Expression And Regulation Of Map Kinase Phosphatases 1 And 2 In Breast Cancer Tamoxifen Sensitivity

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EXPRESSION AND REGULATION OF MAP KINASE PHOSPHATASES 1 AND 2 IN BREAST CANCER TAMOXIFEN SENSITIVITY

by

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DISSERTATION

Submitted to the Graduate School of Wayne State University, Detroit, Michigan

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DOCTOR OF PHILOSOPHY

2013

MAJOR: CANCER BIOLOGY

Advisor Date

Co-Advisor Date
DEDICATION

To Mom and Dad, for not letting me quit piano lessons when I thought they were too hard, for always supporting my dreams, no matter where they might take me, yet always making me feel that “there’s no place like home” and for telling me every day that I am loved more than I could ever imagine.

To Peter, for making me laugh like nobody else can and for always being excited about my work, even when I was frustrated with how it was going. You are definitely my favorite brother.

To Kayte, for encouraging me and inspiring me to always make sure that my work makes a difference. Our family is blessed to have you as part of it.

I love you all more than I could say and I couldn’t have done any of this without you.

You are the best family ever.
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Thank you to my committee members, Dr. Julie Boerner, Dr. Larry Matherly and Dr. Raymond Mattingly, for taking time out of your busy schedules to make sure that my oftentimes high maintenance project was on the right track and for giving helpful suggestions when it wasn’t. Thank you for holding me to a high standard and for being on my side.
Thank you to all the members of the Wu lab, past and present, but especially Drs. Juan Wang and Jing Xu for helping me troubleshoot experiments and telling me all the little secrets to get the protocols to work.

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LIST OF ABBREVIATIONS

AF-1: Activation function-1
AF-2: Activation function-2
DMEM: Dulbecco’s minimum essential medium
DUSP: Dual Specificity Phosphatase
E2: 17-β-estradiol
ER: Estrogen receptor
ERE: Estrogen response element
ERK: Extracellular signal-regulated kinase
FBS: Fetal bovine serum
ICI: ICI 182,780
JNK: c-Jun-N-terminal kinase
kDa: kilodalton
MAPK: Mitogen-Activated Protein Kinase
MCF7-MKP-1: MCF7 cells overexpressing MKP-1
MCF7-MKP-2: MCF7 cells overexpressing MKP-2
MCF7-TAMR: MCF7 tamoxifen resistant cells
MEFs: Mouse embryo fibroblasts
MKK: MAP kinase kinase
MKKK: MAP kinase kinase kinase
MKPs: MAP kinase phosphatases
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide
**NSCLC**: Non-small cell lung cancer

**PBS**: Phospho-buffered saline

**TAM**: 4-OH-tamoxifen
CHAPTER 1
MITOGEN ACTIVATED PROTEIN KINASES, MAP KINASE PHOSPHATASES AND CANCER

Introduction

The tight regulation of signaling is an important mechanism that a cell employs to ensure that messages received both intra- and extracellularly are being interpreted appropriately, allowing the cell to respond to cues from its environment and make the changes necessary to continue to survive or to undergo cell death. In cancer, disruption of normal cell signaling is critical to the continued growth and proliferation of tumor cells. This can happen in a number of ways, including gaining the ability to sustain proliferative signaling as well as the ability to resist cell death. Sustained proliferative signaling often takes the form of increased growth factor activity. This increase can contribute to the cell’s ability to resist cell death, but often the cell is also able to downregulate factors that would lead to apoptosis. One group of signaling molecules with close ties to cancer that contains members involved in both cell growth and cell death is the Mitogen Activated Protein Kinase (MAPK) family.

