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Advances in Brief

Elevated Mitogen-activated Protein Kinase Activity in Estrogen-nonresponsive Human Breast Cancer Cells[1]

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Abstract

The mitogen-activated protein kinase (MAPK) signal transduction pathway plays an essential role in cell cycle progression and can be activated by many growth factor/mitogen pathways including estrogen. MAPK has also been implicated in ligand-independent activation of estrogen receptor-α (ER-α). The development of estrogen-independent growth in breast cancer is likely a first step in progression to hormone independence and antiestrogen resistance. We examined MAPK expression and activity in T5-PRF and T5 human breast cancer cells. T5-PRF is an estrogen-nonresponsive cell line developed from T5 cells by chronically depleting the cells of estrogen in long-term culture. MAPK activity measured *in vitro* was significantly higher ($P < 0.05$) in T5-PRF compared with T5 cells. Western blot analyses showed increased levels of active dually phosphorylated MAPK in T5-PRF cell extracts compared with T5. The increased activity and expression of MAPK may contribute to the estrogen nonresponsive growth phenotype and ligand-independent activity of ER in T5-PRF cells.

Introduction

MAPKs[3] (or ERKs) are a family of protein kinases involved in transmitting signals from a variety of stimuli from the cell membrane to the nucleus (1). The MAPKs, ERK1 and ERK2, are activated by mitogenic stimuli from growth factor receptors such as epidermal growth factor receptor (1). Once activated, ERK1 and ERK2 phosphorylate a variety of proteins, including transcription factors, to effect changes in gene expression. A cascade of protein kinases regulate and activate MAPK via phosphorylation on both threonine and tyrosine residues (1).

Several studies have suggested a potential role for the MAPK signaling pathway in the initiation and pathogenesis of breast cancer. MAPK activity has been shown to be elevated in primary breast cancer compared with benign breast tissue and has also been shown to be overexpressed in metastatic cells within lymph nodes of breast cancer patients (2). Constitutive expression of Raf-1 kinase (an upstream activator of the MAPK pathway) in MCF-7 human breast cancer cells resulted in estrogen-independent growth (3). ER-α, like other members of the steroid-thyroid hormone receptor superfamily, is functionally regulated via phosphorylation by several protein kinases including MAPK (4–8). Phosphorylation is believed to play a role in regulating many aspects of steroid hormone receptor function including DNA binding and transcriptional activation. Ser118 of the ER-α has been shown to be phosphorylated by MAPK in response to receptor activation by growth factors including epidermal growth factor, and mutation of this site to an alanine residue severely diminished ER-α transcriptional ability (4, 7).

Phosphorylation of ER-α by MAPK has been implicated in hormone-independent activation of the ER-α by steroid-independent activators of ER-α (4, 7). The development of estrogen-independent growth in human breast cancer is thought to be one of the initial steps in the progression to hormone independence and resistance to endocrine therapies. However, the mechanisms responsible for the development of estrogen independence and the presence of continued expression of ER-α are poorly understood. To address this, we have developed a breast cancer cell model of apparent estrogen independence (9). T5 human breast cancer cells are ER-α positive, and estrogen treatment in culture results in increased proliferation of these cells. An estrogen-nonresponsive cell line, T5-PRF, was developed from T5 cells by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen seen in the parent cell line while still retaining expression of the ER-α (9). We have characterized and compared MAPK activity and levels in these two cell lines to determine whether differences in MAPK activity could contribute to the estrogen-independent phenotype of the T5-PRF cell line.

Materials and Methods

Reagents. Monoclonal mouse anti-phospho-MAPK antibody (#9105S) was from New England Biolabs (Beverly, MA). Polyclonal rabbit anti-ERK1 (C-16) was from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). [^{32}P]γATP was purchased from ICN (St-Laurent, Quebec, Canada). Estradiol-17β was from Sigma Chemical Co. (St. Louis, MO). PD 98059 was purchased from Calbiochem (La Jolla, CA.). DMEM powder, fetal bovine serum, and myelin basic protein were purchased from Life Technologies, Inc. (Burlington, Ontario, Canada). All other cell culture ingredients were purchased from Flow Laboratories (Mississauga, Ontario, Canada). Cyclic AMP-dependent protein kinase inhibitor peptide was purchased from Bachem (Torrance, CA). ICI 164,384 was a gift from ICI (Macclesfield, Cheshire, United Kingdom). [^{14}C]Chloramphenicol was obtained from NEN (Lachire, Quebec, Canada).

