KERATINOCYTE GROWTH FACTOR AS A SURVIVAL FACTOR IN HUMAN BREAST CANCER

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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2005

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ABSTRACT

Breast cancer is the most common cancer among women and is second leading primary cause of cancer deaths in women in United States. Estrogens are considered to play a central role in human breast carcinogenesis, among the endocrine factors associated with breast cancer. In estrogen-sensitive breast cancer cells, it is widely believed that locally and distally produced growth factors engage intracellular signaling pathways that productively cross-talk with estrogen receptor (ER) and its signaling elements to facilitate tumor cell growth. The inappropriate activation of growth factor signaling cascades, either through an enhanced supply of growth factor ligands, or via up-regulation and increased activation of their target growth factor receptors or their recruited downstream signaling elements, can readily promote anti-hormone failure in breast cancer cells by stimulating cell growth and limiting apoptosis.

KGF is a member of fibroblast growth factor family which is stromal origin and appears to act specifically on epithelial cells. KGF receptor (KGFR), which is a receptor tyrosine kinase belongs to the FGFR family. Our group has shown that KGF stimulates breast cancer cell growth. KGF also increases aromatase activity in primary cultured human breast cells. However, the mechanisms underlying the regulation of breast cancer by KGF is not well defined.
In our study, we found that KGF enhanced breast cancer cell survival through the down-regulation of ER-α expression. Our results demonstrated that KGF could decrease ER-α without changing the mRNA expression of progesterone receptor (PR) and pS2, which indicated that KGF did not bear estrogenic activity. In a cell proliferation assay, tamoxifen (Tam) could induce MCF-7 cell death which could be blocked by KGF treatment. KGF alone did not stimulate MCF-7 cell growth. These findings lead to the hypothesis that KGF can promote anti-hormone failure through the regulation of apoptosis and the KGF-induced signaling pathways.

Our experiments further proved that that the regulation of ER-α by KGF in MCF-7 cells is via PI3K/Akt pathway. We also identified KGF as an anti-apoptotic factor to maintain breast cancer cell survival. KGF increased Akt phosphorylation and decreased ER-α mRNA expression which could be blocked by LY294002, a patent inhibitor of Akt pathway. KGF treatment also induced anti-apoptosis based on the observation of the suppression of DNA fragmentation. Other evidence to support the role of KGF in anti-apoptotic regulation includes variable increase in the expression of the Bcl-2 and Bcl-xL proteins and the decrease of the active-form caspase-9 protein whereas LY294002 blocked the KGF-induced effects. In the cell proliferation assay, KGF maintains the MCF-7 cell survival in the presence of 4OH-Tam, these effects of KGF could be blocked by LY294002. Our data suggested that KGF is able to promote cell survival and anti-estrogenic failure of human breast cancer cells in vitro. The KGF may function as a potential tumor promoter in regulating the process of tumor growth in human breast.

Collectively, these studies provide insight into the role of KGF in the anti-estrogenic resistance of human breast and the underlying signaling pathway in the promotion of
human breast carcinomas. The investigations not only further our understanding of human breast cancer biology but also provide rationales for the development of therapeutic approaches targeting KGF/KGFR signaling as a breast cancer treatment.
ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Young C. Lin. Dr. Lin provides the PhD opportunity plus invaluable scientific guidance, support and mentorship for over the past five years. He has been a constant source of encouragement, advice and financial support for my study and research.

I also would like to appreciate my PhD committee members Dr. Charles Brooks, Yasuko Rikihisa, and Pui-kai Li who advice and direct me throughout my graduate studies.

I would like to show my appreciation to Drs. Yasuro Sugimoto, Samuel K. Kulp and all members in Dr. Lin’s laboratory as well for their technical support, suggestions and sincere friendship.

At last, many thanks to my families for their endless support.
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<td>A.A.</td>
<td>Amino acid</td>
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<tr>
<td>CD:</td>
<td>Cell doubling</td>
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<tr>
<td>DMEM/F12:</td>
<td>Dulbecco’s Modified Eagle Medium/Ham’s F12</td>
</tr>
<tr>
<td>DBD:</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DCC:</td>
<td>Dextran-coated charcoal</td>
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<td>DMSO:</td>
<td>Dimethylsulphoxide</td>
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<tr>
<td>DNA:</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>E2:</td>
<td>$17\beta$-Estradiol</td>
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<td>ER:</td>
<td>Estrogen receptor</td>
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<td>ERE:</td>
<td>Estrogen-responsive element</td>
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<tr>
<td>FBS:</td>
<td>Fetal Bovine Serum</td>
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<td>GFs:</td>
<td>Growth factors</td>
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<td>HBEC:</td>
<td>Human breast epithelial cell</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>HSP</td>
<td>Heat-shock protein</td>
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<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>KGF</td>
<td>Keratinocyte growth factor</td>
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<tr>
<td>KGFR</td>
<td>Keratinocyte growth factor receptor</td>
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<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
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<td>SD</td>
<td>Standard deviation</td>
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Breast cancer is the most common cancer among women except for skin cancer and is second only to lung cancer as the primary cause of cancer deaths in women in United States. Currently, one out of nine American women will develop breast cancer in her lifetime. The rate of breast cancer incidence has increased 2-3% per year over the past decade in both premenopausal and postmenopausal women; however, the mortality rate from breast cancer has not risen as dramatically due to early detection and treatment of breast cancer patients. Every year approximately 200,000 women are diagnosed with breast cancer, of which 40,000 will die from the disease.

The risk factors known to increase the chance of developing breast cancer include gender, age, a family history of breast cancer, an early age at first menstrual period, a late age at menopause, a late age at the time of birth of her first full-term baby, and obesity in
postmenopausal women (figure 1.1). These factors result in changes of genetics and/or local or systemic factors, such as inherited mutations or polymorphism of cancer susceptibility genes, environmental agents that influence the acquisition of somatic genetic changes and several other systemic and local factors, which lead to expression and/or regulation of genes that control normal cellular proliferation and survival. Genes that are altered in cancers can be categorized into two major classes-- proto-oncogenes and tumor suppressor genes. Proto-oncogenes control growth functions such as cell cycle progression via cell signaling. Tumor suppressors maintain cellular homeostasis at many different levels by counter regulation of proto-oncogenes. Upon mutation, proto-oncogenes become oncogenes that act in a dominant-negative manner to permit a disregulation of cell growth. Alteration of tumor suppressor expression and/or regulation can also result in uncontrolled cellular proliferation because of the uncontrolled proto-oncogenes, which can lead to tumor formation. A major obstacle in the cure for cancer is to elucidate the mechanisms by which proto-oncogenes and tumor suppressors regulate and control cellular proliferation.

ESTROGEN AND ESTROGEN RECEPTORS (ER) IN BREAST CANCER

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]. Evidence supporting for the critical role of ovarian estrogens in breast tumorigenesis include the associations
between increased risk of breast cancer and the early onset of menarche, late onset of menopause, and higher age at first birth [3, 4]; as well as the significantly lower frequency at which breast cancer occurs in men and women without functional ovaries during her lifetime when compared to women with intact ovaries [5, 6].

The effects of estrogens are mediated in epithelial cells primarily through interaction with the estrogen receptor (ER) -α. Approximately 70% of all breast cancer patients have hormone-dependent breast cancer, which contains estrogen receptors and requires estrogen for tumor growth. It is believed that estrogens bind with ER to stimulate the cell proliferation rate and thereby increase the number of errors occurring during DNA replication [7]. ER-α is composed of three independent but interacting functional domains: the NH2-terminal or A/B domain, the C or DNA-binding domain, and the D/E/F or ligand-binding domain (figure 1.2).

The mechanism responsible for the ER-mediated pathway has been proposed (figure 1.2). E2 enters the ER-positive cells by diffusion and binds to the ERs in the cells. ER-α becomes hyper-phosphorylated at several serine and tyrosine residues upon estrogen binding [8], and undergoes the conformational changes in structure, dissociation of heat-shock proteins, receptor dimerization, and nuclear localization. These changes facilitate ER-α association with estrogen response elements (EREs) or with proteins bound to other response elements [9] within promoters of target genes. The binding of E2-ER complex to the regulatory elements usually involves a variety of other proteins such as other DNA binding proteins (co-factors) or transcription factors and results in gene activation, i.e., transcription of the gene by RNA polymerase to produce mRNA. The transcribed mRNA in nucleus is translocated to the cytoplasm and translated to
appropriate protein on cytoplasmic ribosomes, which alters cell function, growth, or
differentiation and result in estrogenic effects in breast cells.

A novel member of ERs, termed ER-β, has been identified in cDNA libraries from rat prostate about 10 years after the cloning of the estrogen receptor. The rat ER-β is a protein of 485 amino acids residues with a calculated molecular weight of 54.2 kDa (rat ERα: 595 a.a., 66 kDa). ER-β is highly homologous to ERα, particularly in the DNA-binding domain and in the C-terminal ligand-binding domain. ER-α and ER-β both can bind to estrogen with high affinity. Some functional differences exist between ER-α and ER-β such as aspects of regulation and they may response to some antagonists in different ways due to the lack of structural homology in the amino-terminal domains of these proteins where the activation function-1 (AF-1) resides (Figure 1.2).

ANTI-ESTROGENIC RESISTANCE AND GROWTH FACTORS IN BREAST CANCER

Anti-estrogens primarily compete for ER-α and are widely used for the management of estrogen-responsive human breast cancers. Tamoxifen (Tam), a selective estrogen receptor modulator (SERM), has been widely used in the treatment of breast cancer and reducing the breast cancer incidence in high-risk population [10-12]. Tam has been attributed to promote both growth arrest and apoptosis in breast cancer cells [13]. It is believed that the growth arrest effect of Tam is via down-regulation of estrogen-
regulated growth factors such as epidermal growth factor and transforming growth factor in the ER-α positive cells. The apoptotic effect of Tam has been shown to be mediated by several mechanisms. Tam stimulates caspase-9 and 3 activities in ER-α negative breast cancer cells suggesting that the caspase cascade is a potential regulatory mechanism [14].

The widespread use of Tam contributes to the reduction in breast cancer mortality over the last decade. However, de novo insensitivity and acquired resistance to the selective estrogen receptor modulator tamoxifen severely limit their effectiveness in breast cancer patients. Mechanisms that are proposed to lead to tamoxifen resistance includes changes of ER-α expression, retention of ER-α variants or mutants, and the interactions of signaling pathways between steroid hormones and growth factors. Evidence points the roles of growth factor signaling in the transition of estrogen receptor-positive endocrine-responsive breast cancer cells to anti-estrogen resistant or insensitive status, and the use of anti-growth factor therapies to treat or delay endocrine-resistant states. The network of cross-talk between the ER-α and growth factor signaling pathway may consist of peptide growth factors, tyrosine kinase receptors and down stream signaling proteins such as MAPK and Akt (figure 1.3) [15-17]. The cross-talk between the ER-α and growth factor receptors proceeds in several directions. First, ER-α can generate multiple growth-promoting signals via estrogen-induced genes encoding growth factors, their receptors, and other signaling molecules which can provide cell proliferation and survival stimuli [18, 19]. ER-α can also be phosphorylated by kinase regulated by growth factors, such as MAPK and Akt. The resulting ER-α phosphorylation in the N-terminal region increases gene transcription. Finally,
membrane ER-α can activate growth factor receptor tyrosine kinases, such as members of epidermal growth factor receptor family [20].