Mitogen Activated Protein Kinase Signaling: Overview

There are three major branches of MAPK signaling in mammalian cells: the extracellular signal regulated kinases (ERK), the c-Jun N-terminal kinases (JNK) and the p38 MAP Kinases (Fig. 1). ERKs are activated by growth factors and generally contribute to cell growth. JNK and the p38 MAP kinases can be activated by growth
factors, cytokines and cellular stress. The JNK and p38 pathways can be involved in both cell growth and cell death, depending on the cellular context.\textsuperscript{2-6} All three branches follow a general signaling paradigm in which a stimulus is received at the cell membrane and is transmitted through upstream adaptor molecules and enzymes to a MAP kinase kinase (MKKK). ERK has only one MKKK, Raf.\textsuperscript{2,6,7} B-Raf has been shown to be the predominant isoform involved in activating MEK.\textsuperscript{2} JNK and p38 share several MKKKs, including ASK1 (Apoptosis Signal Regulating Kinase 1), MLK2 (Mixed Lineage Kinase 2), MLK3 (Mixed Lineage Kinase 3), TAK1 (TGF-β-activating Kinase 1) and DLK (Dual Leucine Zipper Bearing Kinase). Additional JNK MKKKs include MEKK1 (MEK Kinase 1), MEKK4 (MEK Kinase 4), MLK1 (Mixed Lineage Kinase 1), MLK4 (Mixed Lineage Kinase 4) and ZAK (Zipper Sterile-α Motif Kinase).\textsuperscript{2,6,7} Phosphorylation of MKKK, in turn, allows for the phosphorylation of specific MAP kinase kinases (MKK). MEK activates ERK, MKK4/7 activates JNK and MKK3/6 activates p38.\textsuperscript{2,6,7} In order to be activated, each MAPK must be dually phosphorylated on threonine and tyrosine residues in a TXY motif.\textsuperscript{5,6,8,9} Once activated, the MAPKs go on to phosphorylate a wide variety of transcription factors, enabling them to affect the transcription of their respective target genes.\textsuperscript{7}

There are multiple isoforms that make up each branch of the MAP Kinase family: six ERKs, three JNKs and four p38s. The ERK branch is made up of ERK1-5 and ERK7/8.\textsuperscript{2} ERK1 and ERK2 are the best characterized and considered the “classical” members of the family. These two kinases share an 83\% sequence homology and are regulated by many of the same factors.\textsuperscript{2} The amino acid activation motif for ERK1 and
ERK2 is T-E-Y. ERK3 and ERK4 are unique in that their activation motif is S-E-G, suggesting that these two kinases do not require dual phosphorylation for activation. Little else is known about either of these kinases.\(^2\) ERK5 has the same activation motif as ERK1 and ERK2 and has been shown to regulate cell survival and proliferation.\(^7\)

Along with ERK3 and ERK4, ERK7/8 is considered an atypical MAPK. It has a T-E-Y activation motif similar to the conventional MAPKs, ERK1/2 and ERK5, but it is unique in that it has been shown to be constitutively phosphorylated, perhaps via autophosphorylation.\(^7\) There are three different JNK isoforms, JNK1, JNK2 and JNK3. These kinases have a T-P-Y activation motif.\(^2\) JNK1 and JNK2 are broadly expressed, with JNK3 displaying a more restricted tissue distribution.\(^7\) JNK1 and JNK2 are known to regulate the cell cycle via its phosphorylation of c-Jun, which forms part of the AP-1 transcription factor. These kinases also play an important role in regulating apoptosis in response to cell stress.\(^7\) The p38 branch of the MAP Kinase family has four members, p38\(\alpha\), p38\(\beta\), p38\(\gamma\) and p38\(\delta\). p38\(\alpha\) is the predominant isoform.\(^7\) The activation motif for p38 is T-G-Y. The p38 MAPKs are known to be strongly activated by cell stress and inflammatory cytokines and to negatively regulate the cell cycle.\(^7\)

