also known as the KGF receptor (KGFR), belongs to the FGF receptor (FGFR) family which consists of four genes including FGFR-1, FGFR-2, FGFR-3 and FGFR-4. These receptors are glycoproteins with two or three immunoglobulin (I)-like domains, a transmembrane region and a tyrosine kinase catalytic site in the cytoplasm. Alternative splicing of the C-terminal half of the third immunoglobulin-like domain produces the FGFR2IIIb and FGFR2IIIc isoforms and changes the ligand-binding properties of FGFR2 (32-34). FGFR2IIIb is expressed in epithelial cells and binds specifically to KGF (33, 35). The findings that normal mammary epithelial ductal tissue is very sensitive to KGF and the presence of KGFRs in breast cancer are sufficient for KGF stimulation of cell proliferation and motility suggest that KGFR could potentially influence the development and progression of breast cancer (31).

KGF contains ten beta strands to form five double-strand anti-parallel beta sheets (36). The region in the beta 4 - beta 5 loop of KGF contributes to KGFR binding and recognition. Therefore, a potential receptor-binding pentapeptide, KGF-13, was constructed using the KGF peptide residues 101-105 (RTVAV) as a template, located within the beta 4 - beta 5 loop. KGF was also evaluated for its biological activity in breast cancer cells. Small receptor-binding peptides are currently the agents of choice for tumor targeting because peptides have a number of distinct advantages over proteins and antibodies. These advantages include: (i) small size, (ii) easy preparation, (iii) tolerance of harsh conditions of chemical modification, (iv) rapid clearance from blood and non-target tissues, (v) high penetration into tumor tissue, and (vi) a high affinity and specificity for receptors (37).

Protein tyrosine phosphatases (PTPases) are a family of proteins which perform the enzymatic roles of removing phosphate groups from phosphotyrosine residues of specific targets inside cells. PTPases regulate important cellular processes like gene expression, cell activation and proliferation, differentiation, development, transport and locomotion, since they counterbalance the growth-promoting effects of protein tyrosine kinases (PTKs), which catalyze the phosphorylation of tyrosine residues (38). Inhibition of PTPases in cell culture leads to increased amounts of phosphotyrosine-containing proteins and cellular transformation. Therefore, alterations in PTPases expression might promote cell growth, neoplastic processes and

transformation (39, 40). PTPγ is a member of the receptor-like family of PTPases originally cloned from human brain stem and placental cDNA libraries using probes derived from the intracellular domain of CD45 (41) or the *Drosophila* PTPase cDNA clone, DPTP12 (42), respectively. Receptor-like PTPγ has a carbonic anhydrase-homologous amino terminus followed by a fibronectin type three domain, a cysteine-free domain, a transmembrane domain and two intracellular PTPase catalytic domains (15, 43, 44). PTPγ has been implicated as a candidate tumor suppressor gene in kidney, ovarian and lung tumors (45, 46). Our results suggested that PTPγ was a potential estrogen-regulated tumor suppressor gene in human breast cancer that may play an important role in neoplastic processes of human breast epithelium (47). In the human breast, PTPγ was expressed in normal and malignant epithelium. PTPγ mRNA expression was lower in cancerous than in normal breast tissues (48). In whole breast tissues, PTPγ mRNA and protein diminished by E_2 or a non-estrogenic agent with estrogenic action, Zeranol, in the epithelium through an ER-mediated mechanism (49).

Our studies investigated KGF as an anti-hormone resistant factor and the potential use of KGF-13 for anticancer therapy. ER-α, PR and PTPγ genes were used as markers for evaluation in MCF-7 cells. We determined that KGF down-regulated the ER-α, PR and PTPγ expression of the MCF-7 cell line. KGF-13 worked as a KGFR agonist to suppress the expression of ER-α, PR and PTPγ. In addition, KGF and KGF-13 increased the resistance of Tam-induced cell death in MCF-7 cells. KGF down-regulation of ER-α and PR suggested a potential mechanism of the growth factor-induced Tam resistance. The involvement of KGF in the down-regulation of PTPγ mRNA and protein suggested that KGF counterbalanced the growth-inhibition of PTPγ *via* regulation of gene expression. To sum up, our experimental data implicated significant steps of KGF-induced anti-hormone resistance in human breast cancer and suggested novel therapeutic strategies for breast cancer *via* interference with KGF signaling.

Materials and Methods

Cell culture. MCF-7 cells, a human breast cancer cell line, were purchased from the American Type Culture Collection (ATCC). The cells were maintained in a Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (DMEM/F12) mixture, containing no phenol red (Sigma Chemical Co., St. Louis, MO, USA), supplemented with 5% (v/v) fetal bovine serum (FBS) (GIBCO Cell Culture™, Grand Island, NY, USA) and 1 X antibiotic-antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) (GIBCO Cell Culture™). MCF-7 cells were seeded and cultured in 75-cm^2 culture flasks in a humidified incubator (5% CO_2: 95% air, 37˚C). The media were replaced every 2 days. When growth had reached approximately 85% confluence, the cells were washed twice with calcium- and magnesium-free phosphate-buffered saline (PBS, pH 7.3). Adherent cells were removed from the flask surface with 1% trypsin-5.3 mM EDTA (GIBCO Cell Culture™) in PBS for 10 min at 37˚C. The trypsinization was stopped after the addition 5% FBS to the culture medium. The cells were removed and collected by centrifugation, and re-suspended in new culture medium, as described above, and subcultured into 75-cm^2 culture flasks at a ratio of 1:3 of the original cell numbers. Recombinant KGF was purchased from PeproTech Inc. KGF-13, a 5-amino acid peptide sequence (RTVAV), was designed in our laboratory and synthesized by Alpha Diagnostic International (San Antonio, TX, USA). KGF-13 is residues 101-105 of the KGF peptide sequence (NCBI protein data base, accession # P21781).