Cell Culture. T5 human breast cancer cells were routinely cultured in DMEM containing 5% v/v FCS, 1% w/v glucose, 2 mM glutamine, and 100 units of penicillin-streptomycin as described previously (9). T5-PRF cells were routinely cultured in PRF/DMEM supplemented with 5% v/v twice charcoal dextran-stripped FCS and 1% w/v glucose, 2 mM glutamine, and 100 units of penicillin-streptomycin as described previously (9).

MAPK Assay. MAPK activity *in vitro* was measured using myelin basic protein as a substrate as described previously (10). For experiments performed in serum-free conditions, cells were plated in PRF/DMEM, and the following day the medium was changed to PRF/DMEM minus serum and changed every day for 7 days. To measure basal activity, T5 cells were passaged once in PRF/DMEM, and both T5 and T5-PRF cells were set up in PRF/DMEM in 100-mm dishes at ~0.2×10^6 cells and harvested in MAPK buffer [100 mM β-glycerophosphate, 1 mM sodium orthovanadate (pH 10), 2 mM EGTA, 20 mM Tris-HCl (pH 7.4), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM 4-[2-aminoethyl]benzenesulfonyl fluoride hydrochloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 0.2 mM benzamidine] 3 days later. Extracts

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[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; ER, estrogen receptor; PRF, phenol red free; CAT, chloramphenicol acetyltransferase; MEK, MAPK kinase.

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for MAPK protein detection were obtained in a similar fashion. For experiments using PD 98059, cells were treated an hour before harvesting with 50 μM inhibitor.

Western Blotting and Immune Detection. Extracts obtained for MAPK assay were analyzed using 7.5% SDS-PAGE with a 4% stacking gel at 200 V for 45 min at room temperature according to the Laemmli method (11). Gels were transferred to nitrocellulose using 3-[cyclohexylamino]-1-propanesulfonic acid transfer buffer [10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11), 20% methanol] and transferred for 1 h at 120 V at 4°C. Blots were blocked overnight in 5% skimmed milk/Tris-buffered saline containing 0.5% Tween-20 (TBS-T). Blots were incubated with mouse anti-phospho-MAPK antibody for detection of dually phosphorylated MAPK (NEB, Beverly, MA; 1:1000 in 1% skimmed milk/TBS-T) or rabbit anti-ERK1 (C-16) for detection of total MAPK protein (Santa Cruz Biotechnology; 1:1000 in 1% skimmed milk/TBS-T) for 4 h at room temperature. Blots were incubated with the appropriate secondary antibody (anti-mouse, Jackson ImmunoResearch Laboratories, West Grove, PA; anti-rabbit, Sigma, St. Louis, MO) for 1 h at room temperature, 1:1000 in 1% skimmed milk/TBS-T. Detection was carried out using the ECL detection system according to the manufacturer's instructions (Amersham, Buckinghamshire, United Kingdom).

Transient Transfections. T5 cells were passaged once in PRF-DMEM, and T5 and T5-PRF cells were set up in PRF-DMEM at 1–2 × 10^6 cells/100-mm dish the day before transfection. Cells were transfected with 2 μg of ERE-tk-CAT (12) and 5 μg of pCH110 (β-galactosidase expression vector; Pharmacia) overnight using the calcium phosphate/glycerol shock method using an equal volume of 2× BBS buffer (50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, and 1.5 mM Na_2HPO_4, pH 6.95). The following day, the cells were subjected to a 2-min glycerol shock (20% v/v), were washed twice with 1× PBS, and given fresh medium plus or minus 10 nM estradiol-17β, plus or minus 50 μM PD 98059 (MEK inhibitor; Calbiochem). Two h later, PD 98059-treated dishes were given a second dose of 50 μM PD 98059, and 18 h later, dishes were harvested and cell extracts were obtained. Cell extracts were assayed for β-galactosidase activity to control for transfection efficiency, and CAT assays were performed as described previously (13).