**MAP Kinase Phosphatases: Overview**

Due to the wide ranging effects of MAP Kinase activation, it is important that there are mechanisms in place to attenuate their signals. This is partially accomplished by a family of dual specificity phosphatases (DUSPs) called MAP kinase phosphatases (MKPs), which are the endogenous negative regulators of MAPKs (Fig. 1).
Figure 1. MAP kinase signaling. The three branches of the MAP kinase signaling family in mammalian cells are activated by stimuli at the cell surface. MAP kinase kinase kinases relay the signal to MAP kinase kinases, which activate ERK, JNK, and p38. The phosphorylation of their respective targets completes the cascade. MAP kinase phosphatases are endogenous negative regulators of MAP kinases. MKPs attenuate the signal by dephosphorylation and prevent MAPKs from carrying out their cellular functions. (Figure reproduced from Haagenson, K. K. & Wu, G. S. The role of MAP kinases and MAP kinase phosphatase-1 in resistance to breast cancer treatment. Cancer Metastasis Rev 29, 143-149, (2010).)
MKPs are dual specificity threonine-tyrosine phosphatases that recognize the TXY motif present in the MAPKs.\textsuperscript{8} There are eleven MKP family members, which can be grouped by subcellular localization and substrate specificity (Table 1). There are four nuclear MKPs: MKP-1 (DUSP1), MKP-2 (DUSP4), PAC-1 (Phosphatase of Activated Cells, DUSP2) and hVH3 (Human VH-1-like Clone 3, DUSP5). MKP-1 has been reported to dephosphorylate ERK1/2, JNK1/2 and p38\textsuperscript{10,11}, but a subsequent study, in which MKP-1 was conditionally expressed from the human metallothionein IIA promoter in U937 cells, revealed a slightly more complex picture. Conditional expression of MKP-1 confirmed that the phosphatase could inhibit ERK, JNK and p38, but when MKP-1 levels were titrated, JNK and p38 were more sensitive to MKP-1 inhibition than ERK, suggesting that these MAPks are the preferred substrates of MKP-1.\textsuperscript{12} MKP-2 has also been shown to dephosphorylate each of the three MAPKs at high concentrations, but when transfected into cells under conditions more closely resembling physiological levels it was shown to be active toward ERK and JNK, but showed little activity toward p38.\textsuperscript{11} PAC-1 shows specificity for ERK and p38, while hVH3 is primarily an ERK specific phosphatase.\textsuperscript{2,5,6,8} A second group of MAPks containing MKP-3 (DUSP6), MKP-4 (DUSP9) and MKP-X, is found in the cytoplasm. With the exception of MKP-4, which demonstrates activity toward both ERK and p38, this group exclusively targets ERKs.\textsuperscript{2,5,6,8,13} The third group of MKPs can claim MKP-5 (DUSP10), MKP-7 (DUSP16) and hVH5 (DUSP8) as its members and can be found in both the nucleus and the cytoplasm. These three MKPs dephosphorylate JNK and p38.\textsuperscript{2,5,6,8,13,14} Although the MKPs might appear to be functionally redundant, they are known to display restricted
<table>
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<td>hVH5 (DUSP-8)</td>
<td>Nucleus/Cytoplasm</td>
<td>JNK, p38</td>
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**Table 1.** MAP Kinase phosphatase family members. The members of the MKP family, along with their subcellular localization and substrate specificity.
tissue expression.²,⁸,¹⁴

The final member of the MKP family is MK-STYX, which is an inactive phosphatase. It is grouped with the other MKPs based on structural similarity.⁵ The predominant features used to classify phosphatases as MKPs (Fig. 2) are the presence of a C-terminal catalytic domain and an N-terminal domain that contains two regions of sequence homology to the catalytic domain of the cdc25 phosphatase.⁵,⁶,¹³,¹⁵ Each of the MKPs has a kinase interaction motif between the two cdc25 homology domains. MKP-1, hVH3 and MKP-7 also have nuclear localization sequences. The three cytoplasmic MKPs, along with MKP-7 and hVH5, contain nuclear export sequences. MKP-7 and hVH5 also have PEST domains.¹³ The active sites of the MKPs contain a catalytically critical cysteine residue present in a signature phosphatase motif: -HCXXXXXR-. These residues work together with a conserved aspartate to facilitate the dephosphorylation of the MAPKs.¹⁶ A substrate induced activation model has been proposed for MKPs in which binding to the MAPK via a MAPK binding domain alters the interaction between the binding domain and the dual specificity phosphatase domain, which contains the active site. This conformational change positions the catalytic domain for optimal dephosphorylation of the MAPKs¹⁶ and the structure/function relationship enables precise termination of MAPK signaling. Thus, the interplay between MAPKs and MKPs helps to maintain the delicate balance of signals present in the cell and allows it to respond to changing environmental cues. The inactivation of MKPs is not an area that has been widely studied. It has been shown that MKP-1¹⁷ and MKP-2¹⁸,¹⁹ are able to be
Figure 2. Structural domains of the MKPs. “Classification, domain structure and phylogenetic analysis of the dual-specificity MAPK phosphatases. (A) Domain structures of the ten catalytically active DUSP proteins and MK-STYX. In addition to the N-terminal non-catalytic domain containing the Cdc25/rhodanese-homology region and the catalytic site, the positions of the conserved kinase interaction motif (KIM), nuclear localization signals (NLS), nuclear export signals (NES) and PEST sequences are indicated. The three subgroups revealed by the phylogenetic analysis are indicated by the background color. (B) DUSP sequence analysis. Human DUSP amino acid sequences were aligned using CLUSTALW and a phylogenetic tree was generated. The three subgroups of DUSP proteins together with defining properties are indicated by the colored ovals.” Figure reproduced with permission from Dickinson, RJ and Keyse, SM. J Cell Sci. 2006 Nov 15;119(Pt 22):4607-15.
degraded by the proteasome. Proteasomal degradation of MKP-1 is enhanced by phosphorylation on Ser 296 and Ser 323.\textsuperscript{20} Conversely, phosphorylation of Ser 359 and Ser 364 protects MKP-1 from degradation.\textsuperscript{17} Similarly, phosphorylation of Ser 386 and Ser 391 of MKP-2 stabilizes the protein and protects it from proteasome-mediated degradation.\textsuperscript{19} In addition to degradation, it has been shown that reactive oxygen species are able to oxidize the cysteine residue in the catalytic site, leading to the inactivation of phosphatase activity. This inactivation can either be reversible or irreversible, depending on how many oxygen molecules react with the cysteine residue. Formation of sulfenic acid via the addition of one oxygen results in reversible inhibition of phosphatase activity, whereas as the formation of sulfinic (two oxygens) or sulfonic (three oxygens) acid resulted in irreversible inhibition of phosphatase activity.\textsuperscript{21}