Cell treatments, total RNA extraction. Before treatments, MCF-7 cells were grown overnight in original culture medium in 24-well culture plates at a density of $2x10^4$ cells/well. The media was replaced by DMEM/F12 supplemented with 0.02% bovine albumin Fraction V (GIBCO Cell Culture™) for 24 h to reduce the effect of the serum. After serum starvation, the MCF-7 cells were treated with either 20 ng/ml KGF, 10 μM KGF-13, 20 ng/ml KGF plus 10 μM KGF-13, or vehicles as the control in DMEM/F12 supplemented with 5% dextran-coated charcoal (DCC) (Dextran T-70; Pharmacia; activated charcoal; Sigma)-treated FBS for 24 h. Total RNA was extracted from the MCF-7 cells by using the Trizol® Reagent (Invitrogen™, Carlsbad, CA, USA).

Relative quantitation of ER-α expression and real-time PCR. The nucleotide sequences of the hybridization probes and primers for the ER-α and 36B4 genes are shown as follows. The ER-α primers were 5'_AGCTCCTCCTCATCCTCTCC_3' (sense), 5'_TCTCCAGCA GCAGGTCATAG_3' (antisense). The 36B4 primers were 5'_CTGGAGACAAAGTGGGAGCC_3' (sense), 5'_ TCGAACAC CTGCTGGATGAC_3' (antisense). The PR primers were 5'_GAA CCAGATGTGATCTATGCAGGA_3'(sense), 5'_CGA AAACC TGGCAATGATTTAGAC_3' (antisense). The PTPγ primers were 5'_GCATCCTCTGCCACATACTACG_3'(sense), 5'_TCATC TTCTGCCAAGCTCTGGT_3'(antisense). All PCR reactions were performed using the SYBR Green I detection chemistry system and detected with the ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). For each PCR run, a master-mix containing 25 μl 2 X SYBR® Green PCR Master Mix (Applied Biosystems), and 5 units/μl of Platinum® *Taq* DNA polymerase (Invitrogen™) was prepared on ice in a total volume of 45 μl. Five μl of each cDNA sample were added to 45 μl of the PCR master-mix. The thermal cycling conditions comprised an initial step at 50˚C for 2 min, 95˚C for 10 min, and 45 cycles at 95˚C for 15 sec and annealing at 60˚C for 1 min.

The comparative C_T method was used for the relative quantitation of ER-α expression. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the TaqMan® probe-amplicon complex formation passes a fixed threshold above baseline. The relative ER-α gene expression level was expressed as:

$$2^{-(\Delta C_T \text{sample} - \Delta C_T \text{control})} = 2^{-(\Delta\,\Delta C_T)}.$$

The ΔC_T was a normalized value of ER-α C_T value to the endogenous control of 36B4 CT value. The results are represented as a percentage of control cells. 36B4 is a cDNA clone for human acidic ribosomal phosphoprotein PO (50, 51).

Immunohistochemical staining. MCF-7 cells were cultured on 24x30-mm cell culture cover-slips (Nalge Nunc Int., Pittsburgh, PA, USA) and washed in PBS three times. After –10˚C methanol fixation for 5 min and air drying, the cells were stained for PTPγ by using VECTASTAIN® Universal Quick Kit and DAB Substrate Kit (Vector Laboratories, Inc., Carpinteria, CA, USA), according to the manufacturer's instructions. The primary antibody was a goat polyclonal IgG C-18 for PTPγ (Santa Cruz Biotech, Santa Cruz, CA, USA). An optimal primary antibody dilution of 1:100 was determined by titration in this system, and omission of the primary antibody served as a negative control.