KERATINOCYTE GROWTH FACTOR (KGF)

Keratinocyte growth factor/fibroblast growth factor 7 (KGF/FGF7) is the seventh member of the FGF family. FGF family comprises over 22 members, such as FGF1 (acidic), FGF2 (basic), FGF3 (int2), FGF4 (hst), FGF5, FGF6, FGF8 (AIGF) and FGF9 (GAF) [21-23]. FGF family members share high affinity for heparin as well as high sequence homology with the central core domain of 120 amino acids, which interact with fibroblast growth factor receptors (FGFR). KGF is an 18.9 kDa protein containing 163 amino acid residues. KGF has a stromal origin and appears to act specifically on epithelial cells to function as a paracrine growth factor in human [24, 25]. KGF has been detected in human normal and cancerous breast tissue [26-28]. KGF controls the growth of mammary epithelium. These include that KGF induced growth of murine mammary epithelial cells in a collagen gel matrix in a heparin-independent manner [29], and systemic administration of KGF to rodents that results in mammary epithelial hyperplasia [30, 31]. These results also suggest that KGF stimulates the migration of breast cancer cells.
FGFR2IIIb, also known as KGFR, belongs to the FGFR family which are glycoprotein with two or three immunoglobulin (I)-like domains, a transmembrane region and a tyrosine kinase catalytic site in cytoplasm (figure 1.4). Four functional genes identified within the FGFR family including FGFR-1, FGFR-2, FGFR-3, FGFR-4 [32]. Alternative splicing of FGFR gene family increases the functional diversity of FGFR. This process regulates the number (two or three) of Ig G domains and the specific sequence of Ig G domains III.

Alternative splicing of the C-terminal half of the third immunoglobulin-like domain on FGFR2 protein produce FGFR2IIIb and FGFR2IIIc isoforms and changes the ligand-binding properties of FGFR2. FGFR2-IIIb, which is expressed in epithelial cells, binds specifically to KGF. The findings that normal mammary epithelial ductal tissue is very sensitive to KGF, that KGFRs are present in breast cancer and that KGF stimulates cell proliferation and motility, suggest that KGFR may influence the development and progression of cancer. The signal transduction of KGF/KGFR can proceed via Ras/MAPK, and PI3K/Akt pathways [33-38]. The findings on KGF stimulation of cell proliferation and motility via Ras/MAPK suggest that KGF/KGFR could potentially influence the growth viability, and progression of human breast tumors.
Akt, also known as protein kinase B (PKB), is a serine/threonine kinase which can be activated by a variety of stimuli including hormones and growth factors. Three Akt members have been identified in human which includes AKT1, AKT2, and AKT [39]. Studies suggest that the overexpression of Akt protein and deregulation of Akt activity might be involved in breast cancer development [40-42]. All three Akt contains a pleckstrin homology (PH) domain in its amino-terminal region, a central kinase domain, and a regulatory domain in the carboxy-terminal region [43]. The kinase catalytic domain of Akt/PKB is a conserved threonine residue (T308 in PKBa/Akt1) whose phosphorylation can partially activate Akt/PKB [40]. The carboxy-terminal tail contains a second regulatory phosphorylation site (S473 in PKBa/Akt1). Phosphorylation at T308 and S473 occurs in response to extracellular stimuli and is essential for full activation of PKB/Akt. The N-terminal PH domain of Akt/PKB interacts with membrane lipid products such as phosphatidylinositol (3, 4, 5) trisphosphate (PIP3) produced by phosphatidylinositol 3-kinase (PI3K). Akt activation occurs in a phosphoinositide-3 kinase (PI3K) dependent manner. The PIP3, product of PI3K, acts as lipid second messenger. PIP3 facilitates the translocation of Akt and phosphoinositide-dependent kinase (PDK) to the plasma membrane, where the PDK mediates Akt phosphorylation on threonine T308 and serine S473 [40, 44, 45].

The PI3K/Akt pathway has a role in breast cancer. Akt3 mRNA is overexpressed and selectively activated by growth factors in hormone-independent breast and prostate
cancer cell lines. The overexpression and constitutive activation of cell surface receptors are also common in numbers of human breast cancers. For example, primary breast tumors, and transgenic mice all show that overexpressed erbB2 is constitutively associated with erbB3; erbB2–erbB3 dimers strongly activate the PI3K/Akt pathway.

One of the major functions of Akt is to promote growth factor-mediated cell survival by blocking apoptosis. Apoptosis in mammalian cells is a multi-step process involved in the loss of mitochondrial integrity associated with release of cytochrome c into cytoplasm, activation of the apoptotic protease-activating factor (Apaf-1), and the cysteine protease, caspase-9, to initiate a caspase cascade [46]. Members of the Bcl-2 family are regulators in the apoptotic pathway. Family members include anti-apoptotic proteins such as Bcl-2 and Bcl-xL, and pro-apoptotic proteins such as Bad, Bid, and Bik (the BH3 subfamily), and Bax and Bak (the Bax subfamily) [47]. Activation of Akt protein is able to regulate cell survival through phosphorylation of pro-apoptotic proteins such as Bad and phosphorylation of transcriptional factors that are responsible for pro- as well as anti-apoptotic genes.

OVERVIEW OF CHAPTERS 2 AND 3

The next two chapters will investigate the regulation and the function of KGF/KGFR signaling in human breast by using MCF-7 cell line as a model. The reason for choosing MCF-7 cell line is that MCF-7 cells retain the ability to process estradiol and KGF via
cytoplasmic ER-α and KGFR, respectively. Chapter 2 looks at the capability of KGF and its derivates to regulate ER-α expression in human breast and provides evidences that KGF has the ability to enhance human breast survival during anti-estrogenic treatment. Chapter 2 also explores the possibility for using small peptides as potential anti-KGFR antagonists for the breast cancer therapy. Chapters 3 focus primarily on signaling transduction and cell survival induced by KGF in human breast. In chapter 3, we elucidate that KGF can enhance cell survival and down-regulate ER-α through the PI3K/Akt pathway in human breast cancer cells. Then, chapter 3 shows that defines the mechanism of cell survival through KGF stimulation is associated with the suppression of apoptosis. There are specific tight relationships between KGF, PI3K/Akt, breast cancer, anti-apoptosis, which indicate that KGF might play an important role in human breast progression. Together, these studies provide a better understanding of the estrogenic ER-α regulation and function of KGF in the human breast and shed a light on the relationship among estrogen, KGF/KGFR signaling and breast cancer, which suggest that KGF is a potential survival factor in human breast.
Figure 1.1: Summary of the interaction between breast cells and risk factors that interfere with the breast cells in the breast microenvironment during breast carcinogenesis. Risk factors contain diet, genetic alterations, hormones (E2), endocrine disruptors, family history and, systemic factors such as growth factors (GFs).
Figure 1.2: The domains and functions of ER-α and ER-β. The figure shows the comparison of rat (r) ERα and ERβ proteins and percent amino acid homology in the functional regions. The ERs consist of six functional domains (A-F). DBD: DNA binding domain; LBD: ligand binding domain; AF: activation function; HSP: heat-shock protein; NLS: nucleus localization sequence.
Figure 1.3: The E$_2$ action in human breast cells through ER-mediated pathway. ERE: estrogen response element
Figure 1.4: The network of cross-talk between the ER-α and growth factor signaling pathway. The network of cross-talk between the ER-α and growth factor signaling may be consisted of peptide growth factors, their receptor tyrosine kinase and down stream signaling proteins such as MAPK and Akt. The cross-talk can generate multiple growth-promoting signals via estrogen-induced genes encoding growth factors, their receptors, and other signaling molecules which can provide cell proliferations and survival stimuli. RTK: receptor tyrosine kinase; GFs: growth factors
Figure 1.5: The protein structure of a receptor tyrosine kinase- human KGFR. The KGFR contains three immunoglobulin (I)-like domains, a transmembrane region and a tyrosine kinase catalytic site in cytoplasm which are highly alternatively spliced and glycosylated. Alternative splicing of Exons IIIb and IIIc in the second half of the third IgG-like domain for FGFR2 affect ligand-binding specificity and affinity for KGF. Exons IIIb and IIIc in the third IgG-like domain are mutually exclusive of each other.
ABSTRACT

There is increasing evidence to establish links between KGF and breast cancer. We have demonstrated that KGF can independently stimulate breast cancer cell growth without estrogens. Also, KGF stimulates aromatase activity in primary cultured human breast cells. This enzyme is a key to the conversion of androgens to estrogens. However, the relationship between growth factors, such as KGF, and estrogen receptor α (ER-α) is not well defined in the human breast cancer.

Real-time PCR and RT-PCR were employed to identify genes expressed in response to KGF, its 5-amino acid fragment KGF-13, and E2. Western blot analysis was used to verify the level of ER-α protein expression in MCF-7 cells. A non-radioactive cell
proliferation assay was applied to determine the growth rate of MCF-7 cells. The results of real-time PCR and cell proliferation assay were analyzed by student-t test and p-values of less than 0.05 were considered statistically significant.

KGF suppressed ER-α mRNA expression. KGF-13 could also act as a KGF agonist to decrease ER-α expression. KGF and KGF-13 both can decrease ER-α without changing the mRNA expression of progesterone receptor (PR) and pS2. In a cell proliferation assay, tamoxifen (Tam) could induce MCF-7 cell death which could be blocked by KGF treatment.

Our results indicated that KGF and KGF-13 might be linked with the tamoxifen resistance by decreasing ER-α and/or interaction of downstream signaling pathways. These data suggest the role of KGF as a breast cancer promoter and its role to be a new therapeutic target.

INTRODUCTION

The steroid hormone estrogen is an essential factor for the human normal breast, and for the development and progression of human breast cancer [1, 2]. The effects of estrogens are mediated primarily through interaction with the ER-α. Upon estrogen binding, the ER-α becomes hyper-phosphorylated at several serine and tyrosine residues and results in dissociation of heat-shock proteins, receptor dimerization and nuclear localization [8]. These facilitate ER-α association with estrogen response elements
(EREs) or with proteins bound to other responsive element within promoters of target genes [9]. Functional analysis of ER-α has shown it to be a modular protein, with two transcriptional activator functions (AF), AF-1 and AF-2 [48, 49]. 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 is the most frequently prescribed anti-estrogenic agent and is also used in prevention for women at high risk of development breast cancer [10, 50]. Despite the relative safety and the beneficial effects of tamoxifen, many breast cancer patients who initially respond frequently acquire tamoxifen resistance [51].

Mechanisms causing endocrine therapy resistance can be categorized: a reduction of ER-α expression, expression of ER-α variants or mutants, and the steroid hormone pathway being modulated by growth factor signaling pathways. Accumulating evidence points an intricate network of cross-talk between the ER-α and growth factor signaling as an important factor in Tam resistance. The network consist of peptide growth factors and their receptor tyrosine kinase, c-Src, Ras/MAPK, protein phosphatase, cyclin and so on [16, 17, 52-54]. The fibroblast growth factors (FGFs) constitute a large family of ligands that signal through a class of cell-surface tyrosine kinase receptors [55].

Keratinocyte growth factor/fibroblast growth factor 7 (KGF/FGF7) is one of 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 appear to act specifically on epithelial cells and are therefore exclusively paracrine growth factors in human [24, 25]. KGF has been detected in
normal and cancerous human breast tissue [26-28]. Reports previously published have
documented the importance of KGF in controlling the growth of mammary epithelium.
These include that KGF induced the growth of murine mammary epithelial cells in a
collagen gel matrix in a heparin-independent manner [29], and systemic administration of
KGF to rodents resulted in mammary epithelial hyperplasia [30, 31].

