Elevated Expression of MAPK Phosphatase 3 in Breast Tumors—A Mechanism of Tamoxifen Resistance

Yukun Cui[1], **Irma Parra**[1], **Mao Zhang**[1], **Susan G. Hilsenbeck**[1], **Anna Tsimelzon**[1], **Toru Furukawa**[3], **Akira Horii**[3], **Zhong-Yin Zhang**[4], **Robert I. Nicholson**[5], and **Suzanne A. W. Fuqua**[1,2,5]

[1]Breast Center Baylor College of Medicine One Baylor Plaza Houston, Texas 77479, USA

[2]Department of Medicine Baylor College of Medicine One Baylor Plaza Houston, Texas 77479, USA

[3]Department of Molecular Pathology Tohoku University School of Medicine Sendai, 980-8575, Japan

[4]Department of Biochemistry and Molecular Biology Indiana University School of Medicine Indianapolis, IN 46202

[5]Tenovus Centre for Cancer Research Welsh School of Pharmacy Cardiff, UK

Abstract

Antiestrogen resistance is a major clinical problem in the treatment of breast cancer. Altered growth factor signaling with estrogen receptor (ER) α has been shown to be associated with the development of resistance. Gene expression profiling was utilized to identify MAPK phosphatase 3 (MKP3) whose expression was correlated with response to the antiestrogen tamoxifen in both patients and in vitro derived cell line models. Overexpression of MKP3 rendered ERα-positive breast cancer cells resistant to the growth inhibitory effects of tamoxifen, and enhanced tamoxifen agonist activity in endometrial cells. MKP3 overexpression was associated with lower levels of activated ERK1,2 phosphorylation in the presence of estrogen, but that estrogen deprivation and tamoxifen treatment decreased MKP3 phosphatase activity, leading to an up-regulation of pERK1,2 MAPK, phosphoserine 118 of ERα, and cyclin D1. The MEK inhibitor PD98059 blocked tamoxifen-resistant growth. Accumulation of reactive oxygen species was observed with tamoxifen treatment of MKP3 overexpressing cells, and antioxidant treatment increased MKP3 phosphatase activity, thereby blocking resistance. Furthermore, PD98059 increased the levels of phospho-JNK in tamoxifen-treated MKP3 overexpressing cells, suggesting an interaction between MKP3 levels, activation of ERK1,2 MAPK, and JNK signaling in human breast cancer cells. MKP3 represents a novel mechanism of resistance which may be a potential biomarker for the use of ERK1,2 and/or JNK inhibitors in combination with tamoxifen treatment.

Keywords

breast cancer; tamoxifen; MAPK; MAPK phosphatase-3

---

[6]To Whom Correspondence Should be Addressed (713) 798-1600, FAX: (713) 798-1673, sfuqua@breastcenter.tmc.edu.

Introduction

The steroid hormone estrogen can stimulate breast tumor growth, and agents that inhibit estrogen synthesis, or antiestrogens like tamoxifen (Tam) which block its receptor, are the standard therapies offered to women with estrogen receptor (ER)α-positive cancer. In many cases, however, these therapies eventually fail. One current hypothesis is that ERα remains essential to the problem of resistance, due to its molecular crosstalk with growth factors, and/or downstream intracellular signaling molecules. There is also a growing body of evidence implicating the mitogen-activated protein kinase extracellular signal-regulated kinases ERK 1,2 MAPKs in the growth factor phosphorylation cascade, and its interaction with ERα signaling in TR (1). Indeed, phosphorylation of ERK 1,2 MAPK has been associated with a poorer quality of response to Tam in breast cancer patients (2).

We know that ERα can be phosphorylated by activated MAPK, resulting in ligand-independent transcriptional activity (3). An emerging area of research in MAPK signaling is the role of specific protein phosphatases in the control of MAPK activation, and their role in specific biological responses. MAPK phosphatase 3 (MKP3, also called dual specificity phosphastase 6 [DUSP6] and Pyst1) is a member of a phosphatase family that inactivates MAPK function [reviewed in (4)]. MKP3 is in a regulatory feedback loop with ERK 1,2 MAPK (5, 6). How MKP3 impacts on ERα and MAPK function in breast cancer cells, and how a resultant feedback loop impacts on the generation of TR is the subject of the present study.

Materials and Methods

Reagents, Hormones, and Antibodies

17β-Estradiol (E2) and 4-hydroxy-tamoxifen (4-OH-Tam) were from Sigma (St. Louis, MO). ICI 182,780 was obtained from Astrazeneca (Macclesfield, UK). The MEK1,2 inhibitor PD98059 and GSH were from Calbiochem (La Jolla, CA). 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) was obtained from Molecular Probes (Eugene, Oregon). p-nitrophenyl phosphate (pNPP) and pNPP assay buffer were obtained from Upstate Biotechnology (Charlottesville, VA).

Construction of siRNA Plasmids

The MKP3 siRNA oligonucleotides containing the sequence GCTCAATCTGTCGATGAAC, corresponding to the MKP3 coding region from #1283 to 1301, the MKP1 siRNA oligonucleotides containing the sequence GGAGGATACGAAGCGTTTT, corresponding to the MKP1 coding region from #615 to 623, or GCATCATCTCTCCCAACTT, corresponding to the MKP1 coding region from #1126 to 1144 were inserted into RNAi-Ready pSiren-Retro-Q vector (Clontech, Pal Alton, CA). The control RNAi oligonucleotide was purchased from Clontech.

Tumor Specimens and Expression Microarray Analysis

A cohort of frozen breast tumor specimens from nine patients who received adjuvant Tam was selected from the tumor bank of The Breast Center, Baylor College of Medicine, for use in the RNA analyses. This study was approved by the Baylor College of Medicine Institutional Review Board in accordance with federal human research study guidelines. Within this cohort, metastatic tumors from five patients who developed their recurrent lesion within 1-11 months while undergoing Tam treatment (Tam-resistant), and four primary tumors that were collected at the time of initial diagnosis from patients who remained disease-free for 93-123 months with a median follow-up of 106 months (Tam-sensitive) were included.

Total cellular RNA was extracted from 100 mg of pulverized tumor powder using Trizol reagent (InVitrogen) followed by Qiagen RNeasy column purification (Qiagen, Valencia, CA). cRNA was hybridized onto Affymetrix HGU95A GeneChips using recommended procedures for hybridization, washing, and staining with streptavidin–phycoerythirin. The GeneChips were scanned, and feature quantitation was performed using MAS5.0 (Affymetrix). Data were normalized using the invariant set method, and modeled to estimate expression using the Perfect-match only model-based expression index as implemented in dChip (7); class comparisons were performed using BRB Array Tools developed by Drs. Richard Simon and Amy Peng (http://linus.nci.nih.gov/BRB-ArrayTools.html), and t-tests were performed with randomized variance modeling (8).

qRT-PCR

Real-time quantitative RT-PCR (qRT-PCR) was performed utilizing the 7700 Sequence Detector (Applied Biosystems, Foster City, CA) Specific quantitative assays for MKP3 were developed using Primer Express software version 1.0 for mMcintosh (Applied Biosystems) following the recommended guidelines based on sequences from Genbank. Primer sequences for human MKP3 were: forward primer, GTGGCACCGACACAGTG, reverse primer, CCCGTATTCTCGTTCCAG, human MKP3 RNA probe sequence, TCTACGACGAGAGCAGCAGCG, assay length, 65 nt; Primer sequences for human 18S rRNA were: forward primer, CGGCTTAATTTGACTCAACAC, reverse primer, ATCAATCTGTCAATCCTGTCC, human 18S rRNA probe sequence, AAACCTCACCCGGCCCG, assay length, 68 nt. Synthetic DNA oligos used as standards (sDNA) encompassed the entire $5' – 3'$ amplicon for the assay (Biosource, Camarillo, CA). Final normalized data are presented as the ratio of MKP3 to 18S RNA.

Cells and Stable Transfection

The paired TR breast cancer cell lines, MCF-7/WT/ and MCF-7/TR or T47D/WT and T47D/TR have been previously described (9). Ishikawa endometrial cancer cells were kindly provided by Dr. Carolyn Smith. MCF-7 cells were originally obtained from Dr. Benita Katzenellebogen, but have been maintained in our laboratory for over 15 years (10). These MCF-7 cells were maintained in Minimal Essential Medium (MEM, InVitrogen) supplemented with 5% fetal bovine serum (Summit Biotechnology, Fort Collins, CO), 200 U/ml penicillin, and 200 μg/ml streptomycin. Cells were incubated at 37 °C in 5% CO_2. To generate MCF-7 and Ishikawa cells stably overexpressing MKP3, pcDNA3-MKP3-V5 (11)

or empty vector (pcDNA3-His-V5, InVitrogen), were transfected as described (10), and positive clones were identified using immunoblot analysis with an anti-V5 antibody (1:5000 dilution). Ishikwa cells was transfected with pcDNA3-MKP3-V5, empty vector (pcDNA3-His-V5, InVitrogen), MKP3 siRNA vector, MKP1 siRNA vectors, or siRNA control vectors and transfectants were selected with either 800μg/ul G418, or 1μg/ul puromycin.