**MAP Kinases and Breast Cancer**

In the United States, breast cancer is the most commonly diagnosed form of cancer in women (not counting skin malignancies), with an estimated 227,000 new cases in 2012. Additionally, approximately 40,000 women are projected to die from their disease, making breast cancer the second-leading cause of cancer death among women behind lung cancer.\textsuperscript{25} Breast cancer is a heterogeneous disease made up of several different subtypes that have been identified through analysis of gene expression patterns.\textsuperscript{26}

The broadest classification of breast tumors is based on the expression of the estrogen receptor (ER), more specifically the expression of ER-\(\alpha\). Approximately 70% of human breast tumors are classified as positive for ER-\(\alpha\) expression.\textsuperscript{27,28} The ER is a
nuclear steroid hormone receptor whose structure is critical for understanding its function. The N-terminal A/B domain contains activation function 1 (AF-1) which is responsible for the ligand independent activation of the receptor via protein-protein interactions and for the transcriptional activation of target genes. The C domain is the ER DNA binding domain which is also involved in receptor dimerization. The D domain is a hinge region. The E domain includes the ligand binding domain, which contains ligand dependent activation function 2 (AF-2). In addition to ligand binding, this domain is involved in nuclear translocation, receptor dimerization and target gene expression. The F domain is involved in the recruitment of co-activators. When ligand binds to the receptor, helix 12, located in the E domain, moves over the ligand binding pocket and creates a surface for co-activator binding. When the ER is bound to an antagonist, helix 12 instead occupies a hydrophobic groove created by helices 3, 4 and 5. This eliminates the ability of the receptor to interact with co-activators.

When ligand is bound to the receptor, the ER forms a dimer with another ER molecule and translocates to the nucleus where it is able to bind to DNA via estrogen response elements (EREs) present in the promoters of its target genes (Fig. 3). The ERE consensus sequence is 5'-GGTCAnnnTGACC-3'. Many genes regulated by estrogen, however, contain imperfect, non-palindromic ER binding sites. Additionally, it has been shown that an ER-α dimer can bind to a half site if stabilized by protein-protein interactions with another transcription factor such as Sp-1. The ability of the ER to regulate transcription of its target genes by binding to consensus sequences and imperfect EREs, as well as its ability to interact with other transcription factors and co-activators
Figure 3. Estrogen and Tamoxifen Mechanisms of Action. Estrogen binding to the estrogen receptor causes its dimerization, which is followed by translocation to the nucleus and DNA binding, followed by the recruitment of co-activators and transcription of the target gene. Tamoxifen competes with estrogen to bind to the receptor, preventing co-activator recruitment and transcription of target genes.
present in the cell, demonstrates that the context in which this signaling occurs is very important in determining its outcome. Another example of how the context of cell signaling influences the transcription of estrogen regulated genes is through phosphorylation of the estrogen receptor. These post-translational modifications are carried out by different kinases and modify ER activity.\textsuperscript{27}\textsuperscript{,}\textsuperscript{29} Several sites have been identified that can be phosphorylated by ERK, including Ser 102, Ser 104, Ser 106, Ser 118, Ser 167 and Thr 311.\textsuperscript{27} Of these sites, phosphorylated Ser 104/106, Ser 118 and Ser 167 have been suggested to have a connection to tamoxifen response in breast cancer.