Western blot analysis. MCF-7 cells were seeded and grown overnight in DMEM/F12 supplemented with 5% FBS in 6-well culture plates at a density of $2x10^4$. The media was replaced with DMEM/F12 supplemented with 0.02% bovine albumin Fraction V (GIBCO Cell Culture™) and grown for 24 h. The MCF-7 cells were treated for 24 h with 20 ng/ml KGF, 10 μM KGF-13, 20 ng/ml KGF plus 10 μM KGF-13 together, or vehicles as control in DMEM/F12 supplemented with 5% DCC-treated FBS. After 24 h, total proteins were extracted from the MCF-7 cells with M-PER® (Pierce Biotechnology, Rockford, IL, USA), according to manual instructions. The protein concentrations were determined by the BCA protein assay kit (Pierce Biotechnology). The lysates were heated to 95-100˚C for 5 min and equal mass amounts of protein were loaded on 4-15% Tris-HCl Ready Gel (BIO-RAD Laboratories, Hercules, CA, USA) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked by immersing the membrane in 5% non-fat milk in PBS containing 0.1% Tween-20 (PBS-T) at 4˚C overnight. The membranes were then incubated in PBS-T for 1 h with the respective primary antibody. A rabbit polyclonal IgG ER-α HC-20 (dilution 1:500) (Santa Cruz Biotech), a mouse monoclonal IgG PR GR18 (dilution 1:1000) (EMD Biosciences, Inc., La Jolla, CA, USA) and a goat polyconal IgG actin C-11 (dilution 1:1000) (Santa Cruz Biotech) were used. After washing in PBS-T, the membranes were incubated with the horseradish peroxidase-linked secondary anti-goat or anti-rabbit immunoglobulin antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) in 5% non-fat dry milk in PBS-T for 1 h at room temperature. After washing in PBS-T, the ER-α, PR and actin proteins were visualized with a chemiluminescent detection system (ECL; Amersham Pharmacia Biotech) and the chemiluminescent image captured on Hyperfilm™ ECL™ (Amersham Pharmacia Biotech).

Cell proliferation assay. Before treatment, MCF-7 cells were grown in DMEM/F12 supplemented with 5% FBS in 96-well culture plates at a density of $1x10^3$ in 100 μl/well overnight. After cells had attached to the wells, the medium was replaced with 100 μl of DMEM/F12 containing 1% of DCC-FBS. The cells were then treated with 4OH-Tam at 1, 3 or 5 μM plus 20 ng/ml of KGF, or vehicles as control in the same fresh medium for 48 h. The experiments were performed in 4 replicates wells for each group. The cell proliferation rate was quantified by using CellTiter 96 AQueous assay (Promega, Madison, WI, USA). Briefly, at the end of treatment, 100 μl of fresh medium with 20 μl of freshly combined MTS/PMS (the ratio of MTS: PMS was 20:1) solution was added to each well. The plates were then incubated for 1.5 h. The intensity of formazan was measured at 490 nm ($OD_{490\ nm}$) by an ELISA plate reader (Molecular Devices Corporation, Menlo Park, CA, USA).

Statistical analysis. The results for the PCR reaction were presented as the mean±standard deviation (S.D.) for three replicates per group. The non-radioactive cell proliferation assay was presented as the mean±S.D for four replicate culture wells per group. Analysis was performed by StatView statistical software for Windows (SAS Institute Inc., Cary, NC, USA). Statistical differences were determined by using the Student's *t*-test for independent groups. *P*-values of less than 0.05 were considered to be statistically significant.

Results

Regulation of ER-α and PR expression by KGF. The presence of estrogen and progesterone receptors predicts the likelihood of benefit from hormonal therapy (52). Estrogen and progesterone receptor-positive breast cancers have a greater chance of responding to hormonal therapy than estrogen and progesterone receptor-negative breast cancers. Reports suggested that growth factors might be associated with decreased PR and ER-α levels in breast cancer patients and cell lines (53, 54). Therefore, we investigated the regulation of ER-α and PR gene expression by KGF. In our study, MCF-7 cells were treated with several concentrations of recombinant KGF (1, 5, 10 or 20 ng/ml) or vehicles as control for 24 h. The total RNA from cells was isolated and ER-α and PR mRNA expressions were measured by real-time PCR. The proteins were also collected and subjected to Western blot analysis. The ER-α and PR proteins were measured on immunoblots by anti-ER-α and anti-PR antibody. β-actin protein served as an internal control. The dependence of ER-α and PR expressions on KGF concentration are presented in Figure 1. The real-time PCR results showed that KGF was able to decrease ER-α and PR mRNA expressions in a dose-dependent manner. The addition of KGF suppressed ER-α and PR mRNA at a dosage of 5 ng/ml and reached maximal inhibition at 20 ng/ml. KGF displayed a significant inhibition of ER-α by 88% and PR mRNA by 43%, respectively at the dose of 20 ng/ml (Figure 1A and B). Also, the ER-α and PR expressions at the protein level were determined by Western blotting assay (Figure 1C). As expected, Western blot analysis revealed that KGF down-regulated ER-α and PR in protein levels in a dose-dependent manner.

Regulation of PTPγ expression by KGF. The balance between PTPases and PTK plays an important role in the regulation of breast cells. Previous data from our laboratory suggest that the overexpression of PTPγ, as a tumor suppressor, inhibits the growth of MCF-7 cells (47). To investigate the regulation of PTPγ expression by KGF, real-time PCR and immunohistochemical staining were used to determine PTPγ