Cell Extracts and Immunoblots

Cells were starved in phenol red–free (PRF), serum-free MEM (Specialty Media, Phillipsburg, NJ) for 48 hours, and were then treated for 2 hours with vehicle (ethanol), estrogen (100 nM), or 4-hydroxytamoxifen (Tam) (100 nM). Equal amount of cell extracts were resolved under denaturing conditions by electrophoresis in 8-10% polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE), and transferred to nitrocellulose membranes by electroblotting (Schleicher & Schuell, Keene, NH). After blocking of the transferred nitrocellulose membrane, they were incubated with primary antibodies either for 1 hour at room temperature (anti-ERα, 1:100 dilution; anti-AIB1, 1:100 dilution; anti-ERK2, 1:500 dilution; anti-Cyclin D1, 1:500 dilution; anti-PR, C-19, 1:200 dilution; anti-p190, 1:1000 dilution; anti-V5, 1:5000 dilution; anti-MKP-1, 1:200 dilution), or overnight at 4°C (anti- phospho MAPK, phospho-JNK, phospho-p38, phospho-Ser118-ERα, MAPK p42/44, JNK, and p38 were used at 1:1000 dilution), and then incubated with secondary antibodies for 1 hour at room temperature and developed with Enhanced Chemiluminescence Reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

ROS Measurements

MKP3 overexpressing MCF-7 cells were treated with vehicle control, estradiol, or Tam for 2 hours as described before, then the cells were trypsinized, washed two times with prewarmed (37°C) PBS, and then resuspended into prewarmed PBS supplemented with either DMSO or 5 μM of a fluurogenic probe for ROS—2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) (Molecular Probes) at 300,000 cells/ml, and incubated at 37°C for another 2 hours. Fluorescence (excitation wavelength: 480 nm, emission wavelength: 530 nm) was measured using a fluorometer in a 96 well plate (300 μl/well) and normalized by subtracting fluorescence of DMSO incubated samples. The experiment was performed in triplicate.

Immunoprecipitation and Phosphatase Assays

MKP3-overexpressing and control transfected MCF-7 cells were treated as described above with ethanol, E2, or Tam, with or without GSH, and lysed in buffer containing 20 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1% Triton X-100, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 0.1% β-mercaptoethanol, 1 mM PMSF, and 1 μg/ml leupeptin . A phosphatase reaction was performed as described {Kim, 2003 #2664and the nonenzymatic hydrolysis of the substrate was corrected by measuring the control vector transfected immunoprecipitates. The amount of product p-nitrophenol was determined from the absorbance at 405 nm. The phosphatase assays were performed in triplicate, n=3 separate experiments.

Xenograft Studies

MCF-7 vector control and MKP3-transfected cells were established as xenografts in ovariectomized 5- to 6-week-old BALB/c athymic nude mice (Harlan Sprague Dawley, Madison, WI) supplemented with 0.25-mg 21 day release E2 pellets (Innovative Research, Sarasota, FL) by inoculating the mice subcutaneously with 5×10^6 cells, as described previously {Osborne, 1995 #2257}. Two vector control animals and 1 MKP3 injected animal failed to develop measurable tumors. These were eliminated from the analysis. When tumors reached ~250 mm^3 (i.e., in 21 days) animals were randomly allocated to continue E2 (n=6 per group), or to estrogen withdrawal plus Tam (n=4-5 per group; 500 µg/animal given subcutaneously in peanut oil, 5 days/week) for another 30 days. Tumor growth was assessed and tumor volumes were measured as described previously (12). Tumor growth curves were fitted to an exponential growth model and tested for whether the growth rates were different for the cell line groups. This was implemented by log-transforming the tumor sizes and using a mixed linear model with fixed main effects for cell line and time, a random subject effect, and an autoregressive convariance structure within subject. Analyses were carried out using SAS (V9.1, Cary, NC).

Anchorage-Independent Growth Assays

Cells were starved for 2 days in PRF MEM (InVitrogen) supplemented with 5% fetal bovine serum (FBS, Summit Biotechnology, Fort Collins, CO), 6 ng/ml insulin, 200 units/ml penicillin, and 200 µg/ml streptomycin. Soft agar assays were performed in six-well plates. After 14 days, the colonies were fixed, and the colony number of colonies >50 cells from quadruplicate assays were then counted. The data shown is the mean colony number of four plates, and is representative of two independent experiments analyzed for statistical significance (p>0.05) using a two-tailed Student's t-test,or one-way Anova test.

Results

MKP3 Overexpression and TR

To identify genes whose expression was associated with the development of TR, we compared primary tumors from patients that did not relapse after Tam treatment despite 7 to 10 years of follow-up with metastatic tumors from patients that progressed during adjuvant Tam treatment using expression microarray analysis. Our rationale was that we might be more likely to identify genes whose differential expression was involved in the initiation of TR, and whose expression might itself be affected by Tam treatment with our selection criteria. Gene expression analysis of our cohort identified MKP3 as being more highly expressed in the Tam-resistant (Tam Res) tumor group as compared to the Tam-sensitive (Tam Sen) tumor group (Fig. 1, *p=0.006*). The median level of MKP3 RNA was 2.5 fold higher in the Tam Res group. Quantitative RT-PCR assay was also used to confirm differential MKP3 RNA expression levels in these tumors; the correlations between Affymetrix MKP3 RNA expression and qRT-PCR levels were very high (data not shown; Rsp=0.83. *p=0.0053*).

We next measured the endogenous levels of MKP3 RNA in two *in vitro*-derived models of TR using qRT-PCR; both of these models were derived by long-term culture in Tam-

containing medium (13). The levels of MKP3 RNA were 19 and 8-fold higher in MCF-7/TR and T47D/TR cells, respectively, compared to the parental WT control cell lines (Fig. 1B). In addition, we measured MKP3 RNA levels in the Ishikawa endometrial cancer cell line where Tam is known to act as an agonist (9). We found very high levels of MKP3 RNA in the Ishikawa line, compared to all of the breast cancer cell lines. Finally, we investigated whether estrogen or Tam treatment of MCF-7 cells modulated MKP3 RNA levels (Fig. 1C), however neither treatment altered MKP3 expression. These data collectively suggest that MKP3 levels may be correlated with the TR phenotype in patients and cell lines.

We next generated several models of TR, first stably transfecting ERα-positive MCF-7 cells with either empty vector plasmid as a control, or a plasmid encoding MKP3. Cells were drug selected for plasmid expression, cloned by limiting dilution, and several resulting clones were screened for expression of MKP3 using an anti-V5 antibody to the V5 tag introduced at the carboy-terminus of MKP3 (11). An immunoblot analysis of two MCF-7-control clones (Fig. 2A, Con 1 and 2) and two MCF-7-MKP3-overexpressing clones (Fig. 2A, MKP3-1 and 2) demonstrated similar ectopic expression of MKP3 in the stable transfectants. The relative MKP3 RNA levels in clone MKP3.1 were approximately 15-fold higher than RNA levels in the vector-alone transfected Con 1 cells (Fig. 2B), a difference which was similar to that found in the MCF-7/TR cells (Fig. 1B).

The growth characteristics of the MCF-7 stable MKP3 transfectants under different hormonal conditions were investigated using anchorage-independent soft agar assays (Fig. 2C). As expected, MCF-7 vector control cells exhibited low colony formation in the absence of E2 (C1 and C2, C treatment); treatment with E2 increased the number of colonies, and Tam treatment reduced the number of colonies of control cells. Ectopic expression of MKP3 reduced the number of colonies formed in soft agar under estrogen-deprived conditions (MKP3-1 and MKP3-2, C) compared to the control transfectants (*p=0.0037*), however estrogen-induced colony formation of the MCF-7-MKP3 clones was equivalent to those obtained with vector control cells. In contrast, the number of soft agar colonies formed in the presence of Tam was increased ~10-fold in the two MKP3-overexpressing transfectants compared to vector control cells (*p=0.001*). Both the steroidal ERα antagonist ICI182,780, and the MEK1,2 inhibitor PD98059 completely blocked Tam-induced colony formation in vector control and MKP3-transfected cells. These results suggest that whereas the basal growth of MCF-7-MKP3 cells may be negatively affected concomitant with MKP3 overexpression, Tam treatment either relieves this growth suppression or acts as an agonist to increase the colony forming efficiency of MKP3-overexpressing breast cancer cells.

We also engineered TR Ishikawa cells to stably overexpress V5-MKP3 or used a siRNA to knockdown MKP3 in this cell line (Fig. 2D and E, respectively). In Ishikawa cells, increased levels of MKP3 did not affect control colony growth, but significantly enhanced growth was seen in the estrogen and Tam-treated MKP3 transfectants (Fig 2D, lower panel, *p= 0.03 and p=0.001*, respectively). Conversely, when relative MKP3 RNA levels were decreased by approximately 45% using siRNA (Fig. 2E, upper panel, *p<0.05*), there was a decrease in Tam colony growth in the Tam treated group (Fig. 2E, lower panel, *p=0.02*). MKP3 siRNA did not affect estrogen-stimulated growth however in Ishikawa cells.

To analyze the endocrine sensitivity of MKP3-overexpressing cells, we next examined their ability to form tumors in athymic nude mice. We established xenografts in ovariectomized mice supplemented with estrogen for 21 days. Mice were then randomized to continue estrogen treatment or subjected to estrogen withdrawal plus Tam (Fig. 2F and G), and tumor growth was monitored over time. The main questions we addressed were whether MKP3-expressing tumors grew differently in the presence of estrogen, and whether they responded similarly to Tam treatment. This was examined by fitting the data in an exponential growth model, and testing whether growth rates were different between the groups; the analyses were done separately for estrogen-treated (Fig. 2F) and Tam-treated animals (Fig. 2G). We found that although MKP3-expressing tumors were slightly larger in size at 21 days compared to control tumors, there was no difference in the estrogen-stimulated exponential growth rate of the MCF-7 vector control transfected and MKP3-overexpressing cells ($P=0.52$). In contrast, the growth rate of MKP3-expressing cells was significantly increased in Tam-treated tumors ($P=0.047$). These *in vivo* data thus recapitulate what we observed in the soft agar assay in MCF-7 and Ishikawa cells, and with our discovery of higher levels of MKP3 RNA in Tam-resistant breast tumors.