FGFR2IIIb, also known as 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 glycoprotein with two or three immunoglobulin (I)-like
domains, a transmembrane region and a tyrosine kinase catalytic site in cytoplasm.
Alternative splicing of the C-terminal half of the third immunoglobulin-like domain
produce FGFR2IIIb and FGFR2IIIc isoforms and changes the ligand-binding properties
of FGFR2 [56-58]. FGFR2-IIIb is expressed in epithelial cells and binds specifically to
KGF [57, 59]. 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 cancer [31].

The stromal-to-epithelial interaction and potential tumorigenesis of KGF/KGFR make
it an attractive model for hormone/growth factor communication in the mammary gland.
Our laboratory has shown that breast malignant transformation may be associated with a
positive feedback stimulation, whereby KGF mRNA expression is elevated by estradiol-
17β (E2). KGF stimulates aromatase mRNA expression and its enzyme activity, which
increase the concersion of andeogens to estrogens and raises the E2 level to produce more
KGF. The purpose of this study is to use MCF-7 human breast cancer cells as a model to
investigate the regulation of ER-α expression and activity by KGF/KGFR signal. We carried out studies to determine that KGF down-regulated ER-α expression of MCF-7 cell line in a does-dependent way. KGF-13, a pentapeptide of the KGF sequence can work as a KGF agonist to suppress the ER-α. The KGF down regulation of ER-α is a mechanism previously not described there the growth factor and estrogen pathways interact with each other. This interaction may be a significant regulatory step in the growth or viability of human breast cancer.

MATERIALS AND METHODS

Cell culture

MCF-7 cells, a human breast cancer cell line, were purchased from the American Type Culture Collection (ATCC). Cells were maintained in 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™) 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-cm² culture flasks in a humidified incubator (5% CO₂: 95% air, 37°C). The media were replaced every 2 days. When growth reached approximately 85% confluence, 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
of culture medium with 5% FBS. Cells were removed and collected by centrifugation,
and re-suspended in new culture medium as described above and subcultured into 75-cm²
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 synthesized by Alpha Diagnostic International (San Antonio, TX). KGF-13 is
residues 101-105 of KGF peptide sequence (NCBI protein data base, accession #
P21781).

Cell treatments, total RNA extraction and reverse transcription-polymerase chain reaction
(RT-PCR)

Before treatments, MCF-7 cells were grown overnight in original culture medium in
24–well culture plates at a density of 2 x 10⁴ cells/well for overnight. The media was
replaced by DMEM/F12 supplemented with 0.02 % bovine albumin Fraction V (GIBCO
Cell Culture™) for 24 hours to reduce the effect of serum. After serum starvation, MCF-
7 cells were treated with either 10 ng/ml KGF, 10 μM KGF-13, 10ng/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 hours. Total RNA was extracted from MCF-7 cells by using the Trizol®
Reagent (Invitrogen™). The procedure of RT-PCR was performed in a gradient
mastercycler (Eppendorf). For cDNA synthesis, 1 μg of total RNA was reverse-
transcribed in a final volume of 50 μl, containing 200 U M-MLV Reverse Transcriptase
(Invitrogen™), 10 μl 5X 1st strand buffer (15 mM MgCl₂, 375μM KCl, 250μM Tris–HCl
pH 8.3), 0.2 mM of dATP, dCTP, dGTP and dTTP, 0.01 M DTT, 1U of RNA Guard Rnase inhibitor (Pharmacia Biotech), and 1 μM random hexamers. These reverse transcription reactions were then incubated at 65 °C for 5 min and 37 °C for 50 min, and heated at 70 °C for 15 min to inactivate reverse transcriptase.

PCR conditions were optimized for MgCl₂ concentration, annealing temperature, and cycle number for the amplification of PCR products. The optimized cycle number within the linear portion of the PCR amplification curve fell between 26 and 39 cycles. The synthesized cDNAs were used as templates for PCR in reagent concentrations of 1.5 mM (pS2), or 3.5 mM MgCl₂ (PR and 36B4), 2.5 μl 10 X PCR Buffer (Invitrogen™), 1 U Platinum Taq DNA polymerase (Invitrogen™), and 0.24 μM primers. The reaction was incubated at 95°C for 5 min. The amplification were performed for thirty-eight cycles (pS2), thirty-three cycles (PR), or thirty cycles (36B4) with denaturation at 95°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1 min in each cycle. The primer sequences pS2 were 5’_TTT GGA GAG AGG AGG CAA TGG_3’ (sense) and 5’-TGG TAT TAG GAT AGA AGC A CC AGG G_3’ (antisense). The primer sequences for PR were 5’-GAT TCAGAA GCC AGC CA G AG-3’ (sense) and 5’-TGC CTC TCG CCT AGT TGA TT-3’ (antisense). The primer sequences for 36B4 were 5’-AAA CTG CTG CCT CAT ATC CG-3’ (sense) and 5’-TTG ATG ATA GAA TGG GGT ACT GAT G-3’ (antisense). The final PCR products (10 μl) mixed with 1 μl of loading buffer were separated on a 1.0 % agarose gel containing ethidium bromide and run in Tris Borate EDTA buffer (TBE) buffer. The specific PCR products were quantified by Alpha Imager (Alpha Inotech Corporation) and the data was analyzed by ImageQuaNT software (Molecular Dynamics). The results were presented as the ratio of each PCR product to
36B4. 36B4 is a cDNA clone for human acidic ribosomal phosphoprotein PO [60], for which mRNA levels have been shown to be unmodified by estradiol treatment [61].

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 and probes are 5’_AGCTCCTCCTCATCCTCTCC_3’ (sense), 5’_TCTCCAGCAGCAGGTCATAG_3’ (antisense) and 5’_TCAGGCACATGAGTAACAAAGGCA_3’ (probe) from TaqMan®. The 36B4 primers and probe are 5’_CTGGAGACAAAGTGGGAGCC_3’ (sense), 5’_TCGAACACCTGCTGGATGAC_3’ (antisense), and 5’_ACGCTGC TGAA CATGCTC AACATCTCC_3’ (probe) from Synthegen.

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 $2^{(\Delta C_T \text{ sample} - \Delta C_T \text{ control})} = 2^{-\Delta C_T}$. The $\Delta C_T$ was a normalized value of ER-α C_T value to the endogenous control of 36B4 C_T value. All PCR reactions were performed using an ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems). For each PCR run, a master-mix contained 5 μl 10 X Real-Time PCR Buffer (Ambion®), 3.5 mM MgCl_2, 0.2 mM dNTP (the mixture of dATP, dCTP, dGTP, and dUTP), 5 μM of each primer, 0.4 mM TaqMan® probe (TaqMan® methodology) and 5 units/μl of Platinum® Taq DNA polymerase (Invitrogen™) was prepared on ice in a total volume of 45 μl. 5 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 minutes, 95 °C for 10 minutes, and 45 cycles at 95 °C for 15 s and annealing at 60 °C for 1 minute.

Western blot analysis

MCF-7 cells were seeded and grown overnight in DMEM/F12 supplemented with 5% fetal bovine serum in 6–well culture plates at a density of 2 x 10^4 for overnight. The media was replaced with DMEM/F12 supplemented with 0.02 % bovine albumin Fraction V (GIBCO) and grown for 24 hours. MCF-7 cells were treated for 24 hours with 10ng/ml KGF, 10 μM KGF-13, 10 ng/ml KGF plus 10 μM KGF-13 together, or vehicles as control in DMEM/F12 supplemented with 5% Dextran-Coated Charcoal (DCC) (Dextran T-70; Pharmacia; activated charcoal; sigma)-treated FBS. After 24 hours, total proteins were extracted from MCF-7 cells with M-PER® (Pierce Biotechnology) according to manual’s instruction. The protein concentrations were determined by the BCA protein assay kit (Pierce Biotechnology). The lysates were heated to 95-100 °C for five minutes and equal mass amounts of protein were loaded on 4 -15% Tris-HCl Ready Gel (BIO-RAD Laboratories) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). 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 one hour with the respective primary antibody. A rabbit polyclonal IgG ER-α HC-20 (dilution 1:500) for ER-α (Santa Cruz Biotech) and a goat polyconal IgG actin C-11 for actin (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) in 5% non-fat dry milk in PBS-T for 1 hour at
room temperature. After washing in PBS-T, the ER-α and actin protein 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% fetal bovine serum in 96–well culture plates at a density of $1 \times 10^3$ in 96-well plates in a volume of 100 μl/well for overnight. After cells were attached to the wells, the medium was replaced with 100 μl of DMEM/F12 containing 1% of DCC FBS. Cells were then treated with tamoxifen at 0, 1.5, 3, 6.25, μM plus 0 or 20 ng/ml of KGF in the same fresh medium for 48 hours. Experiments were performed in 4 replicates wells for each group. Cell proliferation rate was quantified by using CellTiter 96 AQueous assay (Promega). Briefly, at the end of treatment, 100 μl of fresh medium with 20 μl of freshly combined MTS/PMS (the ratio of MTS: PMS is 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) and the relative percentage of survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

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 one group. Analysis was performed by StatView statistical software for Windows (SAS Institute Inc). Statistical
differences were determined by using student-t test for independent groups. *P*-values of less than 0.05 were considered to be statistically significant.

RESULTS

Regulation of ER-α expression by KGF and KGF-13

Previous experiments from our laboratory demonstrate that KGF and E₂ interact with each other [28, 62]. If ER-α expression is increased by KGF, there could be one more additive effect for cell proliferation induced by KGF and E₂. Therefore, we would like to investigate how KGF regulates ER-α gene expression. In our study, MCF-7 cells were treated with several concentrations of recombinant KGF (0-40 ng/ml) for 24 hours. The total RNA from cells was collected and ER-α mRNA expression was measured by real-time PCR. The proteins were also collected and subjected to Western blot analysis. The ER-α proteins were measured on immunoblots by anti-ER-α antibody and actin proteins were served as an internal control. The dependence of ER-α mRNA and protein expression on KGF concentration is presented in Figure 2.1a and 2.1b, respectively. KGF was able to decrease ER-α mRNA expression in a dose dependent manner. KGF significantly suppressed ER-α mRNA starting from the dosage of 20 ng/ml and reaching a maximal inhibition at 40 ng/ml.

KGF-13 was used for this experiment because of its potential binding ability to the KGFR. We would like to determine if this short peptide can act as KGF or as a KGF
antagonist to block the ER-α down regulation by KGF. MCF-7 cells were treated with KGF-13 at the concentration of 0.1, 1 and 10 μM or 24 hours. The 10 ng/ml of KGF were used as a positive control. The ER-α mRNA was measure by real-time PCR. The present of KGF-13 in medium worked like a KGF agonist (Figure 2.2). The addition of KGF-13 displayed a significant inhibition of ER-α mRNA at 10μM.

KGF and KGF-13 modulate ER-α activity

Studies showed that the ER-α activity is up-regulated while ER-α expression is down-regulated through growth factors and their signaling pathway in an E2 deprived environment or the present of E2 alone [15, 16]. To determine whether KGF altered ER-α activity, MCF-7 cells were treated with 10 μM KGF-13, 10 ng/ml KGF, and 20 nM E2 for 24 hours. E2 treatment served as a positive control for its properties of down-regulation of ER-α mRNA expression and up-regulation of ER-α activity. ER-α mRNA was measured by real-time PCR. Two estrogen-regulated genes, PR and pS2, were used as marker for measuring ER-α activity. PR, and pS2 mRNA were measured by RT-PCR. E2 decreased ER-α mRNA expression by ~40%. Similarly, KGF and KGF-13 decreased ER-α mRNA by 37% (Figure 2.3). As E2 induced PR mRNA and pS2 mRNA by over 1.5 fold, KGF did not suppress PR and pS2 significantly. KGF-13 showed similar effects as those of KGF on PR and pS2 mRNA (Figure 2.4 and 2.5).