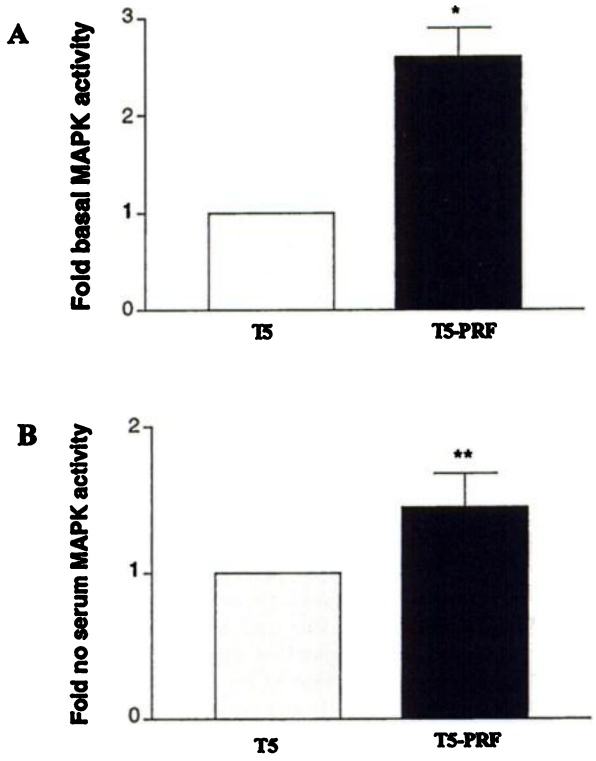

Fig. 1. MAPK activity *in vitro*. Cell extracts for measurement of *in vitro* MAPK activity were obtained as described in "Materials and Methods." Myelin basic protein was used as a substrate to measure kinase activity, and the assay was allowed to proceed for 10 min at 30°C . Histograms represent fold difference in MAPK activity after setting the activity of T5 parent cells to 1.0. *A*, basal activity: fold increase is 2.6 ± 0.3, $n = 3$. *, $P < 0.05$, Student's *t* test. *Bars*, SE. *B*, serum-free activity: fold increase is 1.5 ± 0.2, $n = 3$. **, $P < 0.001$, Student's *t* test. *Bars*, SE.

Results

Increased MAPK Activity in T5-PRF Cells. Previously, we had shown that T5-PRF human breast cancer cells had an elevated level of basal ER activity (in the absence of estrogen), despite the fact that these cells contained 50% less ER[4] (9). We were interested in possible reasons for this apparently estrogen-independent ER activity. Several lines of evidence suggest that MAPK may be involved in ligand-independent activation of ER-α. Therefore, we chose to examine MAPK activity in T5-PRF and the parental T5 human breast cancer cell lines. Our initial experiments examining ER-α transcriptional activity were performed under basal (*i.e.*, estrogen-deplete) conditions with cells setup in PRF/DMEM containing twice charcoal-stripped FCS; therefore, we initially examined *in vitro* MAPK activity under these conditions (Fig. 1*A*). *In vitro* MAPK activity was significantly higher in T5-PRF human breast cancer cells. MAPK activity was 2.6 ± 0.3 (mean ± SE, $n = 3$) fold higher ($P < 0.05$) than the activity assayed in the parental T5 cells. To determine whether this increased activity was reflected in the total amount of MAPK protein and/or the active MAPK pool, Western blotting on cell extracts was performed (Fig. 2). Using an antibody that recognizes total MAPK protein, we found that both cell lines expressed large amounts of both the ERK1 and ERK2 isoforms of MAPK under these conditions (Fig. 2*B*). The active form of MAPK is dually phosphorylated on tyrosine and threonine residues (2), and using an antibody that specifically detects only this active form of the protein, we found that T5-PRF cells had higher levels of activated MAPK protein compared with parental cells (Fig. 2*A*). PD 98059 is a specific inhibitor of MAPK activity because of its specific inhibition of MAPK kinase (also referred to as MEK; Ref. 14). Treating both cell lines with 50 μM PD 98059 for 1 h resulted in a significant inhibition of dually phosphorylated MAPK (Fig. 2*A*), with little or no effect on total MAPK levels (Fig. 2*B*), supporting the conclusion that T5-PRF cells have elevated levels of activated MAPK. Under conditions in which we had serum starved the cells for 7 days, T5-PRF cells still maintained elevated levels of *in vitro* MAPK activity (1.5 ± 0.2, fold, mean ± SE, $n = 3$, $P < 0.001$) compared with parental T5 cells (Fig. 1*B*). Total MAPK protein levels were similar between the two lines (Fig. 3*B*), but activated MAPK protein levels were much higher in T5-PRF cells (Fig. 3*A*, compare *Lanes 1* and *3*). As expected, treating T5-PRF cells with 50 μM PD 98059 for 1 h under serum-free conditions decreased the level of active MAPK detected by Western blotting (Fig. 3*A*, compare *Lanes 3* and *4*), without effecting the total level of MAPK protein expression (Fig. 3*B*, compare *Lanes 3* and *4*). These data support the conclusion that T5-PRF cells contain increased levels of phosphorylated and active MAPK protein.