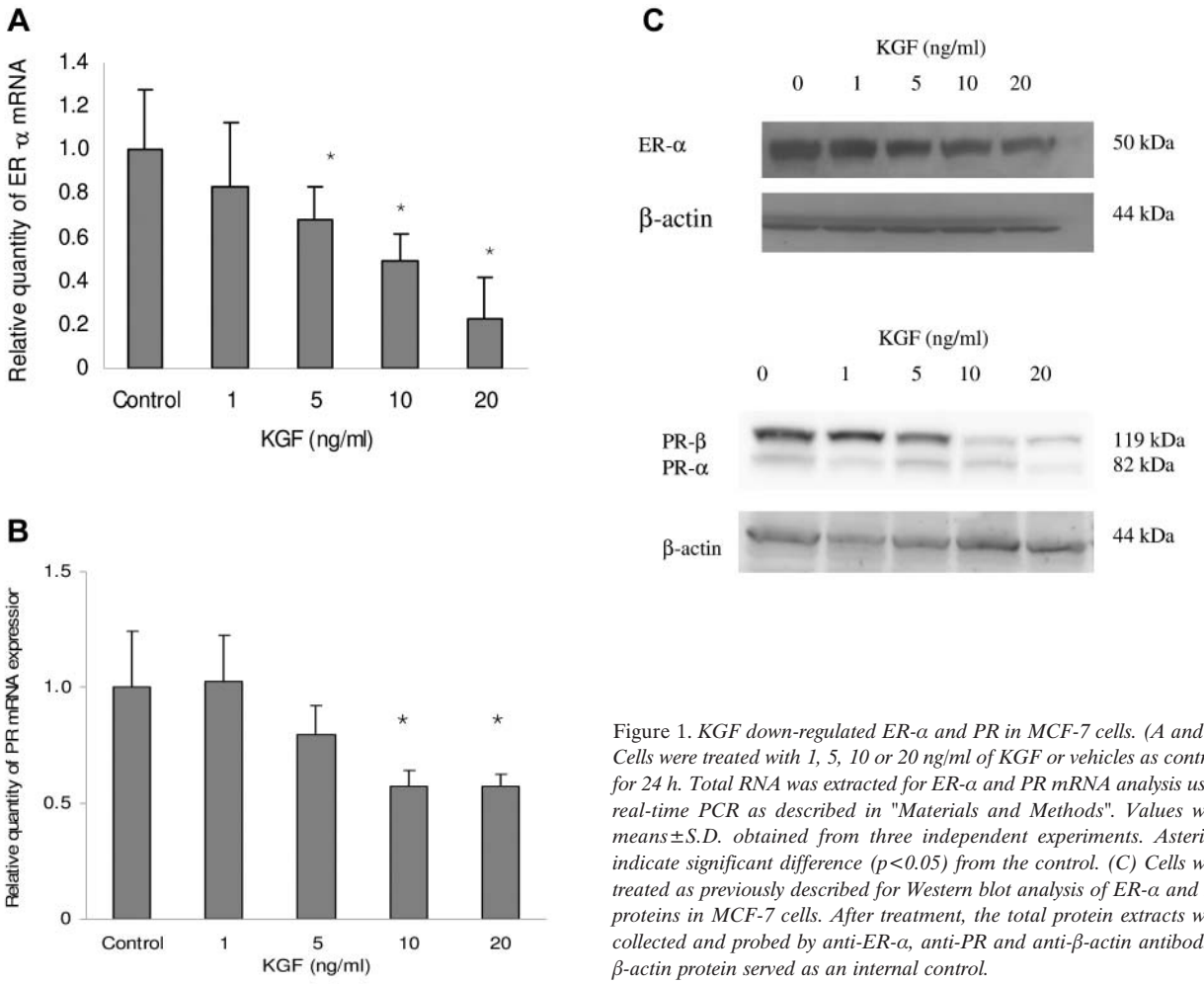

Figure 1. *KGF down-regulated ER-α and PR in MCF-7 cells. (A and B) Cells were treated with 1, 5, 10 or 20 ng/ml of KGF or vehicles as controls for 24 h. Total RNA was extracted for ER-α and PR mRNA analysis using real-time PCR as described in "Materials and Methods". Values were means±S.D. obtained from three independent experiments. Asterisks indicate significant difference ($p<0.05$) from the control. (C) Cells were treated as previously described for Western blot analysis of ER-α and PR proteins in MCF-7 cells. After treatment, the total protein extracts were collected and probed by anti-ER-α, anti-PR and anti-β-actin antibodies. β-actin protein served as an internal control.*

levels in MCF-7 cells. Our data showed that KGF down-regulated PTPγ mRNA expression in the MCF-7 cells. KGF significantly suppressed PTPγ mRNA levels at the dose of 10 and 20 ng/ml by approximately a 57% and 61%, respectively (Figure 2A). The results from immuno-histochemical staining showed that PTPγ staining was observed among MCF-7 cells (Figure 2B). The degree of staining was decreased after 20 ng/ml KGF treatment.

Regulation of ER-α, PR and PTPγ by KGF-13 in MCF-7 cells. A small peptide, KGF-13, was synthesized and evaluated for its potential binding ability to KGFR. KGF-13 was evaluated to determine its role as a KGFR agonist or antagonist for ER-α, PR and PTPγ regulation. MCF-7 cells were treated with KGF-13 at concentrations of 0.1, 1 or 10 μM, or vehicles as control for 24 h. MCF-7 cells treated with 20 ng/ml of KGF were used as a positive control. Real-time PCR results showed that the addition of KGF-13 displayed a significant inhibition of ER-α and PR mRNA by 37% and 51%, respectively (Figure 3A) at a dose of 10 μM. Also, ER-α and PR expressions at the protein level were determined by Western blotting assay. The results further supported ER-α and PR down-regulation by KGF-13 at the protein level (Figure 3D). In addition, it was demonstrated that 10 μM KGF-13 significantly down-regulated PTPγ mRNA by 52% (Figure 3C). Immunohistochemical staining also showed lower PTPγ protein in MCF-7 cells treated with 10 μM KGF-13 than those cells treated with vehicle only (Figure 3E). Our data indicated that the presence of KGF-13 in the medium worked like a KGFR agonist in human breast cancer cells.