Cross-Talk between MKP3, ERK1,2 MAPKs, and the ERα Signaling Pathways

Since there are conflicting reports concerning whether estrogen stimulation activates MAPK in MCF-7 breast cancer cells (14, 15), we next assessed the effects of estrogen and Tam on the activation of MAPK in MCF-7 vector controls and MKP3 transfectants (Fig. 3A, Con 1 and 2, and MKP3-1 and 2). Cells were maintained under estrogen-depleted conditions for 2 days, treated for 2 hours with either estrogen or Tam, and cellular extracts prepared. Immunoblot analysis with anti-V5 was used to detect ectopic MKP3 expression in the two MKP3 transfectants (Fig. 3A, top panel); immunoblotting with an antibody to Rho GDI was used as a loading control in these experiments. In vector-alone transfectants, pMAPK was not induced with either short-term (2-30 minutes, data not shown), or two hours of hormonal treatment (Fig. 3). In contrast, higher levels of pMAPK were seen in the control and Tam-treated MKP3-overexpressing cells compared to that seen in the estrogen-treated cells (Fig. 3A and B). These results were rather paradoxical, in that we predicted to find lower levels of pMAPK in cells concomitant with MKP3 overexpression, but instead observed hormonal influences on the ability of overexpressed MKP3 to modulate pMAPK. Levels of total MAPK did not appear to be affected by MKP3 overexpression. The highest activation of MAPK was observed in the Tam-treated MKP3-overexpresing cells (graphically represented in Fig. 3B).

ERα is a downstream target of pMAPK in breast cancer cells (3). We observed that levels of phosphorylation at ERα serine 118 (pS118 ERα) were highly induced with Tam treatment in the MKP3 transfectants (Fig. 3A and C). The question is whether this dramatic induction with Tam was coupled with changes in the levels of total ERα? It has been demonstrated that ERα undergoes estrogen-dependent down regulation via the proteasomal degradation pathway (16). Down regulation of ERα protein was observed in both control transfected and the MKP3 overexpressing cells in the presence of estrogen. Similarly, it has been reported that Tam stabilizes the receptor (17), and this stabilization was observed in both groups of transfectants. Thus, although ERα hormonal regulation appeared to proceed normally,

higher levels of pERα were induced by Tam in the MKP3 overexpressing cells, which was not associated with higher levels of total ERα as compared to control treated cells.

We next tested the effect of the MEK1,2 inhibitor PD98059 on the ability of Tam to induce phosphorylation of MAPK and ERα in MKP3 overexpressing cells (Fig. 3D). We found that PK98065 effectively inhibited the increase in pMAPK and pS118 ERα in control and Tam-treated MKP3.2 cells. The MEK inhibitor also blocked activation of MAPK in vector control cells under all the treatment conditions. This result suggests that Tam's effects in MKP3 overexpressing cells involve the MEK-ERK MAPK signaling pathway.

As a control we examined the levels of MKP1, which is also a major negative regulator of ERK1/2, but is more specific for JNK and p38, but did not see changes in the levels of MKP1 concomitant with MKP3 overexpression (Fig.3E). We observed an increase in pJNK in MKP3 transfectants under all hormonal conditions. When vector control cells were treated with the MEK1,2 inhibitor, pJNK was elevated, and similarly we found higher levels of JNK activation in MKP3 transfectants in the presence of the inhibitor. These results were not expected, and suggest that the MEK inhibitor might affect the decreased levels or activity of another MKP which could up-regulate pJNK. This possibility is currently under study, but suggests that treatment of breast cancer cells with MEK inhibitors might concomitantly increase JNK signaling in cells, a consequence which might have therapeutic relevance in resistant disease necessitating combination therapy with signal transduction inhibitors.

We next questioned whether either changes in MKP3 phosphatase activity or changes in binding between MKP3 and ERK2 might underlie the changes in pMAPK. Using the artificial substrate pNPP to measure endogenous phosphatase activity in MKP3 and vector control cells, we found an inverse relationship between measured MKP3 phosphatase activity and pMAPK levels in the MKP3 transfectants compared to the vector control. As shown in Fig. 3F, levels of MKP3 phosphatase activity were highest in the estrogen-treated, lowest in Tam, and intermediate in the control-treated cells. These findings in activity were inversely related to the levels of pMAPK in MKP3 overexpressing cells (Fig. 3A). These results suggest that Tam might influence the ability of MKP3 to negatively regulate MAPK. We did not however observe changes in the ability of MKP3 to bind to MAPK as measured by co-immunoprecipitation and immunoblot analysis (Fig. 3G). When the levels of MKP3-bound ERK2 were compared between the different hormonal treatments, no differences were detected (C, E, T, IP:V5 lanes). Control levels of MKP3 and ERK2 were also examined in the pre-IP and post-IP extracts to ensure that adequate pulldown of IP proteins were obtained; no differences were detected. Thus, hormonal modulation of MKP3 phosphatase activity, but not changes in the ability of MKP3 to interact with MAPK may be a determinant of MAPK activation in breast cancer cells.

ER Regulated Gene Transcription

CCND1 is an estrogen-induced protein, and is also a downstream marker of activated MAPK signaling in breast cancer cells (18). High levels of CCND1 were found in Tam-treated MKP3 overexpressing cells using immunoblot analysis (Fig. 4A, top panel); densitometric scanning of the CCND1 immunoblot is shown in Fig. 4B. Thus, the Tam-

stimulated soft agar growth and xenograft growth we observed concomitant with MKP3 overexpression was coupled with the induction of the MAPK downstream marker of proliferation, CCND1. These results suggest that the Tam-resistant phenotype of the MKP3 overexpressing cells is associated with a biomarker of clinical resistance, elevated CCND1, downstream of MAPK activation. We also evaluated the RNA levels of CCND1 and c-fos, that is a known downstream marker of MAPK activation, in our TR patient samples, and found significant correlations between CCND1 (Rsp=0.83, *p=0.0053*), c-fos (Rsp=0.74, *p=0.01*) and MKP3 (data not shown).

We have previously shown that prolonged treatment of MCF-7 cells grown as xenografted tumors in athymic nude mice results in oxidative stress (19), and it is well known that Tam can induce oxidative stress and increase reactive oxygen species (ROS) (20). Furthermore, during oxidative stress MKP3 phosphatase is inactivated in cortical neurons (21). To explore mechanisms associated with MKP3 TR, we therefore measured ROS using a flurogenic probe in MKP3 overexpressing MCF-7 cells after hormonal treatments (Fig. 5A). Levels of ROS were significantly higher in both the control (serum deprived) and Tam-treated, but lowest in the estrogen-treated cells (one-way Anova p=0.0029). Furthermore, treatment with the antioxidant glutathione (GSH) increased MKP3 phosphatase activity in control (*p=0.026*) and Tam-treated MKP3 overexpressing cells (*p=0.0012*) (Fig. 5B), but did not affect total levels of exogenous MKP3 (Fig. 5C). Treatment with GSH also effectively inhibited TR growth in soft agar, but did not affect either control or estrogen-induced growth (Fig. 5D). In agreement with the reversal of resistance after treatment with the antioxidant GSH, we observed inhibition of activated MAPK, and reduction in pS118 ERα levels with combined GSH and Tam treatment of MKP3.2 transfectants (Fig. 3E). These combined results suggest that increases in ROS which can be reversed with an antioxidant may underlie the resistant phenotype in our model, and furthermore that MKP3 activity and downstream signaling in TR cells are sensitive to changes in intracellular redox status in breast cancer cells.

MKP3 is a member of a large family of MAPK phosphatases with different specificities, so we next explored whether this identified TR mechanism extended to the prototypical dual specificity phosphatase MKP1. This is of interest because MKP1 activity can be modulated by mitogens and oxidative stress (4). Using two siRNAs to MKP1 we were able to effectively knockdown MKP1 levels in Ishikawa cells (Fig. 6A). Reduction of MKP1 levels in the two MKP1 siRNA clones si615 and 1126 resulted in increased TR growth of Ishikawa cells in soft agar compared to a control siRNA clone (Fig. 6B, *p=0.0031*). Levels of pMAPK were elevated after both estrogen and Tam treatment in MKP1 siRNA Ishikawa clones (Fig. 6C). Thus, enhanced Tam activation of MAPK was associated with decreased MKP1 levels, but since MKP1 is at least as effective in phosphorylating JNK and p38 as MAPK, the precise role of MKP1 in TR remains to be elucidated. We conclude that activation of MAPK in the presence of Tam is important for the resistant phenotype associated with MKPs.

Discussion

Tam has been the most frequently prescribed antiestrogen for the treatment of women with early-stage and metastatic ERα-positive breast cancer. Although many patients will initially benefit from Tam treatment, the emergence of resistance is a major clinical problem. It has been hypothesized that growth factor receptor pathways might impact on ERα activity, and hence Tam effectiveness. Increased dependence on MAPK signaling has been previously demonstrated to be important for both TR and adaptive resistance to estrogen deprivation in MCF-7 cells (22, 23). We identified MKP3, a negative regulator of MAPK, as being expressed at higher levels in Tam-resistant metastatic lesions, and demonstrated in preclinical studies that its overexpression can confer Tam-resistant growth of cells *in vitro* or as xenografts in athymic nude mice.