KGF influences the tamoxifen resistance of MCF-7 cells

It is interesting to know if the ER-α modulation by KGF has any biological action on MCF-7 cells because loss of ER-α has been proposed to be one of the mechanisms for gaining endocrine therapy resistance. Therefore, the decreasing of ER-α expression in
our model may turn MCF-7 cells to be less sensitive to tamoxifen treatment. A cell proliferation assay was performed in order to prove our hypothesis. MCF-7 was treated with KGF at a concentration of 20 ng/ml combined with several concentrations (0-6.25 μM) of 4-hydroxytamoxifen (4OH-tam). In our experiment, KGF 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 3.1 μM and 6.2 μM, respectively (Figure 2.6).

DISCUSSION

Estrogens play important roles in the development and progression of breast cancer partially through binding with ER-α. Studies have shown quantitative and qualitative changes of ER-α in breast cancer patients with resistance to endocrine therapy. In addition, evidence suggests that endocrine resistance could be associated with the growth factors and cross-talk with ER-α signaling pathway [63-65]. This paper provides evidence that KGF can modulate ER-α expression and ER-α activity in MCF-7 cells. Cells treated with 10 ng/ml KGF decreased ER-α at the mRNA and protein level. KGF did not regulate ER-α activity measured by the estrogen-regulated genes, PR and pS2. KGF-13, a small fragment peptide derived from KGF protein, could act as full length of KGF on ER-α modulation. In a cell proliferation assay, KGF did not stimulate MCF-7 cell growth. However, 20 ng/ml of KGF enhanced the resistance of the estrogen-responsive MCF-7 cells to tamoxifen.

Estrogens exert most of their actions through direct activation of gene expression
(genomic action) by binding and activating ER-α. ER-α is activated by phosphorylation by kinase of diverse of growth factors [66-68]. ER-α genomic activity can also be up-regulated by various growth factors, such as epidermal growth factor and insulin-like growth factor type I [19]. In our study, two estrogen-regulated genes, PR and pS2, were measured as indicators of ER-α genomic activity. Both KGF and E2 could decrease ER-α expression in MCF-7 cells. However, E2 stimulated PR and pS2 mRNA expression whereas KGF did not affect PR and pS2 expression much. The reason for these estrogen-regulated genes being induced by E2 but not by KGF could be that KGF was not able to reactivate or boost the ER-α activity to the same level as E2 does while suppressing ER-α expression. Our results suggest that KGF directed breast cancer cells to be less estrogenic responsive mainly by eliminating ER-α expression without stimulating its genomic activity.

KGF and estrogens produce mitogenic effects on target cells through their signaling pathways or cross talk of many signal transduction components between KGF and estrogens. KGF and estrogen are able to activate many of the effectors, including c-Src, Ras, and MAPK, to trigger its mitogenic activity [69-72]. However, we observed no stimulation of MCF-7 cell growth by KGF in a cell proliferation assay. Besides, the cell proliferation assay indicated that MCF-7 cells treated with KGF at 20 ng/ml combined with tamoxifen at 3.12 μM and 6.25 μM could prevent tamoxifen suppressed growth (figure 2.6). We therefore knew that KGF probably lacked the ability to activate ER-α signaling pathway for cell survival. In stead, the down-regulation of ER-α by KGF makes cells less sensitive to endocrine agents, such as tamoxifen. Tamoxifen is a partial agonist of ER-α, which could lead to either a cytostatic (cell growth arrest) or a cytotoxic
(cell death leading to cancer shrinkage and regression in vivo) end points [73]. Hence, the down-regulation of ER-α in MCF-7 cells should be able to disrupt the anti-estrogenic action of tamoxifen. Taken together, KGF blocked the tamoxifen action through down-regulation of ER-α expression.

The mechanism that is involved in the progress of KGF down-regulation of ER-α and up-regulation of tamoxifen resistance is not understood. Growth factors are found to modulate the expression of nuclear receptor, such as PR and ER, and tamoxifen resistance through PI3K/Akt pathway [16, 74]. The PI3K/Akt pathway is related to the protection of breast cancer cells from tamoxifen [34, 75, 76]. In addition, researches find that cytoprotective activity of FGFs has been associated with various pathways, such as (RAS)/mitogen-activated protein kinase, protein kinase C, and phosphatidylinositol 3-kinase [77-79], in which apoptosis-related factors could be involved, such as Fas/FasL/caspase-3, Bax and Bcl-2 in a variety of systems [80-83]. These studies define a molecular link between activation of the growth factor signaling pathways, activation of ER-α, and inhibition of tamoxifen-induced growth arrest or regression. Base on these results, we suspect that KGF may activate KGFR and the down stream signaling pathway to regulate ER-α expression, then, lead to alterations of cell properties, such as tamoxifen resistance. The signaling pathway involved in KGF modulation of ER-α and tamoxifen 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, 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 [84, 85]. Peptides within this region may be related to the binding activity of KGF to its receptors [84]. In our study, KGF-13 functioned as an agonist of KGF in terms of down-regulating ER-α. These results suggest that KGF-13 may not only bind to receptors but also have functional action. KGF-13 is derived from receptor binding region of KGF peptide with small size of only 5 amino acids. Therefore, we cannot exclude the possibility of nonspecific binding of KGF-13 on KGFR. Thus, it is necessary to further investigate the binding region of KGF-13 on KGFR to regulate the cell function. In addition, our data shows KGF-13 was less potent than the parent peptide base on the observation that the concentration of peptides for inducing activity in our assay was relatively high (10 μM) comparing with full length KGF (0.5 nM). This is probability because the biological half life time of KGF-13 is shorter than that of KGF and because of the non-specific binding of KGF-13. In our results, KGF-13 does not act as a KGFR antagonist; nevertheless, our results shed light on the chance by using KGF-13 as a template to develop KGFR antagonists for breast cancer therapeutic agents in the future.

In human breast micro environment, that 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 observed that KGF increased aromatase activity [86], E2 up-regulates KGF mRNA expression in breast cells [28], and KGFR gene up-regulation have been reported in primary human breast tumor specimens
In this paper, we report that KGF, at nanomolar concentrations, could modulate ER-α gene expression. We also found that KGF increases the resistance of MCF-7 to tamoxifen. Our results predict that the KGFR and its ligand, KGF, both play the important roles in the development of anti-hormone resistance in breast cancer. To sum up, the positive feedback regulation between E₂ and KGF in breast tissue, KGF-induced tamoxifen resistance and ER-α down-regulation may be molecular cascade events which lead breast cancer cells to become estrogen insensitive during breast cancer progression. These results may lead to new prognostic and/or therapeutic targets. Interruption of the KGF/KGFR signal transduction pathways may enhance the effect of anti-estrogens and prevent estrogenic insensitivity on breast cancer cell progression.
Figure 2.1: KGF down-regulates ER-α in MCF-7 cells. A. MCF-7 cell were treated with 5, 10, 20, 40 ng/ml of KGF or vehicles as controls for 24 hours. Total RNA was extracted from MCF-7 cells and analyzed by using real-time PCR with the primers described in “Materials and Methods”. Values are means ± S.D. obtained from 3 independent experiments. Asterisks indicate the significant difference (p<0.05) from the control. B. MCF-7 cells were treated as previously described. Total protein extracts from the cells was used for western blot analysis. Immunoblot membrane was probed by using an anti-ER-α and anti-actin antibody. Actin protein is served as an internal control.
A.

![Bar graph showing the relative quantity of ER-α mRNA with KGF (ng/ml) on the x-axis and relative quantity on the y-axis. Significant increases are indicated by * symbols.]

B.

![Western blot images showing ER-α and β-actin.]

Figure 2.1: continued
Figure 2.2: KGF-13 suppresses ER-α mRNA expression in MCF-7 cells. MCF-7 cells were treated with 0.1, 1, 10 μM of KGF-13, 10 ng/ml of KGF, or vehicles as controls for 24 hours. Total RNA was extracted from MCF-7 cells and analyzed by using real-time PCR with the primers described in “Materials and Methods”. Analysis was performed by using the student’s t-test. Values are means ± S.D. obtained from 3 independent experiments. Asterisks indicate the significant difference (p<0.05) from the control.
Figure 2.3: KGF, KGF-13 and E2 down-regulate ER-\(\alpha\) gene expression in MCF-7 cells. MCF-7 cell were seeded in DMEM/F12 without phenol red supplemented with 5% FBS. Then, the medium were replaced with DM EM/F12 and without phenol red supplemented with 5% DCC FBS and cells were treated with 10 ng/ml KGF, 10 \(\mu\)M KGF-13, and 20 nM E2 for 24 hours. At the end of treatment, total RNA was collected. ER-\(\alpha\) mRNA expression was detected by real-time PCR. Analysis was performed by using the student’s t-test. Values are means \(\pm\) S.D. obtained from 3 independent experiments. Asterisks indicate the significant difference (\(p<0.05\)) from the control.
Figure 2.4: The effects of KGF, KGF-13 and E2 on PR gene expression. MCF-7 cells were treated with 10 ng/ml of KGF, 10μM of KGF-13 and 20 nM of E2 in DMEM/F12 supplemented with 5% DCC FBS for 24 hours. Then the total RNA was collected and the expression of PR mRNA was determined by RT-PCR. A. ethidium bromide-stained PCR products were separated in a 1.0% agarose gel. B. the mRNA ratios of pS2 to 36B4 as measured by densitometry. Analysis was performed by using the student’s t-test. Values are means ± S.D. obtained from 3 independent experiments. Asterisks indicate the significant difference (p<0.05) from the control.
Figure 2.5: The effects of KGF, KGF-13 and E2 on pS2 gene expression. MCF-7 cells were treated with 10 ng/ml of KGF, 10 μM KGF-13 and 20 nM E2 in DMEM/F12 supplemented with 5% DCC FBS for 24 hours. Then the total RNA was collected and the expression of pS2 mRNA was determined by RT-PCR. A. ethidium bromide-stained PCR products were separated in a 1.0% agarose gel. B. the mRNA ratios of pS2 to 36B4 as measured by densitometry. Analysis was performed by using the student’s t-test. Values are means ± S.D. obtained from 3 independent experiments. Asterisks indicate the significant difference (p<0.05) from the control.
Figure 2.6: The effects of KGF and 4-hydroxytamoxifen (4OH-tam) on the MCF-7 cell proliferation. 1000 cells/well were seeded in 96-well plates in DMEM/F12 supplemented with 5% FBS for overnight. The medium was changed to phenol red-free DMEM/F12 with dextran charcoal (DCC) FBS (1%) and cells were treated with 4OH-tam at 0, 1.5, 3.12, 6.25 μM respectively in the presence or absence of KGF (20ng/ml). Cell proliferation rate was determined by CellTiter™ AQueous assay and optical density was measured at 490 nm by an ELISA plate reader. Relative fold change of survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment. Results represent the mean value of four independent experiments ± S.D. Asterisks represent the significant difference (p<0.05) from the control group.
Keratinocyte Growth Factor (KGF) regulates estrogen receptor (ER-α) expression and cell apoptosis via phosphatidylinositol 3-kinase (PI3K)/Akt pathway in human breast cancer cells

Abstract

The PI3K/Akt pathway is involved tamoxifen resistance in the ER-α positive breast cancer cell line, MCF-7. Our previous results demonstrate that KGF down-regulates ER-α expression and increases tamoxifen (Tam) resistance in MCF-7 cells. We therefore hypothesize that the mechanism for developing Tam resistance is the reduction of ER-α and Bcl-2 family protein regulation through modulation of Akt activity.
MCF-7 cells were used as model in our experiments. The regulation of ER-\(\alpha\) expression, anti-apoptotic effects, and the signaling pathway induced by KGF were examined. MCF-7 cells were maintained in phenol red-free DMEM/F12 medium supplemented with 5% FBS or 0.02% BSA. Treatments (vehicle, KGF, LY294002, Tam, KGF plus LY294002, KGF plus LY294002, and KGF plus LY294002 plus Tam ) were administered for either 20 minutes or 24 hours. After treatment, total RNA, low molecular weight DNA and protein were isolated and evaluated by real-time quantitative PCR, DNA fragmentation assay, and western blot analysis respectively. The growth rate of MCF-7 cells was measured by a non-radioactive cell proliferation assay.