KGF and KGF-13 maintained MCF-7 cell survival in 4OH-Tam. Previous data from our laboratory demonstrated that PTPγ was able to inhibit proliferation and anchorage-independent growth of breast cancer cells (47). Furthermore, the down-regulation of ER-α and PR by KGF predicted a better chance of cell survival during endocrine therapy because diminished

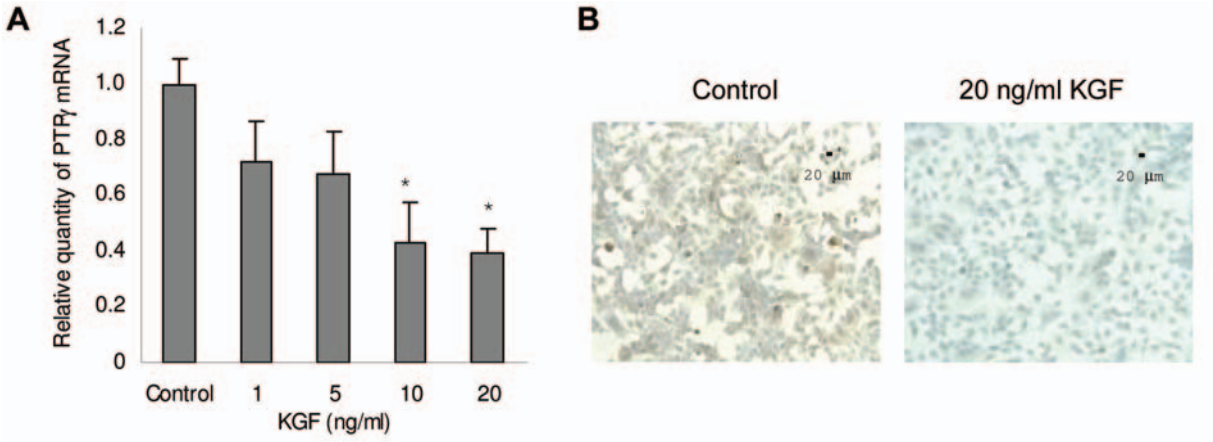

Figure 2. *KGF down-regulated PTP-γ in MCF-7 cells. (A) Cells were treated with 1, 5, 10 or 20 ng/ml of KGF or vehicles as controls for 24 h. Total RNA was extracted for PTPγ mRNA analysis using real-time PCR. Values are means±S.D. obtained from three independent experiments. Asterisks indicate significant difference ($p<0.05$) from the control. (B) Cells were treated with 20 ng/ml KGF or vehicles as control. After treatments, PTPγ protein was analyzed using immunohistochemical staining in MCF-7 cells. Cells were seeded on 24 x 30 mm cell culture cover-slips as previously described. The cells were fixed and stained for PTPγ protein by using VECTASTAIN® Universal Quick Kit and DAB Substrate Kit. Brown staining represents PTPγ immunoreactivity and blue staining represents nuclei.*

ER-α and PR predicted the non response to endocrine therapy. Thus, we hypothesized that the decrease of ER-α, PR and PTPγ expressions by KGF and KGF-13 might make MCF-7 cells less sensitive to endocrine therapy. A cell proliferation assay was performed in order to prove our hypothesis. MCF-7 was treated with either 20 ng/ml KGF or 10 μM KGF-13 combined with several concentrations (1 , 3 and 5 μM) of 4OH-Tam. In our experiment, KGF and KGF-13 alone did not stimulate MCF-7 cell growth. KGF significantly disrupted the 4OH-Tam cell killing effects by 1.2- and 1.3-fold at 4OH-Tam concentrations of 3 μM and 5 μM, respectively (Figure 4A). KGF-13 also significantly disrupted the 4OH-Tam cell killing effects by 1.2- and 1.7-fold at 4OH-Tam concentrations of 3 μM and 5 μM, respectively (Figure 4B).

Discussion

In the present study, we focused on the regulation of ER-α, PR and PTPγ mediated by KGF in breast cancer. We then attempted to correlate the regulation of these molecules with the increase of breast cancer cell survival in the presence of 4OH-Tam. Estrogen and KGF played important roles in the development and progression of breast cancer, although the interactions between estrogen and KGF in breast cancer are not fully clarified yet. Studies have shown quantitative and qualitative changes of ER-α and PR in breast cancer patients with resistance to endocrine therapy. The changes of ER-α and PR levels could be associated with growth factor signaling (55-58). Our laboratory has demonstrated KGF as an estromedin in human breast tissues (28). Besides, the regulation of PTPγ by estrogen was important in the transformation and growth of the human breast (59). This paper provided evidence that KGF down-regulated ER-α, PR and PTPγ expressions in MCF-7 cells. KGF did not stimulate MCF-7 cell growth. However, KGF enhanced the resistance of the estrogen-responsive MCF-7 cells to 4OH-Tam. KGF-13, a small fragment peptide derived from KGF protein, acted as full-length KGF on ER-α, PR and PTPγ modulation. KGF-13 also increased MCF-7 cell survival in the presence of 4OH-Tam.