Antiestrogen Can Reduce Active MAPK Protein Levels. Previous research has suggested that ER-α can directly activate MAPK, and estrogen activation of MAPK can be blocked by antiestrogen in MCF-7 human breast cancer cells (15). T5-PRF cells contain elevated basal ER-α activity that can be inhibited by 85% after treating cells for 24 h with 1 μM ICI 164,384.[4] To examine what effect ICI 164,384 might have on MAPK protein levels under these conditions, T5-PRF cells were treated with 1 μM ICI 164,384 for 24 h, and levels of active and total MAPK protein were examined. ICI 164,384 was able to reduce the amount of active dually phosphorylated MAPK in estrogen-depleted conditions by 30% ($n = 4$), without any apparent effect on

[4] A. S. Coutts, E. Leygue, and L. Murphy. Variant estrogen receptor-α messenger RNA expression in hormone-independent human breast cancer cells, submitted for publication.

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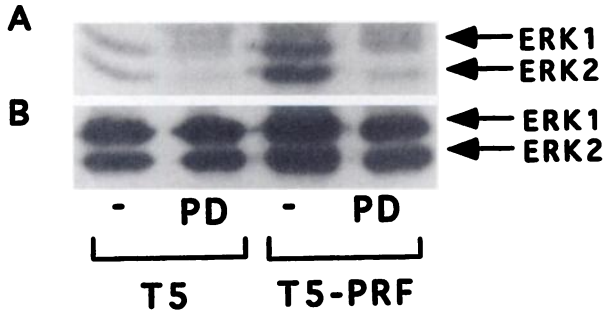

Fig. 2. Western blot analysis of MAPK protein levels under basal conditions. In *A*, 30 μg of cell extract were run on a 7.5% SDS-PAGE. Immune detection was using an anti-phospho-MAPK antibody (NEB). In *B*, 5 μg of cell extract were run on a 7.5% SDS-PAGE. Immune detection was using an anti-ERK1 antibody (Santa Cruz Biotechnology). *PD*, MEK inhibitor PD 98059, 50 μM for 1 h.

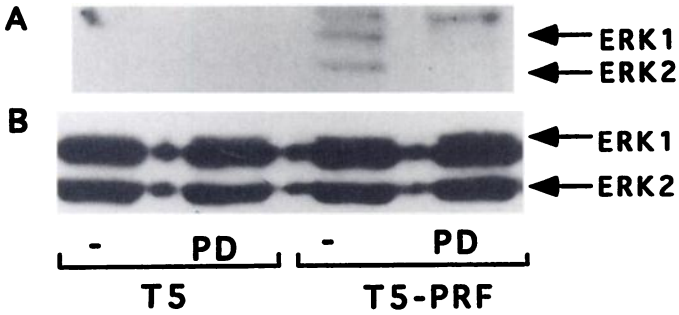

Fig. 3. Western blot analysis of MAPK protein levels under serum-free conditions. In *A*, 30 μg of cell extract were run on a 7.5% SDS-PAGE. Immune detection was using an anti-phospho-MAPK antibody (NEB). In *B*, 5 μg of cell extract were run on a 7.5% SDS-PAGE. Immune detection was using an anti-ERK1 antibody (Santa Cruz Biotechnology). *PD*, MEK inhibitor PD 98059, 50 μM for 1 h.

total MAPK levels (data not shown), suggesting a possible link between the elevated MAPK activity and increased ligand-independent ER-α activity in these cells.