Our results showed that MCF-7 cells treated with KGF increased Akt phosphorylation and decreased ER-\(\alpha\) mRNA expression. These effects could be blocked by LY294002. KGF treatment also reduced apoptosis based on the observation of the suppression of DNA fragmentation, variable increase in the expression of the Bcl-2 and Bcl-xL proteins and the decrease of the active-form caspase-9 protein. These anti-apoptotic effects were blocked by LY294002. In the cell proliferation assay, KGF maintains the MCF-7 cell survival in the presence of 4OH-Tam which could be blocked by LY294002.

We confirmed that KGF regulated ER-\(\alpha\) expression through PI3K/Akt pathway. We also established that KGF inhibited mitochondria mediated apoptotic pathway through PI3K/Akt pathway. These results suggested that KGF could play as a survival factor to facilitate breast cancer cell progression.
INTRODUCTION

Estrogen exposure has been linked with increased breast cancer risk by extensive epidemiologic literature [87]. In addition, evidence derived from in vitro, in vivo and epidemiologic studies have strongly implicated the steroid hormone estrogen as a carcinogen for the development and progression of breast cancer [5, 88]. The tumorigenic effects of estrogens are mediated primarily through interaction with the estrogen receptor-alpha (ER-α). Estrogen binds to ER-α and activates the protein through phosphorylation, dissociates chaperonin proteins such as heat-shock protein 90, and alters its conformation [8]. Hormone-bound ER-α then dimerizes with another receptor, binds to estrogen response elements (EREs) within promoters of estrogen-regulated genes directly or indirectly. Many of the estrogen-regulated genes are important for cell proliferation, inhibition of apoptosis, stimulation of invasion and metastasis, and promotion of angiogenesis [89]. Motivated by the mechanisms of action of estrogen, antiestrogenic agents are designed to work by their interaction with the ER-α for the management of estrogen responsive breast cancers.

Tamoxifen (Tam), a selective estrogen receptor modulator (SERM) has been widely used in the treatment of breast cancer and reduce breast cancer incidence in high-risk populations [10-12]. Tam influences both growth arrest and apoptosis in breast cancer cells [13]. It is believed that the growth arrest effect of Tam is via down-regulation of estrogen-regulated growth factors such as epidermal growth factor and transforming growth factor in ER-α positive cells. Tam has been implicated to mediate apoptotic
effects via several mechanisms. Tam stimulates caspase-9 and 3, and activities in ER-\(\alpha\) negative breast cancer cells suggest the caspase cascade as a potential regulatory mechanism [46, 90].

In spite of the widespread use of Tam and its significant contribution to the reduction in breast cancer mortality over the last decade, acquired and primary Tam resistance exists in breast cancer patients [91]. Mechanisms which lead to tamoxifen resistance includes changes of ER-\(\alpha\) expression, retention of ER-\(\alpha\) variants or mutants, and the interactions of signaling pathways between steroid hormones and growth factors. Accumulating evidence points an intricate network of cross-talk between the ER-\(\alpha\) and growth factor signaling. The network may consiste of peptide growth factors, their receptor tyrosine kinase, and down stream signaling proteins such as c-Src, Erk1/2, MAPK, protein phosphatase and Akt [16, 17, 54]. The cross-talk between the ER-\(\alpha\) and growth factor receptors occurs in several directions. First, ER-\(\alpha\) can generate multiple growth-promoting signals via estrogen-induced genes encoding growth factors, their receptors, and other signaling molecules which can stimulate cell proliferation and survival [18, 19]. ER-\(\alpha\) can also be phosphorylated by signal proteins such as MAPK and Akt [92]. The resulting ER-\(\alpha\) phosphorylation in the N-terminal region increases gene transcription. Finally, membrane ER-\(\alpha\) can activate growth factor receptor tyrosine kinases, such as members of epidermal growth factor receptor family [20].

Keratinocyte growth factor/fibroblast growth factor 7 (KGF/FGF7) is one of FGF family, which comprises at least 22 members and share high affinity for heparin as well we high sequence homology with the central core domain of 120 amino acids, which interact with fibroblast growth factor receptors (FGFR). KGF has a stromal origin and
appears to act specifically on epithelial cells and is therefore exclusively a paracrine growth factor in human tissues [24, 25]. KGF has been detected in normal and cancerous breast tissue [26, 27]. Reports have documented the importance of KGF in controlling mammary epithelium. Data include that KGF stimulates normal human breast and human breast cancer epithelial cell proliferation in a dose-dependent manner (21). KGF induced the growth of murine mammary epithelial cells in a collagen gel matrix in a heparin-independent manner, and systemic administration of KGF to rodents resulted in mammary epithelial hyperplasia [29-31]. Data suggests that KGF stimulates the migration and of breast cancer cells [36].

FGFR2IIIb, also known as KGF receptor (KGFR) belongs to the FGFR family which are glycoprotein with two or three immunoglobulin (I)-like domains, a transmembrane region and a tyrosine kinase catalytic site in cytoplasm. Four functional genes have been identified within FGFR family including FGFR-1, FGFR-2, FGFR-3, FGFR-4 [32]. Alternative splicing of FGFR gene family has increased the functional diversity of FGFR. This process regulates the number (two or three) of Ig G domains and the specific sequence of Ig G domains III.

Alternative splicing of the C-terminal half of the third immunoglobulin-like domain on FGFR2 protein produce FGFR2IIIb and FGFR2IIIc isoforms and changes the ligand-binding properties of FGFR2 [56-58]. FGFR2-IIIb, which is expressed in epithelial cells, binds specifically to KGF [57, 59]. The signal transduction of KGF/KGFR can proceed via Ras /MAPK, and PI3K/Akt pathways [33, 35-38, 72]. The findings on KGF stimulation of cell proliferation and motility via Ras/MAPK suggest that KGF/KGFR could potentially influence the development and progression of tumor [36, 93].
However, the PI3K /Akt pathway by which KGF and KGFR are involved in human breast cancer is not fully understood yet.

Akt, also known as protein kinase B (PKB), is a serine/threonine kinase (which can be activated by a variety of stimuli including hormones and growth factors). Three Akt members have been identified which includes AKT1, AKT2, and AKT3 in humans [39]. Studies suggest that the overexpression of Akt protein and deregulation of Akt activity might be involved in breast cancer development [40-42]. Each Akt isoform contains a pleckstrin homology (PH) domain in its amino-terminal region, a central kinase domain, and a regulatory domain in the carboxy-terminal region [43]. The catalytic kinase domain of Akt/PKB contains a conserved threonine residue (T308 in PKBa/Akt1) whose phosphorylation can partially activate Akt/PKB activity [40]. The carboxy-terminal tail containing a second regulatory phosphorylation site (S473 in PKBa/Akt1). Phosphorylation at T308 and S473 occurs in response to extracellular stimuli and is essential for full activation of PKB/Akt. The N-terminal PH domain of Akt/PKB interacts with membrane lipid products such as phosphatidylinositol (3,4,5) trisphosphate (PIP3) produced by phosphatidylinositol 3-kinase (PI3K). Akt activation occurs in a phosphoinositide-3 kinase (PI3K) dependent manner. The PIP3, product of PI3K, acts as lipid second messenger for the translocation of Akt and phosphoinositide- dependent kinase (PDK) to the plasma membrane, where the PDK phosphorylates Akt on threonine T308 and serine S473 [40, 44, 45].

One of the major functions of Akt is to promote growth factor-mediated cell survival through apoptosis regulation. Apoptosis in mammalian cells is a multistep process involved in the loss of mitochondrial integrity followed by cytochrome c release,
activation of the apoptotic protease, activating factor (Apaf-1) and the cysteine protease, caspase-9 to initiate a caspase cascade [46]. Members of the Bcl-2 family are regulators in the apoptotic pathway, and include anti-apoptotic proteins such as Bcl-2 and Bcl-xL and pro-apoptotic proteins such as Bad, Bid, and Bik (the BH3 subfamily) and Bax and Bak (the Bax subfamily) [47]. Recent studies have shown that activation of Akt protein is able to regulate cell survival. Akt phosphorylates pro-apoptotic proteins such as Bad and transcriptional factors that are responsible for pro- as well as anti-apoptotic genes [39].

Hormones and growth factors such as estradiol (E2), herregulin beta1 (HRG), Epithelial growth factor, and insulin-like growth factor-I (IGF-I) activate PI 3-K/Akt pathway and regulate ER-α expression in the hormone dependent breast cancer cell line, MCF-7 [46, 94, 95]. Our lab has shown that KGF can regulate ER-α expression and tamoxifen resistance in MCF-7 cells. However, the regulatory pathway for the KGF-induced phenomenon is not known yet. The goal of the present study was to identify the mechanisms for the KGF-induced tamoxifen resistance displayed by ER-α positive tumor. In this paper, we now show that KGF can rapidly activate Akt and modulate ER-α expression and apoptosis in MCF-7 cells. Inhibitors of PI 3-K inhibit the effect of KGF on ER-α expression and apoptosis of MCF-7 cells. Selective inhibitor experiments suggest that the effects of ER-α expression and tamoxifen resistance induced by KGF are Akt mediated and KGF-mediated tamoxifen resistance is via anti-apoptotic regulatory pathway.
MATERIALS AND METHODS

Cell culture

MCF-7 cells, a human breast cancer cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in 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% fetal bovine serum (FBS) (GIBCO Cell Culture™) and antibiotic-antimycotic (100 U/ml penicillin G sodium, 100 \( \mu g \)/ml streptomycin sulfate and 0.25 \( \mu g \)/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. As growing to about 85% confluence, cells were washed twice with calcium- and magnesium-free phosphate-buffered saline (PBS, pH 7.3). Cells were then trypsinized with 1% trypsin-5.3 mM EDTA (GIBCO Cell Culture™) in PBS for 10 min at 37°C. The trypsinization was stopped by addition of culture medium with 5% FBS. Then, cells were centrifuged and resuspended in the original cultured medium as described above and subcultured into 75-cm\(^2\) culture flasks at a ratio of 1 : 3. The recombinant KGF is purchased from PeproTech Inc. LY294002 (LY), an inhibitor of Akt activity was purchased from Cell Signaling Technology Inc. And, 4-hydroxy tamoxifen (4OH-Tam) was purchased from Sigma Aldrich Inc.