PTPγ has been suggested as a molecular biomarker for investigating the estrogen and/or non-steroidal estrogenic agents such as Zeranol, in controlling the etiological process of tumorigenesis in human breast. PTPγ has been implicated as a candidate tumor suppressor gene which can be suppressed by estrogens through an ER-α-mediated mechanism (49). Here, we demonstrated that KGF down-regulated PTPγ expression. We propose that the loss of PTPγ in our experiment as a potential biomarker for poor prognosis and endocrine resistance in breast cancers for the following reasons. First, PTPases regulate cellular processes through counterbalancing the growth-promoting effects of PTKs, which catalyze the phosphorylation of tyrosine residues (38). Therefore, the loss of PTPγ suggests the loss of balance between cell growth promotion and inhibition. Second, 4OH-Tam is a partial agonist of ER-α which is able to inhibit the mitogenic activity of growth factors on hormone-responsive MCF-7 cells *via* increasing the activity of membrane-associated PTPases in a ER-α-dependent way (60). Hence, the down-regulation of ER-α and PTPγ in MCF-7 cells may be able to disrupt the anti-estrogenic action of 4OH-Tam *via* both loss of PTPγ and ER-α.

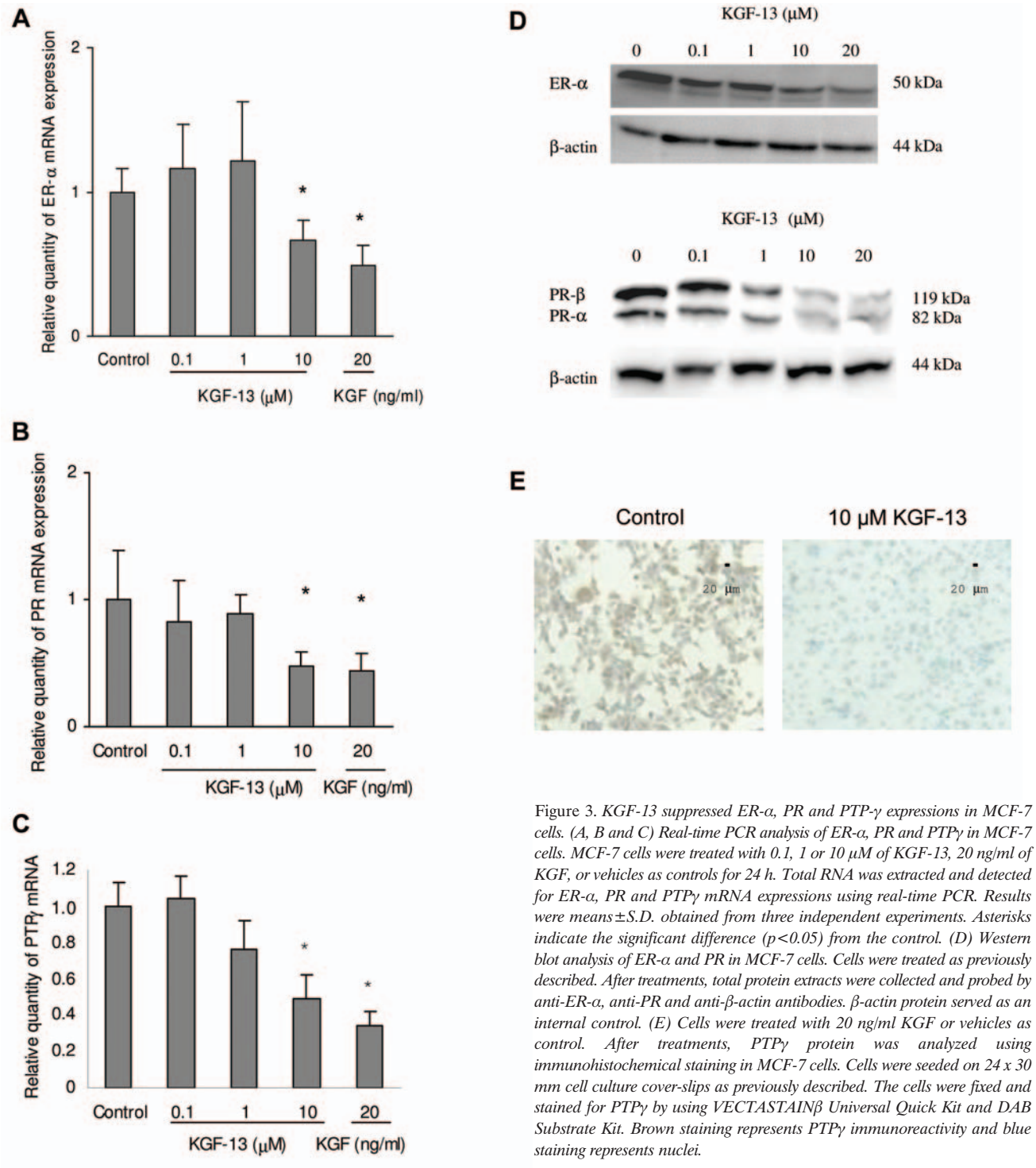

Figure 3. *KGF-13 suppressed ER-α, PR and PTP-γ expressions in MCF-7 cells. (A, B and C) Real-time PCR analysis of ER-α, PR and PTPγ in MCF-7 cells. MCF-7 cells were treated with 0.1, 1 or 10 μM of KGF-13, 20 ng/ml of KGF, or vehicles as controls for 24 h. Total RNA was extracted and detected for ER-α, PR and PTPγ mRNA expressions using real-time PCR. Results were means±S.D. obtained from three independent experiments. Asterisks indicate the significant difference ($p<0.05$) from the control. (D) Western blot analysis of ER-α and PR in MCF-7 cells. Cells were treated as previously described. After treatments, total protein extracts were collected and probed by anti-ER-α, anti-PR and anti-β-actin antibodies. β-actin protein served as an internal control. (E) Cells were treated with 20 ng/ml KGF or vehicles as control. After treatments, PTPγ protein was analyzed using immunohistochemical staining in MCF-7 cells. Cells were seeded on 24 x 30 mm cell culture cover-slips as previously described. The cells were fixed and stained for PTPγ by using VECTASTAINβ Universal Quick Kit and DAB Substrate Kit. Brown staining represents PTPγ immunoreactivity and blue staining represents nuclei.*