We employed microarray expression profiling to identify genes associated with Tam resistance in breast cancer patients as a means of exploring new regulatory mechanisms operative during the selective pressure of Tam treatment. Recently a two-gene expression Tam prediction model was developed using microarray analysis comparing primary breast tumors from patients treated with adjuvant Tam who remained disease-free, to those patients who developed distant recurrence (24). Although these results have been recently challenged (25), microarray technologies have shown great promise in identifying molecular features of hormone responsiveness (26). Our study differed in both experimental design and goal compared to the microarray study of Ma et al. (24). Although we used a similarly-defined Tam sensitive group of primary tumors, we chose to compare these to metastatic lesions from patients who recurred while on Tam, with the goal to identify gene candidates that we could then examine further for a mechanistic role in resistance. Neither Ma et al. (24), or a similarly designed microarray study reported by Jansen et al. (26) found MKP3 RNA levels to be differentially associated with the outcome of Tam-treated primary tumors. We attribute this difference to be due to either experimental design, or in the diverse array platforms utilized between the studies. Interestingly, we found that the levels of pMAPK protein were highest in Tam-treated and lowest in the presence of estradiol when MCF-7 cells were engineered to overexpress MKP3. We found that MEK inhibition reversed Tam-induced soft agar growth of MKP3 overexpressing MCF-7 cells, further implicating MAPK signaling in resistance in our model. These results suggest that activated MAPK remains a common component of Tam-resistant growth in preclinical models.

We demonstrated that MKP3 enzymatic activity was particularly sensitive to regulation by hormones, and we propose that its hormonal modulation may be an alternative and novel mechanism by which ERK1,2 can be activated and regulated in breast cancer cells. Mechanistically our data demonstrate that this hormonal regulation is associated with alterations in the redox state of the cell, with high ROS coincident TR growth and low MKP3 activity. The use of an antioxidant to reverse resistance confirms the role of oxidative stress in the process. Several of the MKPs are known to be induced following exposure to stress and/or growth factor stimulation (4), however MKP3 has not been previously demonstrated to be regulated by these stimuli. Recently, it has been shown that the activity of MKP3, rather than its expression, was downregulated by oxidative signals (21), possibly through oxidizing critical cysteine residues.

There is also evidence for multiple, temporally discrete pathways which differentially regulate MAPK depending on the external stimulus. For instance, fibroblast growth factor (FGF) 1 and heregulin β1-induced TR in MCF-7 cells is also associated with prolonged MAPK activation that is incompletely susceptible to MEK inhibitors (27). Our results suggest that Tam increases ERK1,2 activity via the loss of MKP3 phosphatase activity, an alternative "off-off" mechanism of resistance which remains sensitive to MEK inhibition. Therefore, patients with Tam-resistant disease and elevated MKP3 may be markedly sensitive to MEK inhibitors or antioxidants.

ERα expression is lost in a minority of recurrent metastatic lesions after Tam treatment (28). Thus, our model is different from hyperactivation of the Raf1/MEK1 signaling pathway which leads to a down-regulation of ERα, and potentially reflects the more common resistance mechanisms associated with continued ERα expression in patients. We have shown that PKA signaling induces ERα S305 phosphorylation, which is coupled to acetylation at K303 within the hinge domain and estrogen sensitivity (29). This site has been demonstrated to be important for Tam resistance (30) and expression of CCND1 (31). The ERα S118 site is phosphorylated in response to epidermal growth factor signaling, possibly via pMAPK (32), and by activated ERK1,2 in breast cancer cells (3). ERα S118 was phosphorylated in response to Tam in MKP3 overexpressing cells, which was associated with higher pMAPK levels. However, high levels ERα pS118 have been shown to be associated with a better disease outcome in breast cancer patients treated with Tam in one clinical study (33). Therefore the usefulness of pS118 as a clinical marker of resistance requires further study.

We hypothesize that MKP3 may be a novel target of Tam action. It is possible that breast tumors may compensate for chronic activation of MAPK by up-regulation of phosphatases, such as MKP3, that control these pathways. The emergence of Tam resistance may therefore involve the disruption of this regulatory compensatory loop by inactivation of MKP3 phosphatase activity explaining the seemingly paradoxical up-regulation of MKP3 levels, but down-regulation of its activity in Tam-treated MKP3 overexpressing cells.

We also detected activation of JNK with MEK1 inhibitor treatment of MCF-7 cells, with further enhanced JNK phosphorylation noted in our MKP3 overexpressing cells. Whether down-regulation of other MKPs may be associated with this effect, and whether combined MAPK inhibitors with Tam treatment are efficacious in our model is currently under study in our laboratory. It is tempting to speculate that MKP3 expressing tumors might be sensitive to estrogen deprivation (aromatase inhibitor treatment), given our finding that there was limited growth of MKP3 overexpressing breast cancer cells in the absence of estrogen. In summary, this study suggests that MKP3 may be an attractive new diagnostic and potentially therapeutic target in breast cancer.

Acknowledgements

References

1. Kurokawa H, Lenferink AE, Simpson JF, et al. Inhibition of HER2/neu (erB-2) and mitogen-activated protein kinass enhances tamoxifen action against HER2-overxpressing, tamoxifen-resistant breast cancer cells. Cancer Res. 2000; 60:5887–94. [PubMed: 11059787]

2. Gee JM, Robertson JF, Ellis IO, et al. Phosphorylation of ERK1/2 mitogen-activated protein kinase is associated with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. Int J Cancer. 2001; 95:247–54. [PubMed: 11400118]

3. Kato S, Endoh H, Masuhiro Y, et al. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science. 1995; 270:1491–4. [PubMed: 7491495]

4. Camps M, Nichols A, Arkinstall S. Dual specificity phosphatases: a gene family for control of MAP kinase function. Faseb J. 2000; 14:6–16. [PubMed: 10627275]

5. Zhou B, Wu L, Shen K, et al. Multiple regions of MAP kinase phosphatase 3 are involved in its recognition and activation by ERK2. J Biol Chem. 2001; 276:6506–15. [PubMed: 11104775]

6. Nichols A, Camps M, Gillieron C, et al. Substrate recognition domains within extracellular signal-regulated kinase mediate binding and catalytic activation of mitogen-activated protein kinase phosphatase-3. J Biol Chem. 2000; 275:24613–21. [PubMed: 10811804]

7. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. Genome Biology. 2001; 2:32.1–32.11.

8. Wright GW, Simon RM. A random variance model for detection of differential gene expression in small microarray experiments. Bioinformatics. 2003; 19:2448–55. [PubMed: 14668230]

9. Shah YM, Rowan BG. The Src kinase pathway promotes tamoxifen agonist action in Ishikawa endometrial cells through phosphorylation-dependent stabilization of estrogen receptor (alpha) promoter interaction and elevated steroid receptor coactivator 1 activity. Mol Endocrinol. 2005; 19:732–48. [PubMed: 15528270]

10. Fuqua SAW, Wiltschke C, Zhang QX, et al. A hypersensitive estrogen receptor-α mutation in premalignant breast lesions. Cancer Research. 2000; 60:4026–29. [PubMed: 10945602]

11. Furukawa T, Sunamura M, Motoi F, et al. Potential tumor suppressive pathway involving DUSP6/MKP-3 in pancreatic cancer. Am J Pathol. 2003; 162:1807–15. [PubMed: 12759238]

12. Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, et al. Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. J Natl Cancer Inst. 1995; 87:746–50. [PubMed: 7563152]

13. Hiscox S, Morgan L, Green TP, et al. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. Breast Cancer Res Treat. 2005; 1:12.

14. Lobenhofer EK, Marks JR. Estrogen-induced mitogenesis of MCF-7 cells does not require the induction of mitogen-activated protein kinase activity. J Steroid Biochem Mol Biol. 2000; 75:11–20. [PubMed: 11179904]

15. Migliaccio A, Di Domenico M, Castoria G, et al. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO Journal. 1996; 15:1292–300. [PubMed: 8635462]

16. Nawaz Z, Lonard DM, Dennis AP, et al. Proteasome-dependent degradation of the human estrogen receptor. Biochemistry. 1999; 96:1858–62.

17. Wijayaratne AL, McDonnell DP. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem. 2001; 276:35684–92. [PubMed: 11473106]

18. Doisneau-Sixou SF, Sergio CM, Carroll JS, et al. Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocr Relat Cancer. 2003; 10:179–86. [PubMed: 12790780]

19. Schiff R, Reddy P, Ahotupa M, et al. Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors in vivo. J Natl Cancer Inst. 2000; 92:1926–34. [PubMed: 11106684]

20. Mandlekar S, Kong AN. Mechanisms of tamoxifen-induced apoptosis. Apoptosis. 2001; 6:469–77. [PubMed: 11595837]

21. Levinthal DJ, Defranco DB. Reversible oxidation of ERK-directed protein phosphatases drives oxidative toxicity in neurons. J Biol Chem. 2005; 280:5875–83. [PubMed: 15579467]

22. Larsen SS, Egeblad M, Jaattela M, et al. Acquired antiestrogen resistance in MCF-7 human breast cancer sublines is not accomplished by altered expression of receptors in the ErbB-family. Breast Cancer Res Treat. 1999; 58:41–56. [PubMed: 10634517]

23. Song RX, Santen RJ, Kumar R, et al. Adaptive mechanisms induced by long-term estrogen deprivation in breast cancer cells. Mol Cell Endocrinol. 2002; 193:29–42. [PubMed: 12160999]

24. Ma XJ, Wang Z, Ryan PD, et al. A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. Cancer Cell. 2004; 5:607–16. [PubMed: 15193263]