Cell treatment, total RNA extraction, and reverse transcription-polymerase chain reaction (RT-PCR)
Before treatment, MCF-7 cells were seeded in DMEM/F12 supplemented with 5% fetal bovine serum in 24–well culture plates at a density of 2 x 10^4 for overnight. The media was changed to DMEM/F12 supplemented with 0.02 % bovine albumin Fraction V (GIBCO Cell Culture™) for 24 hours. Subsequently, MCF-7 cells were treated with KGF, LY, 4-OH-Tam, KGF with LY, KGF with LY and 4-OH Tam or vehicles as control in DMEM/F12 supplemented with 5% Dextran-Coated Charcoal (DCC) (Dextran T-70; Pharmacia; activated charcoal; Sigma Aldrich)-treated FBS for 24 hours. Total RNA was extracted from MCF 7 cells by using the Trizol® Reagent (Invitrogen™). RT-PCR was performed in a gradient mastercycler (Eppendorf, Scientific Inc.). For cDNA synthesis, 1 μg of total RNA from was reverse-transcribed in a final volume of 50 μl containing 200 U M-MLV Reverse Transcriptase (Invitrogen™) 10 μl 5X 1st strand buffer (15 mM MgCl2, 375 mM KCl, 250 mM Tris–HCl pH 8.3), 0.2 mM of dATP, dCTP, dGTP and dTTP, 0.01 M DTT, 1U of RNA Guard Rnase inhibitor (Pharmacia Biotech) and 1 mM random hexamers. Samples were incubated at 65 °C for 5 min and 37 °C for 50 min, and reverse transcriptase was inactivated by heating at 70 °C for 15 minutes.

Relative quantitation of ER-α expression and Real-time PCR

The nucleotide sequences of the hybridization probes and primers for the ER-α and acidic ribosomal phosphoprotein P0 (36B4) genes are shown as follows. The nucleotide sequences of the hybridization probe and primers for the ER-α genes are sense AGCTCCTCCTCATCCTCTCC, anti-sense TCTCCAGCAGCAGGTGTCATAG and probe FAM-TCAGGCA CATGAGTAAC AAAG GCA-TAMRA from TaqMan®. The primer and probe for 36B4 gene are: sense CTGGAGACAAAGTGGGAGCC, anti-sense
TCGAACACCTGCTGGATGAC and probe FAM-ACGCTGCTGAACATGCTCA
ACATCTCC-TAMRA from Synthegen.

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} = 2^{-\left(\Delta C_{T_{\text{sample}}} - \Delta C_{T_{\text{control}}}}\right)\]. The $\Delta C_T$ was a normalized value of ER-α $C_T$ value to the endogenous control of 36B4 $C_T$ value. All PCR reactions were performed using an ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems). For each PCR run, a master-mix in a total of 45 μl was prepared on ice containing 5 μl 10 X Real-Time PCR Buffer (Ambion®), 3.5 mM MgCl$_2$, 0.2 mM dATP, dCTP and dGTP, and dUTP, 5 mM of each primer, 0.4 mM TaqMan® probe (TaqMan® methodology) and 5 units per micro litter of Platinum® Taq DNA polymerase (Invitrogen™). 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 minutes, 95 °C for 10 minutes, and 45 cycles at 95 °C for 15 seconds and annealing at 60 °C for 1 minute.

Western blot analysis

MCF-7 cells were seeded in 6–well culture plates with DMEM/F12 supplemented with 5% fetal bovine serum at a density of $2 \times 10^5$ for overnight. The media was changed to DMEM/F12 supplemented with 0.02 % bovine albumin Fraction V (BSA) (GIBCO®) for 24 hours. Subsequently, MCF-7 cells were treated with KGF, LY, 4-OH-Tam, KGF with LY, KGF with LY and 4-OH Tam or vehicles as control in DMEM/F12
supplemented with 0.02% BSA for 24 hours. Total proteins were extracted from MCF 7 cells with M-PER® (Pierce Biotechnology) according to manual’s instruction. The protein concentrations were determined by the BCA protein assay kit (Pierce Biotechnology). The lysates were heated to 95-100°C for five minutes, loaded on a 4 - 15% Tris-HCl Ready Gel (BIO-RAD laboratories) equal mass amounts of protein for electrophorosis. Then, proteins were transferred onto the polyvinylidene fluoride (PVDF) membrane (Millipore). 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 and incubated for one hour with the primary antibody. Primary antibodies used for immunoblotting were to ER-α HC-20) actin C-11, Bcl-2 (Santa Cruz Biotech), Akt, phospho-Akt, cleaved caspase -9, phospho-Bad, and Bcl-xL (Cell Signaling Technology, Inc.). After washing in PBS-T, the membranes were incubated with the horseradish peroxidase-linked secondary antibody or anti-rabbit immunoglobulin antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hour at room temperature and followed by PBS-T wash. After washing in PBS-T, the proteins were visualized with a chemiluminescent detection system (ECL; Amersham Pharmacia Biotech) and exposed to a cooled CCD camera (Fujifilm Medical System).

Cell proliferation assay

MCF-7 cells were seeded in 96–well culture plates with DMEM/F12 supplemented with 5% fetal bovine serum at a density of 1 x 10³ in 96-well plates in a volume of 100 µl/ well for overnight. After cells were attached to the wells, the medium was replaced with 100 µl of DMEM/F12 containing 1% of DCC FBS. Then, cells were treated with 10
μM 4OH-Tam, 20 ng/ml KGF, 10 μM LY, 4OH-Tam plus KGF, the combination of 4OH-Tam, KGF and LY, and the vehicle as control in the same fresh medium for 48 hours. Experiments were performed in 4 replicates wells for each group. Cell proliferation rate was quantified by using CellTiter 96 AQueous assay (Promega). Briefly, at the end of treatment, 100 μl of fresh medium with 20 μl of freshly combined MTS/PMS (the ratio of MTS: PMS is 20 : 1)) solution was added to each well. Then, the plates were incubated for 1.5 h. The optical density was read at 490 nm (OD$_{490\text{nm}}$) by an ELISA plate reader (Molecular Device) and the relative percentage of survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

Statistical analysis

The results for the PCR reaction were presented as the mean ± S.D. for three replicate as one group. The nonradioactive cell proliferation assay was presented as the mean ± S.D. for four replicate culture wells as one group. Analysis was performed by using StatView statistical software for Windows (SAS Institute Inc.). Statistical differences were determined by using student-t test for independent groups. $P$-values of less than 0.05 were considered to be statistically significant.

RESULTS

KGF stimulated AKT phosphorylation

We investigated the phosphorylation status of Erk1, 2, or, Akt in MCF-7 cells because Erk1, 2, or Akt kinase activities can be triggered by tyrosine kinase signaling of KGF.
In this study, MCF-7 cells were serum-starved for 24 hours. Cells were then treated with KGF for the time courses and doses indicated (figure 3.1A and B). Western blot analysis on activation of Erk 1, 2, and Akt was performed by using an antibody that specially recognizes Erk 1, 2 phosphorylation site at Thr 202/Tyr 204 and Akt phosphorylation site at Ser 473. The cells were lysed and the amounts of total phosphorylated Erk1, 2 and Akt were measured on immunoblots. We used total-Akt, whose expression was not changed by KGF as an internal controls to measure the fold changes of Akt phosphorylation. We found that KGF significantly induced increases of Akt phosphorylation in 2.6 and 2.4 fold at concentration of 10 and 20 ng/ml respectively (Figure 3.1A). The time course of the activation of Akt by 20 ng/ml KGF is presented in Figure 3.1B. KGF induced Akt activation rapidly in 5 minutes and reached to the peak at 15 minutes. Concentrations of KGF that stimulated Akt activations did not trigger the Erk 1, 2 activities (data not shown). To sum up, our western blot analyses showed that KGF rapidly stimulates phosphorylation of Akt but not that of Erk1, 2 in MCF-7 cells.

Akt induction by KGF required PI3K

In order to confirm that phosphorylation of Akt required the activation of PI3K, we evaluated KGF-induced Akt phosphorylation in the presence of the PI3K inhibitor (LY 294002) or the Akt inhibitor (Akt inh), respectively in MCF-7 cells. MCF-7 cells were serum-starved for 24 hours. Subsequently, MCF-7 cells were pretreated under the presence or absence of either the LY294002 (10 μM) or Akt inh (10 μM) for 30 minutes. MCF-7 cells were then treated with 20 ng/ml KGF for 10 minutes. Inhibition of PI3K by LY294002 and Akt inh resulted in a substantial reduction of Akt kinase phosphorylation.
compared to vehicle alone (Figure 3.2). These data provided the evidence of activation of Akt kinase by KGF via a PI3K mediated pathway.

Akt inhibitors blocked KGF-induced ER-α gene expression

Our lab has reported that KGF acted as a survival factor via modulating ER-α gene expression in the breast cancer cell line, MCF-7. To determine the involvement of PI3K/Akt pathway in the KGF-induced ER-α expression, MCF-7 cells were treated with 20 ng/ml KGF for 24 hours in the presence or absence of 10 μM Akt inh. ER-α protein was measured by using the western blot assay and ER-α mRNA expression was determined by Real-Time PCR (Figures 3.3 and 3.4). Our results demonstrated that 20 ng/ml KGF decreased ER-α protein and mRNA by 55%. Akt inh reversed the effect of KGF on ER-α expression (P<0.05) (Figure 3.3 and 3.4). These results demonstrated that KGF regulated ER-α expression through the PI3K/Akt pathway which could lead to the resistance of MCF-7 cell to the 4OH-Tam induced cell killing effect.

PI3K/Akt dependency of KGF protection of MCF-7 cells from apoptosis

Tamoxifen resistance is associated with growth factors and kinase pathways of these factors [8, 96]. Previous results from our laboratory demonstrated that the KGF pathway mediated the effects of Tam on the survival in the MCF-7 cells in a cell proliferation assay. These results implied that KGF may protect cells from 4OH-Tam-induced cell killing. To further determine that the resistance of 4OH-Tam-inducing cell killing after KGF treatment was due to the inactivation of the apoptotic pathway, we investigated the internucleosomal DNA fragmentation, one of the characteristics of cell apoptosis. MCF-7 cells were first treated with three doses of 4OH- Tam (2.5, 5, 10 μM) for either 24 or 48 hours. The appearance of DNA ladder was observed in cells treated with 10, but not 2.5
and 5 μM of 4OH-Tam at 48 hours, while untreated cells did not (Figure 3.5A). We also found that a significant decrease in DNA ladder formation was observed when cells were treated with the combination of 20 ng/ml KGF and 10 μM 4OH-Tam. We next investigated the mechanisms involved in the anti-apoptotic effect of KGF. Studies have shown that KGF induced Akt kinase activity and inhibited apoptosis in A549 lung epithelial cells [33]. Therefore, we would like to know whether Akt kinase was also involved in KGF-induced anti-apoptotic effects in MCF-7 cells. To address this question, we incubated the cells in the presence or absence of Akt inh 30 minutes pretreatment at a concentration of 10 μM. Our results showed that Akt inh abolished the protective effect of KGF in cells exposed to 4OH-Tam (Figure 3.5B). These results implied that KGF contributed to the acquired tamoxifen resistance in breast cancer cells via PI3K/Akt pathway.