MAPK Is Involved in ER-α Transcriptional Activity. To further examine the role elevated MAPK activity might play in terms of the elevated basal (*i.e.*, estrogen-independent) ER-α transcriptional activity in T5-PRF cells, transient transfections were carried out. Previously, we had determined that 50 μM PD 98059 for 1 h was sufficient to significantly reduce the levels of dually phosphorylated MAPK under basal conditions (Fig. 2*A*). Because our transient transfection experiments required 24-h treatments, we determined conditions under which PD 98059 was able to maintain low levels of dually phosphorylated MAPK over this time period (data not shown). It was determined that adequate inhibition could be achieved by treating cells initially with 50 μM PD 98059, followed 2 h later by an additional 50 μM treatment, with the cells being harvested 18 h later. This resulted in an ~85% inhibition of dually phosphorylated MAPK protein levels (data not shown). When T5-PRF cells were transfected with an estrogen-responsive CAT reporter gene and treated with PD 98059, there was a significant reduction of 44.2% ± 8.1 (mean ± SE, $P < 0.05$, $n = 6$) in the basal ER-α transcriptional activity seen (Fig. 4). Interestingly, there was also a significant and equivalent reduction in the estrogen-induced transcriptional activity of 46.3% ± 12.4 (mean ± SE, $P < 0.05$, $n = 6$) by PD 98059, suggesting a role for MAPK activation in estrogen-induced transcriptional activation also.

Discussion

We have found elevated levels of activated MAPK in a human breast cancer cell line, T5-PRF, that is nonresponsive to estrogen in terms of growth and has elevated levels of estrogen-independent ER transcriptional activity. The ER-α, like other members of the steroid hormone receptor superfamily, is a phosphoprotein. The function of phosphorylation is not clear, but it has been suggested to play a role in many aspects of receptor activity, including DNA binding and transcriptional activation. In MCF-7 human breast cancer cells, the ER-α is phosphorylated on serine-118, serine-154, and serine-167 in response to estradiol binding (6, 16, 17). In COS-1 cells, additional hormone-induced phosphorylation sites, serine-104, serine-106, and serine-118, on the ER-α have been identified (8). It has also been demonstrated that serine-118 and tyrosine-537 on ER-α are phosphorylated independently of estradiol binding in MCF-7 cells (5, 16) and that src family tyrosine kinases are capable of estradiol-independent phosphorylation of the ER-α *in vitro* (5). The activation of the MAPK pathway through an estrogen-independent mechanism (*i.e.*, EGF) can result in transcriptional activation of the ER-α, and phosphorylation of the ER-α on serine-118 is required for this activity (4, 7, 17).

Estrogens are known mitogens for breast cancer cells, but how estrogen promotes cell proliferation is unknown. The MAPK signal transduction pathway plays an essential role in cell cycle progression and can be activated by many growth factor/mitogenic pathways including estrogen. In several cell types, including MCF-7 human breast cancer cells, estradiol has been shown to rapidly increase MAPK activity (15). That this activation requires ER-α was demonstrated in cells via transient transfection experiments showing an absolute requirement for ER-α for activation, and the addition of antiestrogen blocked estrogen-induced MAPK activation (15). A recent report was able to show growth factor, but not estrogen-induced, activation of MAPK in MCF-7 cells (17). The discrepancy between this report and a previous report demonstrating estrogen activation of MAPK in MCF-7 cells (15) is unclear, but may be due to differences in the experimental conditions under which activation was assayed between the two papers.

Peptide growth factor signaling pathways can cross-talk with the ER-α. Indeed, it has been demonstrated that growth factors can result in ligand-independent activation of ER-α (7). Several studies have demonstrated that overexpression of a growth factor, or its receptor, which can activate the MAPK cascade, or a component of the MAPK pathway (*e.g.*, Raf) can result in estrogen-independent growth in cells in culture and in some cases tumorigenesis *in vivo* in the absence of estrogen. For example, overexpression of a constitutively active Raf