25. Reid JF, Lusa L, De Cecco L, et al. Limits of predictive models using microarray data for breast cancer clinical treatment outcome. J Natl Cancer Inst. 2005; 97:927–30. [PubMed: 15956654]

26. Jansen MP, Foekens JA, van Staveren IL, et al. Molecular classification of tamoxifen-resistant breast carcinomas by gene expression profiling. J Clin Oncol. 2005; 23:732–40. [PubMed: 15681518]

27. Thottassery JV, Sun Y, Westbrook L, et al. Prolonged extracellular signal-regulated kinase 1/2 activation during fibroblast growth factor 1- or heregulin beta1-induced antiestrogen-resistant growth of breast cancer cells is resistant to mitogen-activated protein/extracellular regulated kinase kinase inhibitors. Cancer Res. 2004; 64:4637–47. [PubMed: 15231676]

28. Gutierrez MC, Detre S, Johnston S, et al. Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. J Clin Oncol. 2005; 23:2469–76. [PubMed: 15753463]

29. Cui Y, Zhang M, Pestell R, et al. Phosphorylation of estrogen receptor α blocks its acetylation and regulates estrogen sensitivity. Cancer Research. 2004; 64:9199–208. [PubMed: 15604293]

30. Michalides R, Griekspoor A, Balkenende A, et al. Tamoxifen resistance by a conformational arrest of the estrogen receptor alpha after PKA activation in breast cancer. Cancer Cell. 2004; 5:597–605. [PubMed: 15193262]

31. Balasenthil S, Barnes CJ, Rayala SK, et al. Estrogen receptor activation at serine 305 is sufficient to upregulate cyclin D1 in breast cancer cells. FEBS Lett. 2004; 567:243–7. [PubMed: 15178330]

32. Bunone G, Briand PA, Miksicek RJ, et al. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. Embo J. 1996; 15:2174–83. [PubMed: 8641283]

33. Murphy LC, Niu Y, Snell L, et al. Phospho-serine-118 estrogen receptor-alpha expression is associated with better disease outcome in women treated with tamoxifen. Clin Cancer Res. 2004; 10:5902–6. [PubMed: 15355923]
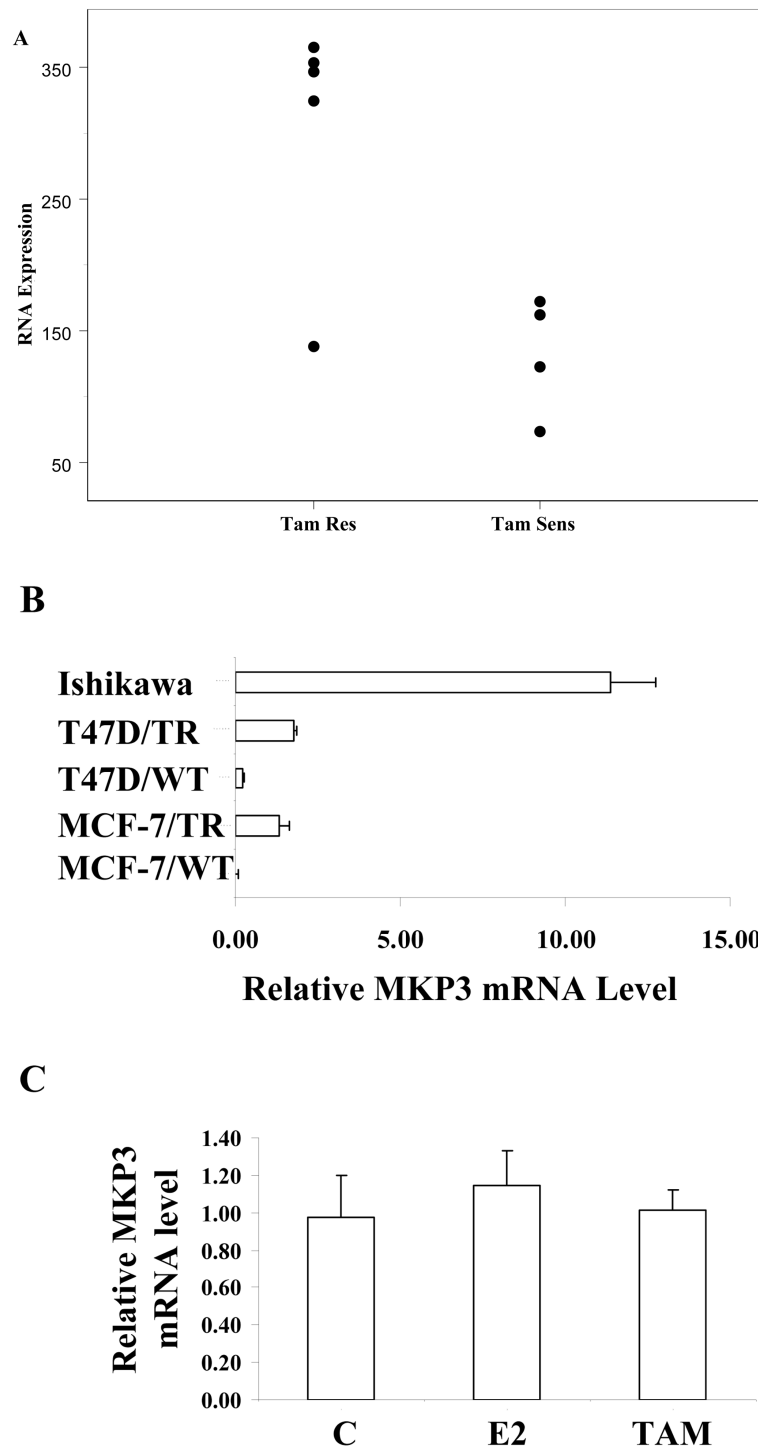
A

B

C

Fig. 1.

A. Scatter plot of MKP3 RNA expression in 9 tumors; 4 tumors were Tam sensitive and 5 were Tam-resistant. Circles show the individual RNA values in arbritary units. Parametric p-value = 0.006; permutation p-value adjusted for multiple comparisons = 0.09. B. Relative MKP3 mRNA levels were determined using qRT-PCR in various cell lines. Relative levels
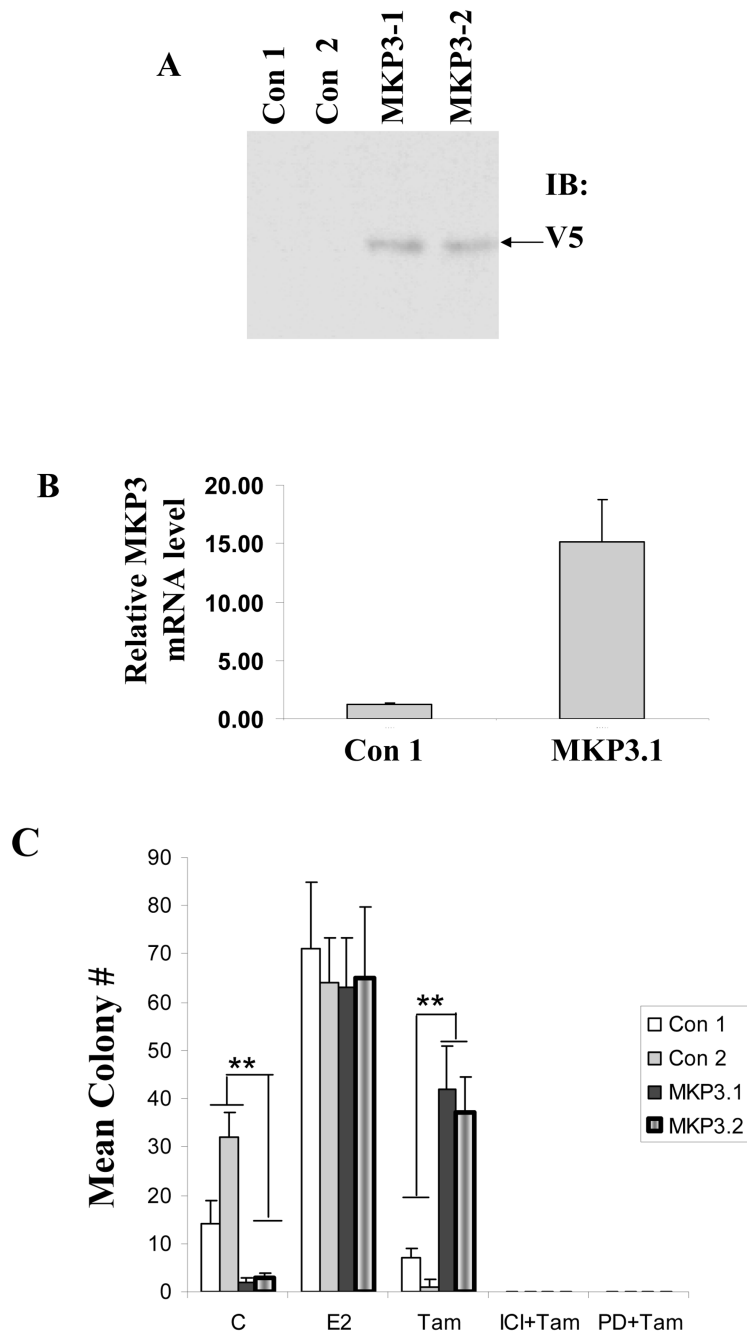
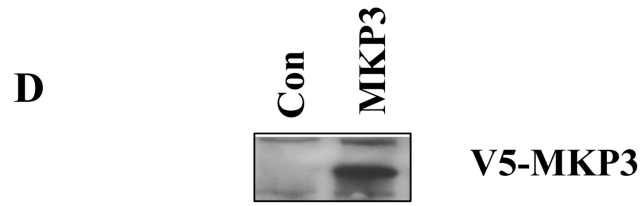were normalized to the levels of 18S ribosomal RNA in each RNA sample. C. Relative MKP3 mRNA levels of MCF-7 cells treated with estrogen (E2, 10 nM) or Tam (Tam, 100 nM) for 24 hours. Relative MKP3 RNA levels were normalized to the levels of 18S ribosomal RNA in each RNA sample.