KGF produces mitogenic effects on target cells through many signal transduction components. These components include c-Src, Ras and MAPK (61-64). In our study, no stimulation of MCF-7 cell growth by KGF was observed. However, 20 ng/ml KGF prevented 4OH-Tam-suppressed growth (Figure 4A) in MCF-7 cells. This indicates that mechanisms other than growth stimulation were involved. One possible mechanism is the regulation of ER-α and PR expressions by KGF. Both ER-α and PR were prognostic factors in breast cancers. Estrogen and progesterone

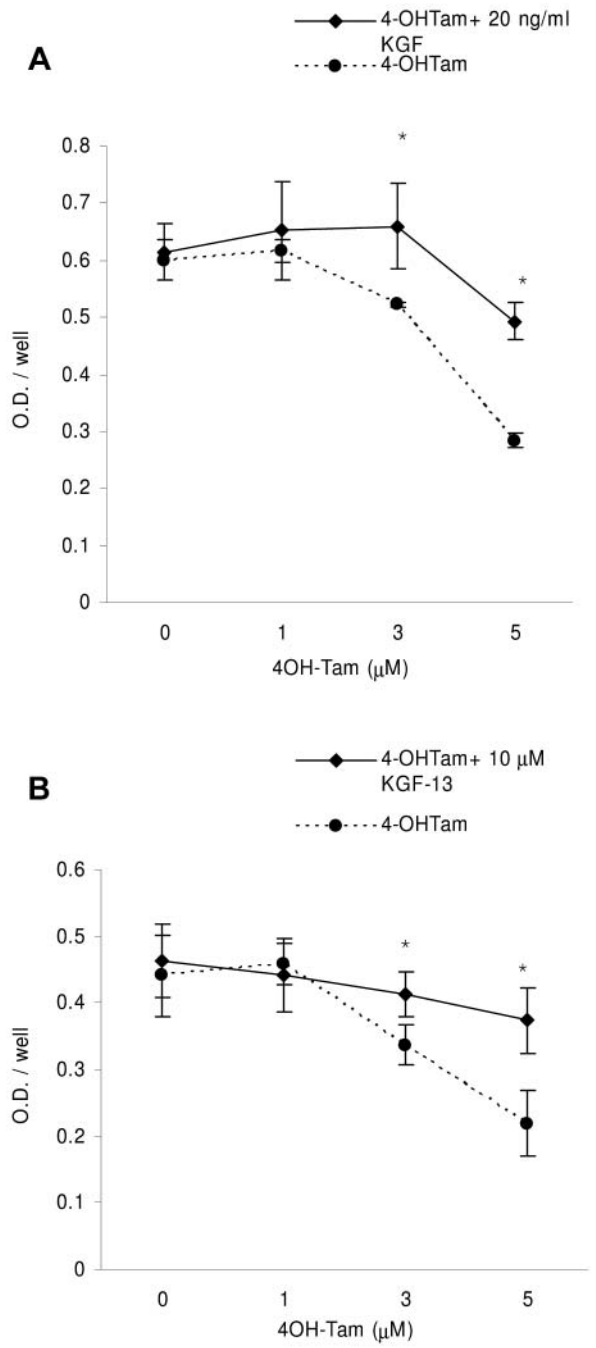

Figure 4. *KGF and KGF-13 protect MCF-7 cells from 4OH-Tam-induced cell killing of MCF-7 cells. 1000 cells/ well were seeded in 96-well plates in DMEM/ F12 supplemented with 5% FBS and left overnight. The cells were then treated with 4OH-Tam at 1, 3 or 5 μM, or vehicles as control in the presence or absence of KGF (20 ng/ml) (A) or KGF-13 (10 μM) (B) in phenol red-free DMEM/ F12 with DCC FBS (1%) for 2 days. The cell proliferation rate was determined by CellTiter™ AQueous assay and optical density was measured at 490 nm by an ELISA plate reader. The results represent the mean value±S.D of four replicate culture wells. Asterisks represent significant difference ($p<0.05$) from the control group.*

receptor-positive breast cancers are more likely to be highly differentiated and less malignant than estrogen and progesterone receptor-negative breast cancers (45, 46). The presence of estrogen and progesterone receptors also predicts the possibility of benefit from endocrine therapy (47). Our results showed that KGF down-regulated ER-α and PR expressions and maintained MCF-7 cell survival in the presence of 4OH-Tam. These observations support the idea that loss of ER-α and PR predicts poor response to endocrine therapy. These data also imply that KGF acts as a potential ER-α and PR regulatory factor which leads to the ER-α-negative /PR-negative phenotype and resistance to endocrine therapy in breast cancer cells.