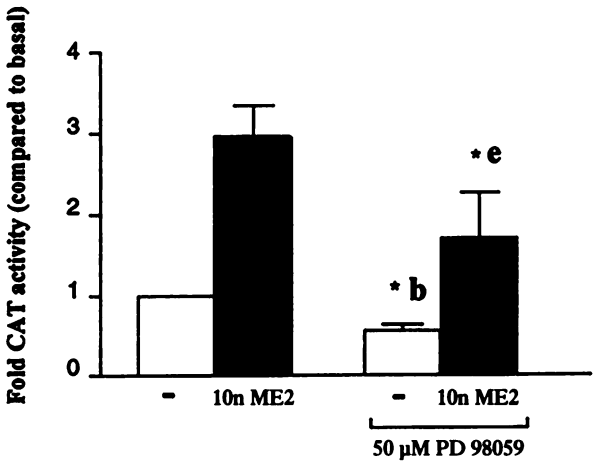

Fig. 4. ER-α transcriptional activity in the presence of PD 98059. Transient transfections were performed as described in "Materials and Methods." Results are expressed as fold CAT activity compared with untreated T5-PRF cells. Histograms represent means (*bars*, SE), $n = 6$. **b*, compared with basal without PD 98059; **e*, compared with estrogen treatment without PD 98059, $P < 0.05$, Student's *t* test.

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kinase in MCF-7 cells allows for growth in the absence of estrogen (3). Expression of constitutively activated MEK in NIH 3T3 cells resulted in the ability of the cells to proliferate in conditions of low serum and also resulted in morphological transformation (18). These data demonstrate the importance of the MAPK pathway in cell growth regulation and likely tumorigenesis.

What role the elevated MAPK activity might play in the estrogen-independent growth phenotype of T5-PRF cells is not clear. Our previous studies have shown that these cells no longer respond to estrogen by increasing their proliferation rate. It is likely that elevated MAPK activity could confer an additional growth advantage to these cells, thereby allowing maximal proliferation in the absence of estrogen. Our transfection experiments using PD 98059 suggest that the MAPK activity may play a role in the elevated basal ER activity we see in these cells but may not account for all of the increased basal activity. At a concentration of 50 μM, PD 98059 can almost completely abolish the level of active dually phosphorylated MAPK protein (85% decrease), whereas a similar concentration resulted in slightly less than 50% reduction in basal ER-α transcriptional activity. This suggests that other mechanisms, along with elevated MAPK, contribute to the ligand-independent ER-α activity. Our previous study has identified a variant ER-α in T5-PRF cells that may contribute to the increased basal ER-α activity.[4] Studies have also shown that cells grown long-term in the absence of estrogen can develop supersensitivity to residual estrogens in the growth medium (19). It is also likely that the residual dually phosphorylated MAPK protein remaining, even after treating cells with PD 98059 (~15%), is sufficient to contribute to the basal ER-α activity.

We were also able to see an inhibition of estrogen-dependent ER-α transcriptional activity after treating cells with PD 98059. This was of a similar magnitude as the effect on basal transcription (~50%), supporting the hypothesis that MAPK plays an important role in both ligand-dependent and -independent ER-α transcriptional activity.

Our experiments with the antiestrogen ICI 164,384 also suggest that the link between MAPK activity and ER-α transcriptional activity is likely not a straightforward one. As mentioned, T5-PRF cells treated with 1 μM ICI 164,384 under basal conditions show an ~85% reduction in the ligand-independent ER-α transcriptional activity,[4] whereas the same dose and time of antiestrogen treatment results in an ~30% inhibition of dually phosphorylated MAPK protein.

Elevated levels of MAPK activity and expression have been associated with the malignant phenotype and have been shown in breast tumors compared with normal tissue and benign breast conditions (2). Breast tumors also have been shown to contain elevated tyrosine kinase activities compared with benign breast tumors and normal breast tissues (20). These data suggest that an increase or deregulation of growth controlling signals, such as those contributed by MAPK, may be involved in the etiology and pathogenesis of breast cancer. During the course of breast cancer progression, tumors become hormone-independent and refractory to endocrine therapies directed at blocking the activity of ER-α. The development of estrogen-independent growth is believed to be an initial step in the progression to a hormone-independent phenotype, and estrogen-independent growth is a characteristic of a more aggressive breast cancer cell phenotype. Our data support the hypothesis that elevated levels of MAPK could confer a growth advantage to breast cancer cells, perhaps leading to estrogen-independent growth.

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