A

Con 1 Con 2 MKP3-1 MKP3-2

IB:

← **V5**

B

Relative MKP3 mRNA level

- 20.00
- 15.00
- 10.00
- 5.00
- 0.00

Con 1   MKP3.1

C

Mean Colony #

- 90
- 80
- 70
- 60
- 50
- 40
- 30
- 20
- 10
- 0

C   E2   Tam   ICI+Tam   PD+Tam

**
**

☐ Con 1
☐ Con 2
■ MKP3.1
▣ MKP3.2

D

E

F

G
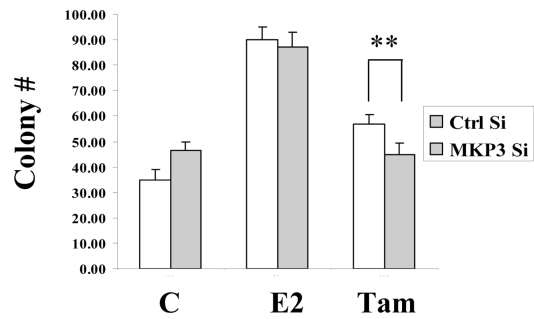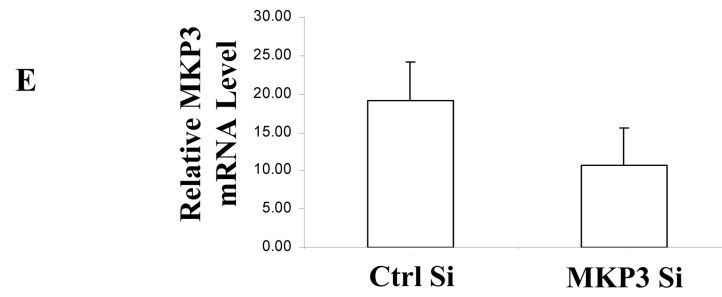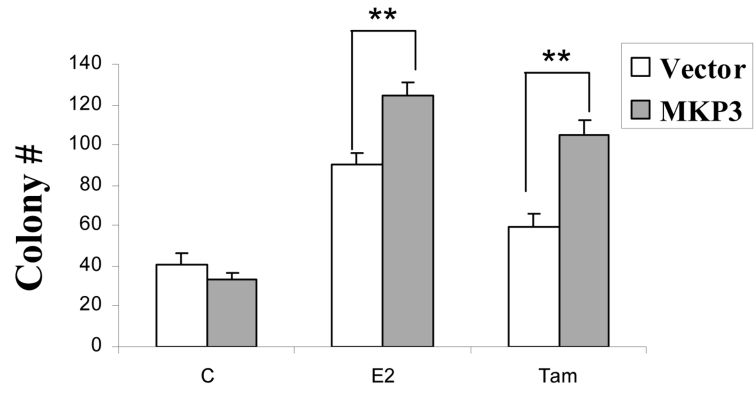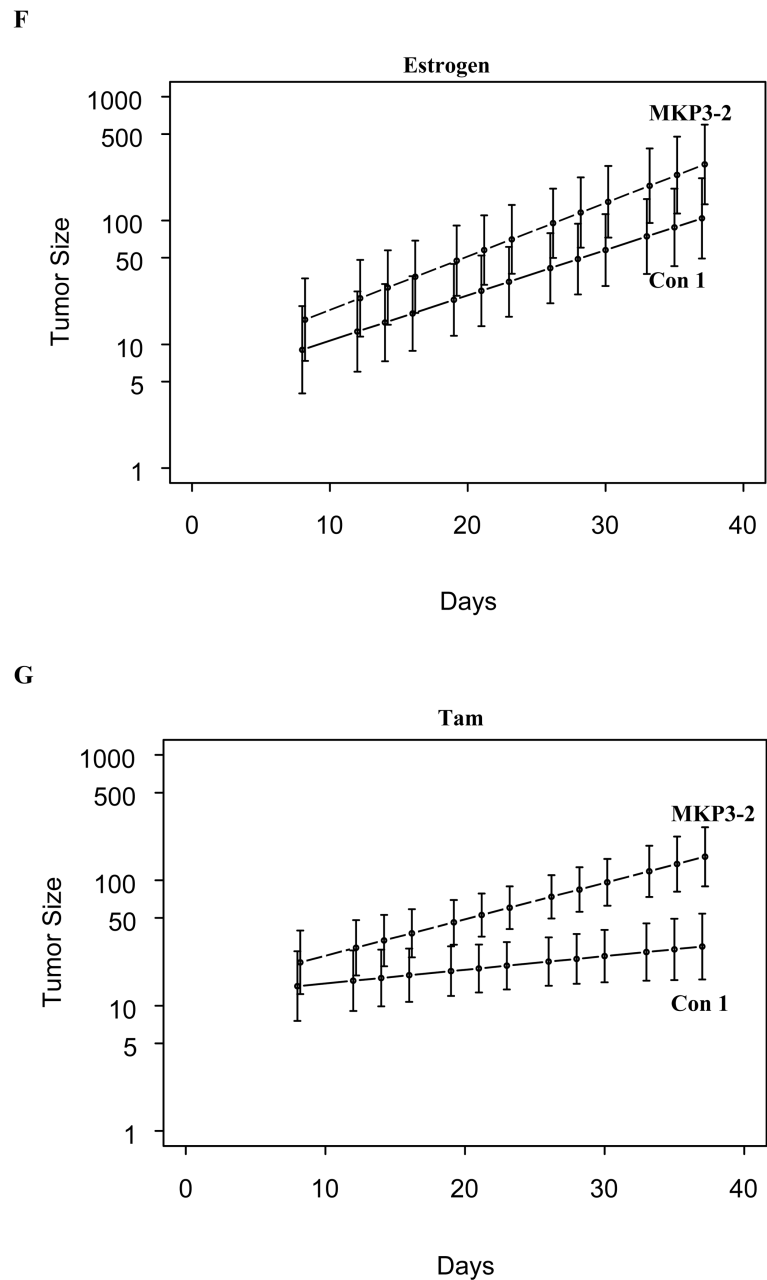
Fig. 2.
A. Immunoblot (IB) analysis of two vector-alone, control transfected MCF-7 clones (Con 1 and 2), and two MKP-3 transfected clones (MKP3-1 and 2). A V5 epitope tag was included in the expression vector and was used for visualization of MKP3 levels. B. Relative MKP3 levels in Con1 and MKP3.1 transfectants were determined using qRT-PCR. Levels were normalized to 18S ribosomal RNA in each sample. C. Anchorage-independent colony formation assay with the above transfectants in the presence of 1.0 nM estrogen (E2), 100 nM tamoxifen (Tam), 100 nM Tam + 10 nM ICI 182,780, or 100 nM Tam + 20 nM PD98059. The mean colony number was assayed after growth under the respective treatment conditions for 14 days and statistical significance was assessed using a two-tailed Student's

t-test; **significance level at p<0.05. D. Immunoblot analysis of vector-alone, control transfected Ishikawa cells (Con), and a MKP-3 transfected Ishikawa cell (MKP3) (upper panel). A V5 epitope tag was included in the expression vector and was used for visualization of MKP3 levels. Anchorage-independent colony formation assay with the Ishikawa transfectants in the presence of ethanol (C), estrogen (E2) or Tam. The mean colony number was assayed after growth under the respective treatment conditions for 14 days and statistical significance was assessed using a two-tailed Student's t-test; **significance level at p<0.05. E. Knockdown of MKP3 in Ishikawa cells was performed using a siRNA to MKP3. Relative MKP3 levels in control siRNA transfected (Ctrl Si) and MKP3 siRNA transfectants were determined using qRT-PCR (upper panel). Colony formation assays were performed in the presence of ethanol vehicle (C), 1.0 nM E2 and 100 nM Tam (lower panel). ** significance level at p<0.05 using a two-taileld Student's t-test. F. and G. MCF-7-vector control 1 and MCF-7-MKP3-2 cells were injected into athymic nude mice supplemented with E2 for 21 days, and then randomly assigned to either continue E2 treatment (F.), or to have their E2 pellets removed and were treated with Tam (G.). There were n=6 animals each for the E2-treated vector control, and MKP3-overexpressing cells; there were n=4 for Tam-treated vector control, and n=5 animals for the Tam-treated MKP3-overexpressing cells.