Pro- and Anti-apoptotic targets of (KGF/PI3K/Akt) on MCF-7 cells

Akt has direct and indirect effects on the apoptosis pathway via the phosphorylation of the pro-apoptotic Bcl-2 related protein, Bad, at Ser136 to facilitate its dissociation from Bcl-2 and Bcl-xL which lead to the promotion of cell survive. Non-phosphorylated Bad protein forms complexes in the mitochondria with the anti-apoptotic family members Bcl-2 and Bcl-xL to inhibit their function and lead to apoptosis. The phosphorylation of Akt is also able to regulate cell survival through transcriptional factors that are responsible for pro- as well as anti-apoptotic genes. To investigate whether the KGF-induced Akt pathway affects pro- and/or anti-apoptotic proteins in breast cancer cells, MCF-7 cells were treated with serial doses of KGF and various members of the Bcl-2 family were subjected to western blot analysis including anti-apoptotic effectors such as
Bcl-2 and Bcl-xL and their pro-apoptotic counterparts such as Bad, Bax. KGF resulted in variable increase in the expression of the Bcl-2 and Bcl-xL proteins with the exception of Bax protein (Figure 3.6A). KGF also stimulated the phosphorylation of Bad protein with antibody against p-Bad at Ser-136 in the western blot analysis in MCF-7 cells (Figure 3.6B). These effects could be either partially or totally inhibited by Akt inh.

Effects of 4OH-Tam and KGF/PI3K/Akt on the activation of caspase-9 in MCF-7 cells

The involvement of Bcl-2 and Bad implied that induction of anti-apoptosis by KGF could be via the mitochondrial pathway. Therefore, we next examined whether caspase-9, a molecule that plays a role as an initiator caspase in this pathway, was activated. To address this question, we used an antibody that could detect the active-form of caspase-9 protein in MCF-7 cell lysates for western blot analysis. MCF-7 cells were treated with either KGF 20ng/ml, Akt inh 10 μM, 4OH-Tam 10 μM, KGF plus 4OH-Tam together, the combination of KGF, 4OH-Tam and Akt inh, or vehicle for 48 hours before cell collection. Our data demonstrated that 4OH-Tam increased expression of active-form of caspase-9 by 1.7 fold after 2 days of treatment in comparison to control (Figure 3.7). The KGF plus 4OH-Tam treatment decreased the active-form caspase-9 protein to about the same extent as control. The combination of Akt inh with KGF and 4OH-Tam was able to increase the active-form caspase-9 protein which can be down regulated by KGF. Taken together, our data suggested that the inhibitory effects of 4OH-Tam on cell growth could be through the apoptotic signaling pathway which was likely to be mediated via the mitochondrial pathway. KGF could act as a survival factor to block the active-form caspase-9 formation induced by 4OH-Tam to inhibit apoptosis. This anti-apoptotic pathway triggered by KGF was PI3K/Akt mediated.
Involvement of Akt in the survival effect of KGF

It is interesting to know if the ER-α modulation by KGF has any biological action on MCF-7 cells because the down-regulation of ER-α and anti-apoptotic effects induced by KGF/PI3K/Akt could be the possible mechanisms for KGF-induced tamoxifen resistance in breast cancer cells. To demonstrate the role of KGF/PI3K/Akt in the cell survival action against 4OH- Tam in MCF-7 cells, MCF-7 was treated by 20 ng KGF / ml, 10 μM 4OH- Tam, 10 μM Akt inh, or the vehicle for 3 days the non-radioactive cell proliferation assay was used. In figure 3.8, 4OH- Tam decreased 30% of MCF-7 cells compared to control. KGF alone did not stimulate MCF-7 cell growth, however, the combination of KGF and 4OH- Tam maintained the cell survival at the same level as the control. Akt inh alone did not inhibit cell growth significantly. Our data suggested that KGF disrupted the cell killing effect of 4OH- Tam and this effect was regulated via PI3K/Akt pathway.

DISCUSSION

The regulation of ER-α expression and apoptosis is a fundamental feature in the progression of cancer. The role of KGF in these processes in breast cancer is not well documented. Previously, we report that KGF down regulated ER-α expression and up regulated anti estrogenic resistance in MCF-7 cells. To expand our understanding upon these observations, we turned our focus to the PI3K/Akt signaling axis, a known pathway with protective effects that would inhibit cell apoptosis and affect ER-α expression after
being activated. In this experiment we have addressed the role of KGF in the model of anti-estrogen-induced apoptosis in hormone-responsive human breast cancer cells. We determine that KGF quickly induces Akt phosphorylation in a dose-dependent manner in MCF-7 cells. We revealed the suppression of apoptosis by KGF in Tam resistance. In particular, KGF increases Bcl-2 and Bcl-xL expression and triggers the phosphorylation of Bad protein. The activation caspase-9 protein further confirms that the induction of anti-apoptosis by KGF treatment is via the mitochondrial pathway. We also determined that the inhibitory effect on apoptosis provided by KGF and the down regulation of ER-α expression is dependent on activation of PI3K/Akt pathway. These data support our hypothesis that KGF preserves breast cancer cell viability by anti-apoptosis and adjusting ER-α expression via the PI3K/Akt mediated pathway.

Tam has been used in the treatment of breast cancer for over a decade. The primary anti-tumor mechanism of Tam is believed to be through the inhibition of estrogen receptor. Recent studies have implicated the role of caspases in Tam-induced apoptotic signaling. One of the caspases, caspases-9, is intracellular cysteine-containing proteases which are believed to be the terminal executors of apoptosis. Its activation is mediated through the mitochondrial pathway. We showed that 4OH-Tam induced apoptosis on DNA fragmentation assay and increased the active-form of caspase-9 expression in the breast cancer cell line MCF-7. Our data suggest that 4OH-Tam-induced apoptosis may be partially through mitochondrial pathway.

Several studies have shown that growth factors inhibit anti-estrogenic effects on cell proliferation and survival. In many cell lines, the importance of the PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways in cell proliferation and survival
have been demonstrated. In our experiment, KGF did not stimulate MCF-7 cell growth. However, KGF maintained cell survival in the presence of 4OH-Tam. KGF-stimulated the phosphorylation of Akt protein is further supported by our inhibitor experiments. Our results suggest the predominant role of PI3K/Akt pathway for the inhibition of anti-estrogenic action triggered by KGF.

Apoptosis is an important mechanism for removing unwanted cells. The Bcl-2 family is comprised of both anti-apoptotic and pro-apoptotic proteins which have been established to be upstream of the apoptotic mitochondrial pathway. Our experiment use two anti-apoptotic proteins, Bcl-2, Bcl-xL, two pro-apoptotic proteins, Bax and Bad, and caspase-9 to measure the effect of KGF on MCF-7 cell apoptosis. We observe up-regulation of Bcl-2 and Bcl-xL proteins by KGF. We also detected the activation of caspase-9 after 4OH-Tam treatments and the inhibition of 4OH-Tam induced-caspase-9 activity by KGF. The increase in Bcl-2 and Bcl-xL expression and the decrease in the active-form of caspase-9 might be interpreted as an attempt of KGF to protect the breast tumor cells from apoptosis through the apoptotic mitochondrial pathway because it has been suggested by numerous studies that the survival or death of cells are determined by an altered balance between pro-apoptotic and anti-apoptotic proteins such as the ratio of Bcl-2 to Bax [97]. Focusing further on KGFR/PI3K/Akt kinase signaling, its inhibition by LY-294002 resulted in a decrease of Bcl-2 and Bcl-xL expression and an increase of the active of caspase-9 expression. Our observations are in agreement with previous reports [98, 99] to show that Bcl-xL and Bcl-2 are downstream of PI3K/Akt kinase signaling. The down-regulation of these mitochondrial proteins are responsible for the pro-apoptotic response caused by suppression of Akt kinase.
Bad protein is one of the Akt targets with implication for cell survival regulation. Non-phosphorylated Bad protein will inhibit Bcl-2 and other anti-apoptotic Bcl-2 family by direct binding. Phosphorylation of Bad protein neutralizes its pro-apoptosis activity. Our experiments suggest that the phosphorylation of Bad protein is also involved in the survival regulation of MCF-7 cells. Investigation of KGF regulation of ER-α and apoptosis in MCF-7 cells suggests that reduced Tam sensitivity could be caused by the down-regulation of ER-α and the anti-apoptotic effects mediated through the PI3K/Akt pathway. Our data provide evidence of KGF as an important survival factor in the control of tumor survival during breast tumor progression. These results show the potential benefits for developing therapeutic agents targeting the KGF signaling pathway in the treatment of human cancer.
Figure 3.1: KGF stimulates Akt phosphorylation.  A) MCF-7 cells were serum-starved for 24 hours and treated with 10-100 ng/ml KGF for 20 minutes.  B) MCF-7 cells were serum starved for 24 hours and treated with 100 ng/ml for the times indicated. Western blot of cell extracts are probed using anti-phospho-Akt (p-Akt, Ser 473) or total anti-Akt antibody. Total Akt was used as a control for equal loading and transfer. The ratio between phosphorylated and total Akt was determined by densitometry. Results are present as a fold change of control. Results represent the mean value of three independent experiments ± S.D. Asterisks represent the significant difference (p<0.05) from the control group (continued).
Figure 3.1: Continued
Figure 3.1: Continued
Figure 3.2: LY and Akt inh inhibit KGF-induced Akt phosphorylation in MCF-7 cells. Cells were serum starved for 24 hours and treated with 20 ng/ml KGF for 10 minutes in the presence or absence of the 10 μM of LY 294002 or Akt inh 30 minutes pretreatment. Western blot of cell extracts are probed using anti-phospho-Akt (p-Akt, Ser 473) or total anti-Akt antibody. Total Akt was used as a control for equal loading and transfer. The ratio between phosphorylated and total Akt was determined by densitometry. Results are present as a fold change of control. Results represent the mean value of three independent experiments ± S.D. Symbols (* and +) represent the significant difference (p<0.05). “*” compares with control group. “+” compares with KGF group.
Figure 3.3: PI3K inhibitors block KGF induced ER-α gene expression. MCF-7 cell were seeded in DMEM/F12 without phenol red supplemented with 5% FBS. Then, the medium were replaced with DMEM/F12 and without phenol red supplemented with 5% DCC FBS and cells were treated with 10 ng/ml KGF in the present or absence of the 10 μM Akt inh for 24 hours. At the end of treatment, total RNA were collected. ER-α mRNA expression was detected by real-time PCR. Analysis was performed by using the student’s t-test. Results represent the mean value of four independent experiments ± S.D. Symbols (* and +) represent the significant difference (p<0.05). “ * ” compares with control group. “ + ” compares with KGF group.
Figure 3.4: The Akt inhibitor block KGF induced ER-α protein expression. MCF-7 cells were seeded in DMEM/F12 without phenol red supplemented with 5% FBS. Then, the medium were replaced with DMEM/F12 and without phenol red supplemented with 5% DCC FBS and cells were treated with 20 ng/ml KGF in the present or absence of the 10 μM Akt inh for 24 hours. At the end of treatment, total proteins were collected. ER-α protein was detected by western blot. The ratio between ER-α and actin was determined by densitometry. Results are present as a fold change of control. Results represent the mean value of three independent experiments ± S.D. Symbols (* and +) represent the significant difference (p<0.05). “*” compares with control group. “+” compares with KGF group.
Figure 3.5: DNA fragmentation in MCF-7 cells. A): DNA fragmentation in MCF-7 cells after exposure to three concentrations of Tam for either 24 hours or 48 hours in DMEM/F12 medium supplemented with 0.2 % bovine serum albumin. B): DNA fragmentation in MCF-7 cells after exposure to 20 ng/ml KGF, 10 μM 4OH-Tam Akt inh or the combination of KGF and Tam, KGF and Akt inh or KGF plus 4OH-Tam and LY for 48 hours. The genomic DNA was isolated from same number of both untreated and treated cells and separated on a 0.8% agarose gel at 90 volts for 3 hours. The left lane of each graph is a 500 bp DNA ladder.
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Figure 3.5: Continued
Figure 3.6: The effects of KGF on the expression and phosphorylation of anti- and pro-apoptotic proteins. KGF up-regulates Bcl-xL and Bcl-2 protein expression through Akt mediated pathway. MCF-7 cells were serum starved for 24 hours and treated with KGF for the doses indicated or the combination of 20 ng/ml of KGF and 10 μM of Akt inh for 48 hours. Western blot of cell extracts are probed using the indicated antibodies (anti Bcl-xL and Bcl-2 antibodies). Actin was used as a control for equal loading and transfer. The ratio between each target protein and actin was determined by densitometry. Results are present as a fold change of control. Results represent the mean value of three independent experiments ± S.D. Asterisks represent the significant difference (p<0.05) from the control group. Single asterisk compares with Bcl-xL control. Double asterisks compare with Bcl-2 control (continued).
Figure 3.6: Continued.