The PR gene is estrogen-regulated through an ER-α-dependent pathway. The phosphorylation and genomic activity of ER-α can be activated by kinases, or growth factors, such as epidermal growth factor and insulin-like growth factor type I (65-68). In the present study, PR was shown to be down-regulated by KGF. Our results suggest an ER-α-independent regulatory mechanism of loss of PR expression because of KGF-induced ER-α down-regulation. However, a growth factor-mediated down-regulation of PR, which is not mediated *via* a reduction of ER levels, is suggested. In the growth factor-mediated PR expression, the down-regulation of PR expression involves the activation of the PI3K/Akt pathway and repression of the PR promoter (44, 60, 69). Therefore, further studies are needed to fully understand how KGF inhibits PR expression.

The molecules that are involved in the KGF-induced down-regulation of ER-α, PR and PTPγ expressions and increase of 4OH-Tam resistance are not well understood. Growth factors and molecular cross-talk between ER-α, PR and growth factor-receptor signaling provides possible explanations for the reduced Tam sensitivity in MCF-7 cells with low ER-α and PR expressions. PI3K/Akt signaling, which is related to the protection of breast cancer cells from Tam (70-72), has been implied as an important pathway for modulation of PR and ER expressions and Tam resistance (19, 58). In addition, the cytoprotective activity of FGFs has been associated with various signaling components, such as (RAS)/mitogen-activated protein kinase, protein kinase C and phosphatidylinositol 3-kinase (73-75), in which apoptosis-related factors could be involved, such as Fas/FasL/caspase-3, Bax and Bcl-2 in a variety of systems (76-79). These studies define a molecular link between activation of growth factor signaling, activation of ER-α and inhibition of Tam-induced growth arrest or regression. Based on these results, we believe that KGF may activate KGFR and down-stream signaling pathways to regulate ER-α and PR expressions, leading to alterations of cell properties, such as Tam resistance. The investigation of signaling transduction components involved in KGF

modulation of ER-α and Tam resistance in MCF-7 cells are in progress in our laboratory.

Peptides have a number of distinct advantages over proteins and antibodies. These include: small size, easy preparation, high penetration into tumor tissue and a high affinity and specificity for receptors. A short peptide, KGF-13, was synthesized for our experiment. This 5 amino acid length peptide is derived from KGF protein and locates in a loop between β strand 4 and 5 of the KGF protein structure. KGF-13 shares no amino acid sequence with the FGF family except KGF (NCBI Protein Blast). In KGF protein, the peptide sequences responsible for receptor binding and biological function activation may locate in different sequence sites (80, 81). Peptides within this region may be related to the binding activity of KGF to its receptors (80). In our study, KGF-13 functioned as an agonist of KGFR in terms of down-regulating ER-α, PR and PTPγ. These results suggest that KGF-13 may not only bind to receptors, but also have functional action. KGF-13 is a short peptide of only 5 amino acids. Therefore, we cannot exclude the possibility of non-specific binding of KGF-13 to KGFR. Thus, it is necessary to further investigate the binding ability of KGF-13 on KGFR for regulation of breast cancer cells. In addition, our data showed that KGF-13 was less potent than the parent peptide based on the observation that the concentration of KGF-13 for activity induction was relatively high (10 μM) compared with full length KGF (0.5 nM). This is probably because of the shorter biological half-life of KGF-13 and the non-specific binding of KGF-13. In our study, KGF-13 did not act as a KGFR antagonist; nevertheless, our results shed light on the possibility of using KGF-13 as a template to develop KGFR antagonists for breast cancer therapeutic agents in the future.

In the human breast micro-environment, the growth and progression of human breast cancer cells may be closely regulated by steroid hormones, growth factors, their receptors, as well as their downstream targets. We have established that KGF increased aromatase activity (82) and E_2 up-regulated KGF mRNA expression in breast cells (28). KGFR gene up-regulation has been reported in primary human breast tumor specimens (83). In this paper, we report that KGF, at nanomolar concentrations, modulated ER-α gene expression. We also found that KGF increased the resistance of MCF-7 to 4OH-Tam. Our results predict that the KGFR and its ligand, KGF, play the important roles in the development of anti-hormone resistance in breast cancers. To sum up, the positive feedback regulation between E_2 and KGF in breast tissues, KGF-induced 4OH-Tam resistance and ER-α down-regulation may be molecular cascade events which lead breast cancer cells to become estrogen-insensitive during breast cancer progression. Interruption of the KGF/KGFR signal transduction pathways may enhance the effect of anti-estrogens and prevent estrogenic insensitivity on breast cancer cell progression.

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