A

B

C

D
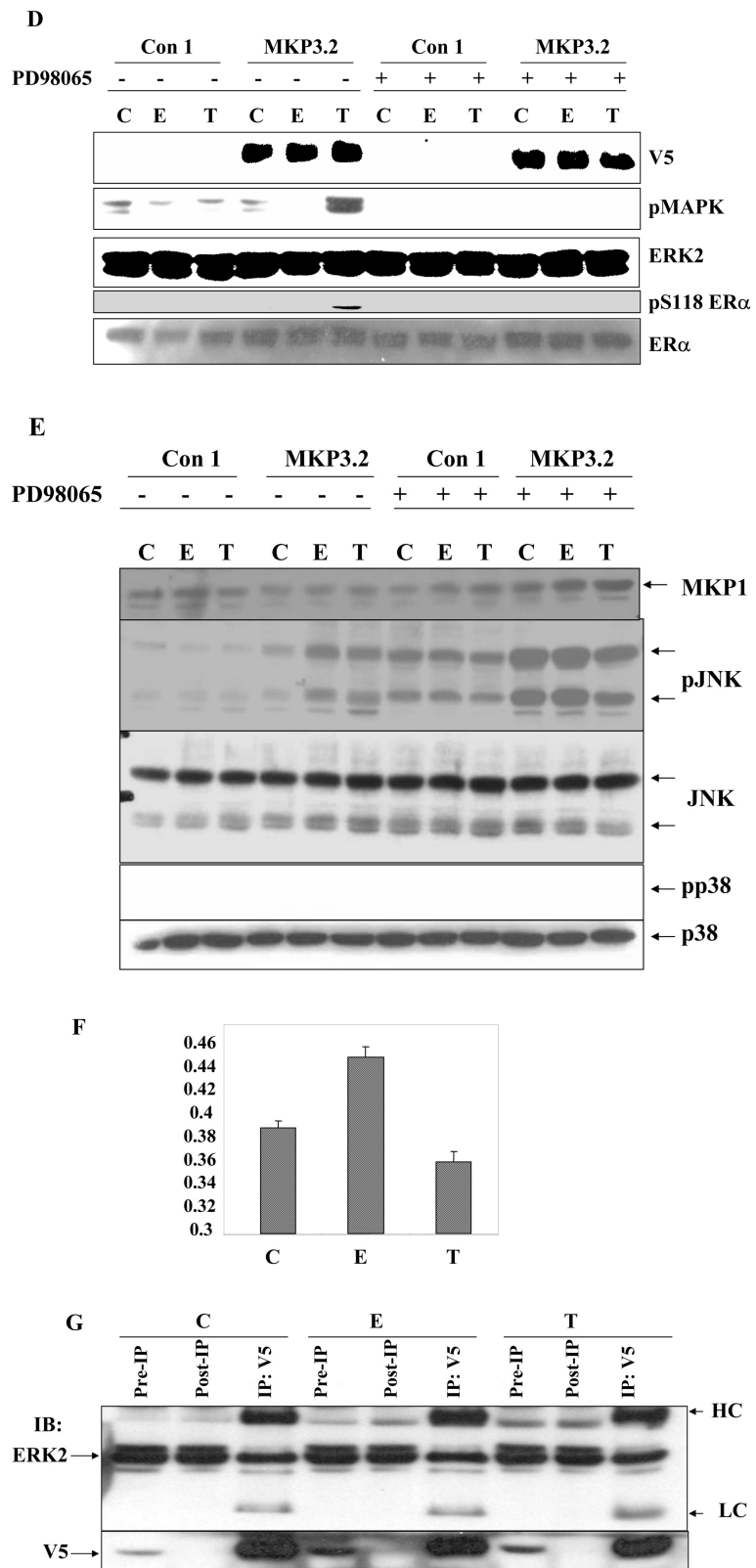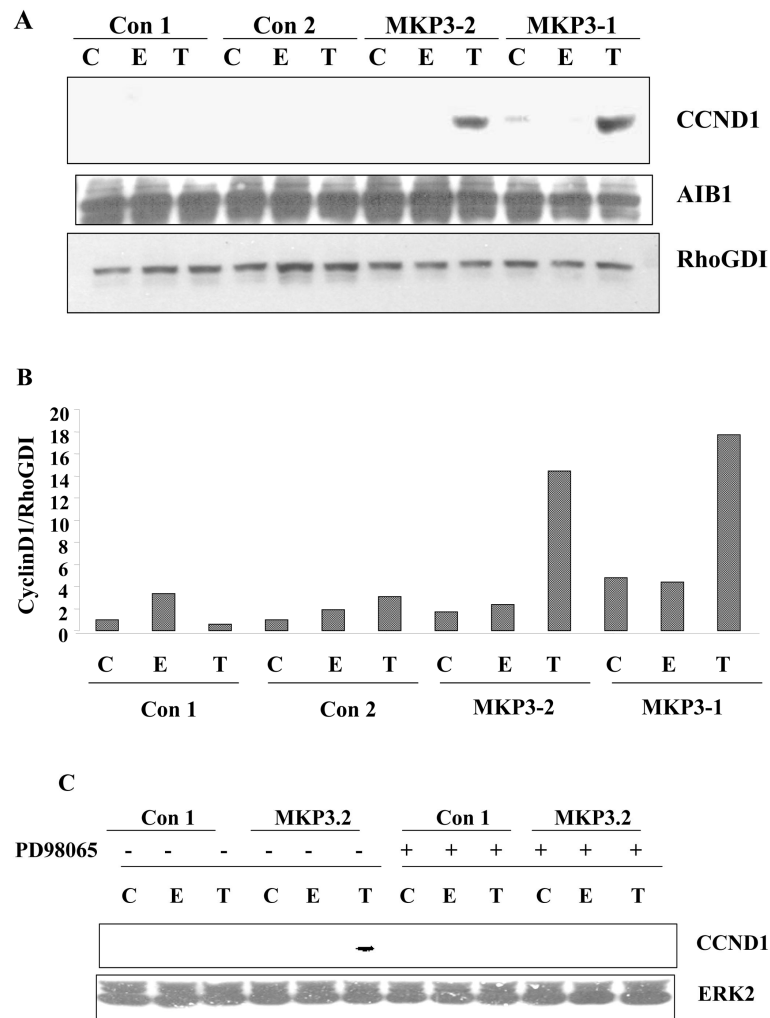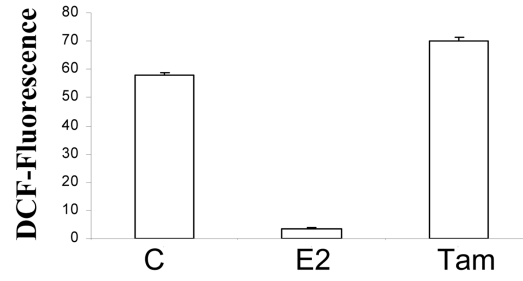

E

F

G
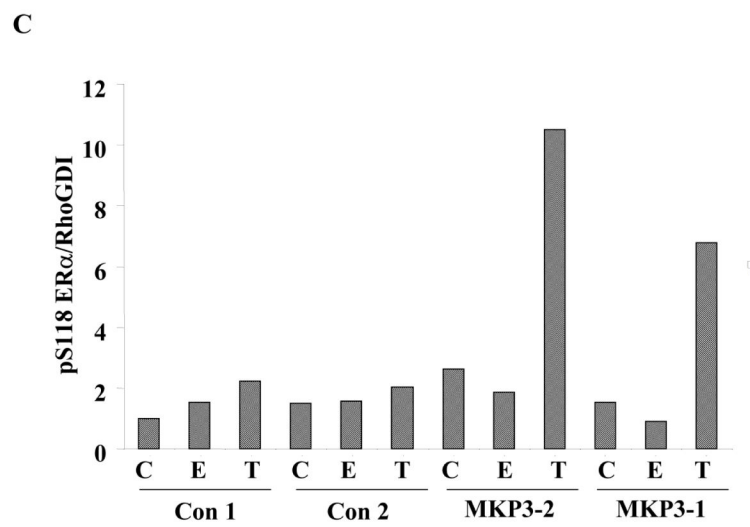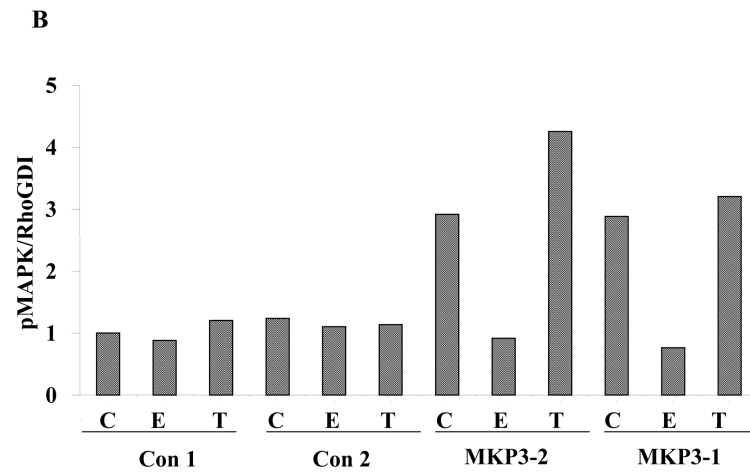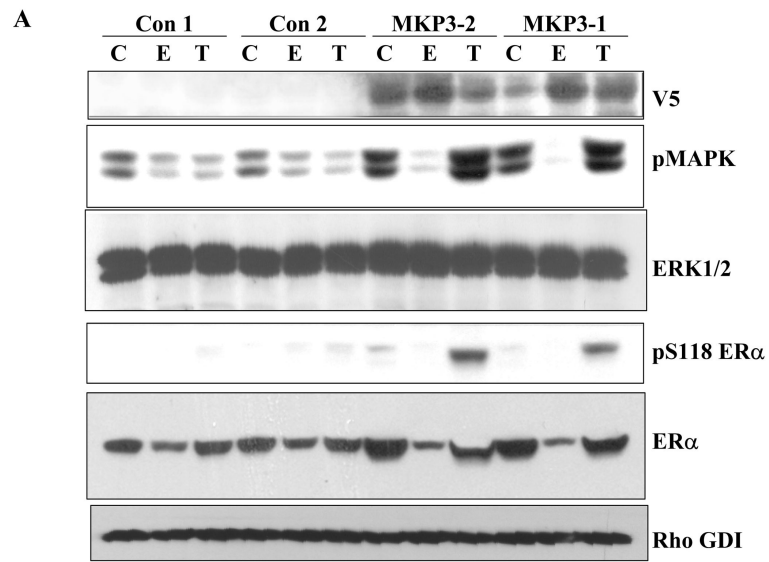
Fig. 3.