Figure 3.6: Continued.
Figure 3.6: Continued.
Figure 3.7: Western blot analysis of active-form caspase-9 expression at MW 35kD in MCF-7. MCF-7 cells were serum starved for 24 hours and treated with KGF, KGF plus 4OH-Tam, or the combination of KGF and Akt inh for the doses indicated for 48 hours. Western blot of cell extracts are probed using the indicated antibodies. 4OH-Tam increase active-form caspase-9 by 1.7 fold respectively after 2 days of treatment. KGF inhibits 4OH-Tam-induced expression of active-form of caspase-9. The Akt inhibitor blocks the effect of KGF on Tam treatment. Actin was used as a control for equal loading and transfer. The ratio between active-form caspase-9 and actin was determined by densitometry and results are represented as a fold change of control. Results represent the mean value of three independent experiments ± S.D. Symbols (* and +) represent the significant difference (p<0.05). “ * ” compares with control group. “ + ” compares with KGF+ 4OH-Tam.
Figure 3.8: The effect of KGF, Akt inh and 4OH-Tam in cell proliferation assay. 1000 cells/well were seeded in 96-well plates in DMEM/ F12 supplemented with 5% FBS for overnight. The medium was changed to phenol red-free DMEM/ F12 with dextran charcoal (DCC) FBS (1%) and cells were treated with 4OH-tam at 10 μM respectively in the presence or absence of either 20 ng/ml KGF or Akt inh 10 μM. Cell proliferation rate was determined by CellTiter™ AQueous assay and optical density was measured at 490 nm by an ELISA plate reader. Relative fold change of survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment. Results represent the mean value of four independent experiments ± S.D. Asterisks represent the significant difference (p<0.05) from the control group.
KGF and anti-hormone resistance in human breast

Estrogens exert most of their actions by binding that activated gene expression (genomic action). In estrogen-sensitive breast cancer cells, it is believed that growth factors signaling pathways that cross-talk with estrogen receptor (ER) signaling elements facilitate tumor cell growth, with anti-estrogenic drugs acting to disrupt such events and promote growth inhibition. Growth factor pathways can enhance ER phosphorylation in a ligand-independent manner. ER-α transcriptional activity and cell growth can also be up-regulated by various growth factors, such as epidermal growth factor and insulin-like
growth factor type I. Paradoxically, a decline in ER expression is also a possible outcome when growth factor signaling is sustained (Gee et al. 2004).

In our study, we have shown that challenge of MCF-7 cells with KGF is able to generate cells’ refractory to the growth-inhibitory effects of tamoxifen suggesting that growth factor signaling is uncoupled from ER signaling. Two estrogen-regulated genes, PR and pS2, were measured as indicators of ER-α genomic activity. Both KGF and E2 could decrease ER-α expression in MCF-7 cells. However, E2 stimulated PR and pS2 mRNA expression whereas KGF did not affect PR and pS2 expression significantly. These observations probably involve ER turn over rate, ligand binding of ER, and cross-talk between ER and growth factor receptor signaling pathways. The reason for the expressions of estrogen-regulated genes responding differently by E2 and by KGF is not clear yet. Our results suggest that KGF directed breast cancer cells to be less estrogenic responsiveness by eliminating ER-α expression. These findings provide a putative link between KGF and risk of anti-hormone resistance in human breast cancer. Our concern is that the production of KGF in the tissue surrounding the breast cancer lesion may influence the development of hormone insensitivity in human breast cancer.

KGF activate Akt phosphorylation in human breast

Keratinocyte growth factor (KGF) (FGF-7) is primarily produced by stromal-derived cells in many tissues and acts as an epithelial cell-specific mitogen. In particular, KGF mediates mesenchymal-epithelial interactions in many tissues, including the ovary, placenta, endometrium, and seminal vesicle. KGF is unique because it interacts only through the KGF receptor (KGFR, also known as FGFR2IIIb), which is expressed exclusively by epithelial cells, indicating that it may have target cell specificity.
Our previous results indicated an intriguing relationship between breast cancer and KGF. We speculate that KGF is a survival factor that possesses cancer promoter activity in breast. Therefore, defining the mechanism(s) of KGF/KGFR signaling and elucidating its role in breast cancer progression is important. Although KGF expression in the breast has been reported, the mechanism of intracellular signaling of the KGF and KGFR system has not been investigated. MAPK and PI3K have been indicated as the down stream pathways triggered by KGF and involved in the proliferation and differentiation of some cells. We investigated possible pathways for KGF stimulation. Our data indicated a phosphorylation of Akt induced by KGF in MCF-7 cells. KGF recognizes only KGFR with high affinity, precluding the possibility that Akt is activated by other members of the FGFR family. The activation of Akt is associated with the stimulation of cell proliferation, motility, or anti-apoptosis via transcription factor activation in the nucleus. Therefore, we hypothesize that there must be some transcriptional factors responsible for the tumor promoter gene expression. In the future, we need to search for such regulatory components and evaluate of transcription factors through a series of immuno-precipitation immuno-blotting studies that will allow us to identify regulatory mechanism(s) of the KGF/KGFR signaling.

KGF inhibits Tam-induced cell survival of human breast cancer cells

KGF stimulated DNA synthesis and proliferation of epithelial cells in breast tissue [62]. In vitro experiments also demonstrated that KGF induced mammary hyperplasia and mammary adenocarcinoma [31]. KGF has also been suggested to promote motility of breast cancer cells and stimulates the migration and proliferation of breast cancer line in a culture wounding model [36]. As described in Chapter 3 in this dissertation, we
examined the effect of KGF on the growth of human breast cancer and examine the underlying mechanisms of KGF/KGFR regulation in human breast cells. We found that KGF itself does not promote MCF-7 cell growth. Instead, KGF blocks the Tam-induced cell death in MCF-7 cells through the regulation of apoptosis in MCF-7 cells. In the future, *in vivo* experiments need to be done to further support our *in vitro* experiment.

KGF exposure plays an important role in the control of hormone independent tumor survival. Not only will these data shed light on investigating the inhibitors of KGF signaling pathway but also anti-apoptotic protein agents in controlling the tumor growth in human breast and for developing therapeutic agents that may be beneficial in the treatment of human cancer.

**FUTURE WORKS AND CLINICAL IMPLICATIONS**

Mechanism(s) of KGF-induced cell survival in human breast – characterization and analysis of the cross talk between KGF and ER signaling

Breast cancer growth is regulated by steroid hormones, polypeptide hormones, and growth factors. Endocrine therapies for breast cancer have been designed to interrupt estrogen signaling by either blocking ER-α or by lowering the estrogen availability. However, de novo and acquired resistance to anti-estrogens are common in many patients. Cross-talk between ER and growth factor receptor pathways contributes to the development of this resistance. The work in this dissertation has shown that KGF can
down-regulate ER-α, increase ER-α regulated genes and inhibit Tam-induced cell killing via the KGF-induced Akt cell signaling pathway. These results suggest cross-talk between KGF and ER mediated signaling pathways. Future goals will focus on characterization and analysis of the ER function in KGF/KGFR signaling for the involvement of membrane and nuclear ER-α in the growth factor receptor signaling. ER located in the cell membrane also can activate the growth factor receptor pathways. This work will allow us to identify the possible molecules involved in the regulation of KGF signaling by ER in human breast and define the mechanisms for this regulation.

Investigations of the tumorigenicity of KGF in nude mice

The inappropriate activation of growth factor signaling cascades, either through an enhanced supply of growth factor ligands, or via up-regulation and increased activation of their target growth factor receptors can readily promote anti-hormone failure in breast cancer cells. During this dissertation, in combination with our previous results, we have shown that KGF is an estrogen-regulated factor which can enhance the aromatase mRNA expression and breast cancer cell survival *in vitro*. Aromatase is an enzyme that converts testosterone to estrogen. Our results demonstrate that there is an intriguing relationship between, estrogen and KGF and apoptosis in breast cancer cells, and further suggest a potential positive feed back loop model between estrogen and KGF for the stimulation of breast cancer growth. These findings also suggest the existence of a KGF-driven paracrine regulatory loop which leads to tamoxifen resistance of breast cancer via down-regulation of ER-α. It will be interested to know whether these results can be obscured *in vivo*. We expect that KGF/KGFR signaling can stimulate mammary gland tumor growth through the regulation of ER-α and anti-apoptotic process in the presence of anti-
estrogens in nude mice. The *in vivo* model can help us to further prove our concepts that increased KGF/KGFR signaling promotes anti-apoptosis, reduces ER levels, and facilitates insensitivity to anti-estrogen treatment.

Clinical relevance of KGF in human breast cancer patients

Our studies conclude that there will be some clinical relevance for KGF/KGFR signaling to serve as a molecular target for breast cancer patient treatment. Our studies not only demonstrate that increased growth factor signaling is a potent mechanism for the promotion of anti-estrogen resistance and cell survival but also show small synthesized peptide fragment as the native KGF protein. These demonstrate a clinical potential use for short peptides as breast cancer therapies. This pentapeptide can serve as a template for as the design of KGFR inhibitors for the blockade of KGF induced-cell survival. In the future, KGF signaling pathway might be able to be manipulated in clinical trials for cancer therapy.

In light of the above, the combination therapies of anti-estrogens and KGFR inhibitor also appear to be possible breast cancer treatment. We expect combination therapies to be more effective than mono-therapies in terms of significantly prolong the survivability of human breast cancer patients by providing a more complete inhibition of ER and growth factor signaling.
CONCLUDING REMARKS

The studies in this dissertation were carried out to determine if KGF is a survival factor in human breasts and to identify the mechanisms and signaling pathway driven by KGF in human breast. This information increases our understanding of the basic mechanisms of growth factor signaling and resistance to selective estrogen receptor modulators and contributes to our overall understanding of using anti-growth factor therapies to treat or delay endocrine resistance in breast cancer. But, there are still many questions to be answered with more investigations in the future. The primary goal of this research was to provide rationale for the treatment of human breast cancer with some novel target sites. Therefore, we hope that it should be possible to design new therapies targeted to this novel tumor viability factor and lead to the improved treatment of human breast cancer based on our study, and hope that these studies can be used as a basis for the similar investigations in other human cancers.
LITERATURE CITED


66. Arnold SF, Obourn JD, Jaffe H, Notides AC. Phosphorylation of the human


76. Faridi J, Wang L, Endemann G, Roth RA. Expression of constitutively active Akt-3 in MCF-7 breast cancer cells reverses the estrogen and tamoxifen


98. Kandasamy K, Srivastava RK. Role of the phosphatidylinositol 3'-kinase/PTEN/Akt kinase pathway in tumor necrosis factor-related apoptosis-