A. Immunoblot analysis of two vector control (Con 1 and 2), and two MKP3-overexpressing transfectants (MKP3-1 and 2) treated for 2 hours with ethanol vehicle (C), 100 nM E2 (E), or 100 nM Tam (T). Immunoblots were stained with antibodies to V5 to demonstrate MKP3 levels, or to pMAPK, total MAPK, ERα S118, total ERα antibodies, and anti-Rho GDI as a loading control. B. Densitometric scan of the immunoblot in panel A showing levels of pMAPK normalized to Rho GDI levels. C. Densitometric scan of the immunoblot in panel A showing levels of pS118 normalized to Rho GDI levels. D. An immunoblot analysis of MKP3 Con 1 and MKP3-2 transfectants treated with vehicle, E2, or Tam for 2 hours in the absence(-) or presence of 20 nM PD98059. Immunoblots were stained with antibodies to V5, phospho-pMAPK and S118 ERα, or total MAPK and ERα. E. An immunoblot analysis of MKP3 Con 1 and MKP3-2 transfectants treated with vehicle, E2, or Tam for 2 hours in the absence(-) or presence of PD98059. Immunoblots were stained with antibodies to MKP1, phosphoJNK, and total JNK. F. Phosphatase assay using pNPP as a substrate using extracts prepared from MKP3 vector 1 and MKP3-2 cells treated for 2 hours with vehicle, E2, or Tam. The nonenzymatic hydrolysis of the substrate was corrected (absorbance at 405 nm) by subtracting the control vector transfected immunoprecipitates, from MKP3 levels and expressed as MKP3-Vector 405 nM. Phosphatase assays were performed in triplicate, n=3 separate experiments shown. G. MKP3/MAPK binding assay was performed with MKP3 Con 1 and MKP3-2 transfectants treated for 2 hours with ethanol vehicle (C), E2 (E), or Tam (T). Pre- and Post-V5 immunoprecipitated extracts (Pre-IP and Post-IP) were immunoblotted with antibodies to ERK2 and V5 to demonstrate levels of MAPK and MKP3, arrows respectively. Immunoglobulin heavy chain (HC) and light chain (LC) are shown. The specificity of the V5 and ERK2 antibodies are shown in the previous figures.
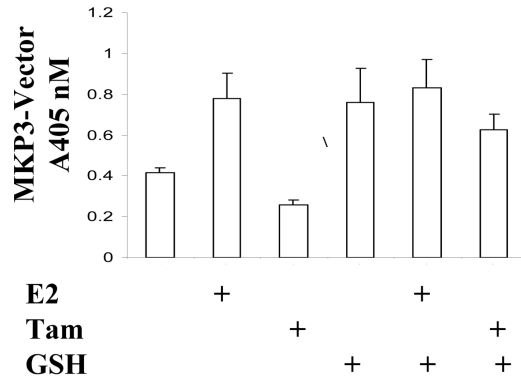
A

B

C

Fig. 4.
A. Immunoblot analysis of two vector control (Con 1 and 2), and two MKP3-overexpressing transfectants (MKP3-1 and 2) treated for 2 hours with ethanol vehicle (C), 100 nM E2 (E), or 100 nM Tam (T). Immunoblots were stained with antibodies to CCND1, and anti-Rho GDI as a loading control. B. Densitometric scan of the immunoblot in panel A showing levels of CCND1 normalized to Rho GDI levels.
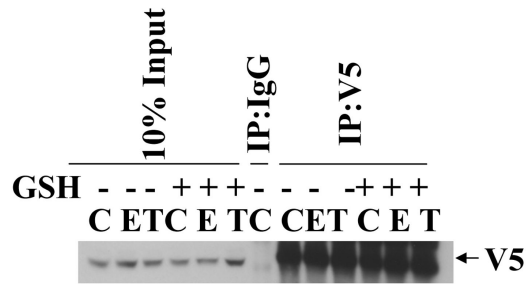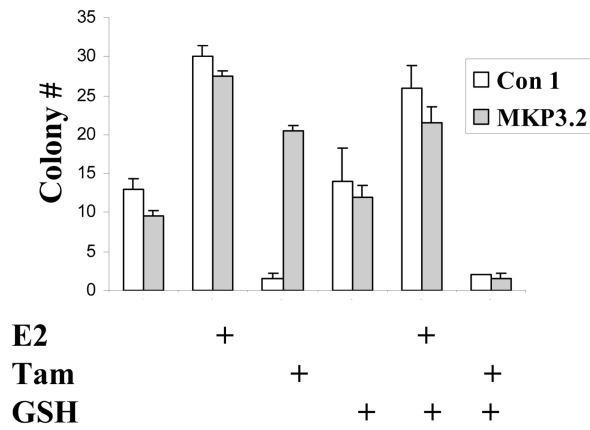
A

B

E2		+			+	
Tam			+			+
GSH				+	+	+

C

GSH - - - + + + - - - -+ + +
C ETC E TC CET C E T ← V5

D

E2		+			+	
Tam			+			+
GSH				+	+	+

E

	Con 1			MKP3.2			Con 1			MKP3.2		
GSH	-	-	-	-	-	-	+	+	+	+	+	+
	C	E	T	C	E	T	C	E	T	C	E	T

pMAPK

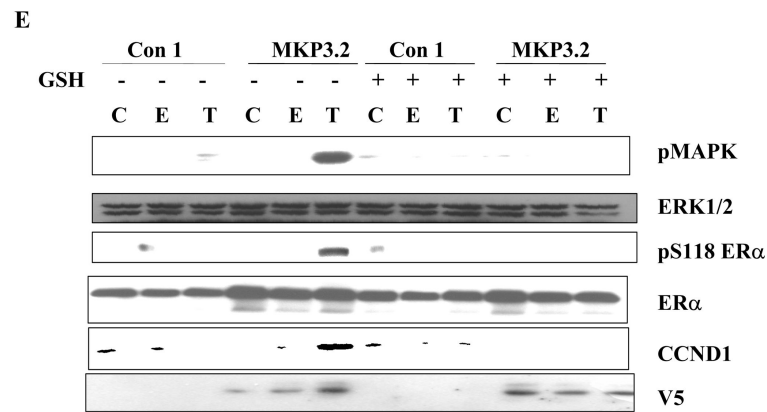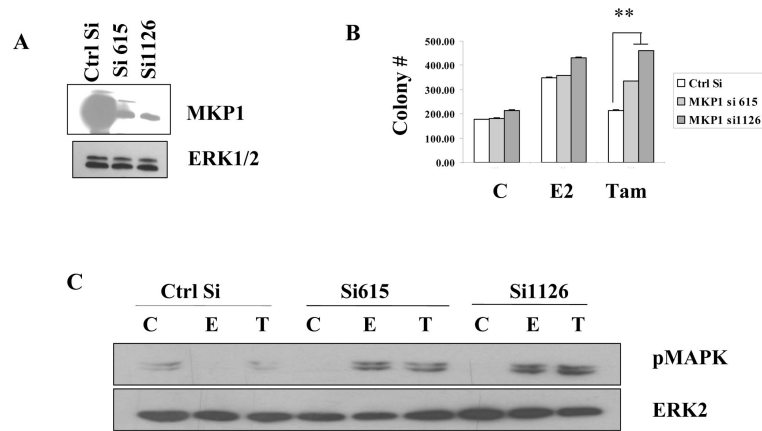ERK1/2

pS118 ERα

ERα

CCND1

V5

Fig. 5.
A. ROS levels in MKP3.2 MCF-7 cells treated for 2 hours with ethanol vehicle (C), 100 nM E2, or 100 nM Tam. were measured using the fluorogenic DCF. B. MKP3 phosphatase activity was examined in MKP3.2 and vector Con 1 control cells in the presence of hormonal treatments and GSH. C. Immunoblot of exogenous MKP3 levels in cells from panel B using an antibody to the V5 tag. D. Anchorage-independent colony formation assay with the above transfectants in the presence of vechicle, 1.0 nM E2, 100 nM Tam +/− 100 μM GSH. The mean colony number was assayed after growth under the respective treatment conditions for 14 days. E. An immunoblot analysis of MKP3 Con 1 and MKP3.2 transfectants treated with vehicle, E2, or Tam for 2 hours in the absence(-) or presence of GSH Immunoblots were stained with antibodies to pMAPK, pS118 ERα, total Erk1/2, total ERα, and V5 to detect exogenous MKP3.

Fig. 6.
A. Immunoblot analysis of vector-alone, control siRNA transfected Ishikawa cells (Ctrl Si), and two MKP1 siRNA transfected Ishikawa cell polls (Si615 and 1126). MKP1 and total Erk1/2 levels were measured. B. Anchorage-independent colony formation assay with the above Ishikawa transfectants in the presence of ethanol (C), 1.0 nM E2 or 100 nM Tam. The mean colony number was assayed after growth under the respective treatment conditions for 14 days and statistical significance was assessed using a two-tailed Student's t-test; **significance level at $p<0.05$. C. Immunoblot analysis of Ctrl SiRNA and two MKP1 siRNAs after treatment with ethanol (C), 100 nM E2, or 100 nM Tam using antibodies to pMAPK and total Erk2.