It has been shown that many breast cancers contain an increased proportion of cells with activated ERK.\textsuperscript{22} A 1997 study by Sivaraman \textit{et al.} assessed the activity of ERK in 37 breast tissue samples, 11 of which were determined to be breast carcinomas. Each of the breast tumors showed increased ERK activity, measured by an \textit{in vitro} kinase assay, compared to samples taken from patients with benign breast disease. Subsequent analysis of the breast carcinomas showed that ERK mRNA expression was elevated in malignant epithelial cells, but not in stromal cells in both the primary tumors, as well as in lymph node metastases, suggesting that the overexpression and increased activity of ERK might play a role in tumor initiation and progression.\textsuperscript{23}

Overexpression of both p38 and JNK1 has also been noted in primary breast tumors. An examination of 14 breast tumor samples paired with non-malignant breast tissue samples from the same patients revealed that p38 expression and activity were both increased three-fold in the malignant tissues. A similar three-fold increase in JNK expression was also observed.\textsuperscript{24}
MAP Kinases and Endocrine Therapy Resistance

For women diagnosed with ER-positive breast cancer, first line therapy involves treatment with tamoxifen, a selective estrogen receptor modulator that mimics the binding of estrogen to the ER (Fig. 3). In breast tissue, tamoxifen is known to act as an antagonist. When tamoxifen is bound to ER, co-activators are unable to bind, repressing the transcription of estrogen responsive genes. In bone and uterine tissue, however, tamoxifen has been shown to act as an agonist and has been associated with an increased risk of endometrial cancer. This effect must be taken into consideration when using the drug in the clinic. When used in an adjuvant setting, tamoxifen treatment reduces the odds of developing recurrent disease by approximately 40 to 50\%.\textsuperscript{31} Approximately 30 to 50\% of patients presenting with metastatic disease will experience temporary remissions while on tamoxifen therapy.\textsuperscript{31,32} However, almost all of these women will go on to develop recurrent disease that is resistant to tamoxifen treatment. This fact makes it clear that tamoxifen resistance is a major problem in the clinical setting and underscores the need to establish biomarkers to identify patients who will benefit the most from TAM therapy, as well as for development of novel drug targets.

Due to the heterogeneous nature of breast cancers and the complexity of signaling that occurs in tumors, it is not realistic to expect that a single mechanism would be identified as the cause of resistance in patients. Therefore, it is important to continue to explore all possible causes in an attempt to determine which patients will experience the greatest benefit from tamoxifen treatment. Even in the presence of tamoxifen, it is still possible to have activation of estrogen responsive genes (Fig. 4). One such mechanism is
through the hypersensitization of the ER to ligand binding. Following estrogen deprivation, the receptor is able to become activated in response to much lower concentrations of estrogen compared to conditions when ligand is present in abundance. Many molecular mechanisms of tamoxifen resistance have been suggested, including, but not limited to, several that involve MAP Kinase signaling. In another scenario, tamoxifen is able to act as an agonist via ER interactions with transcription factors such as AP-1 or Sp-1. For example, AP-1, which is a downstream target of MAPK signaling, binds to its response element and is able to crosstalk with the tamoxifen-bound ER, changing the context for the regulation of its target genes. Yet another mechanism involves the ligand independent activation of the ER. As noted earlier, ERK is able to directly phosphorylate several residues on the estrogen receptor. Phosphorylation of Ser 118, which is located in the ligand independent AF-1 portion of the ER A/B domain, is perhaps the best characterized, but most enigmatic of the sites targeted by ERK. A 2006 study done by Sarwar et al used immunohistochemistry to examine Ser 118 phosphorylation in 301 breast tumor biopsies and found that 83% of the tumors that were positive for ER-α were also positive for phospho-Ser 118. Additionally, there was a correlation between positive Ser 118 phosphorylation and low tumor grade, indicating that phospho-Ser 118 might be associated with more favorable prognosis.³³ When phosphorylation of Ser 118 was examined in a smaller group of patients (n=21) that
Figure 4. Regulation of E2 responsive genes in the presence of tamoxifen. Following estrogen deprivation, the estrogen receptor becomes responsive to lower concentrations of ligand than when ligand was plentiful. This is called hypersensitization. Ligand independent activation of the receptor can occur when kinases such as ERK phosphorylate residues in the ER, such as Ser 118, in AF-1 of the A/B domain. Tamoxifen bound ER binds to other transcription factors, like AP-1 or SP-1, using them as a scaffold to drive the transcription of genes that were not previously transcriptionally regulated by ER.
had relapsed while on tamoxifen, a statistically significant difference in the levels of phospho-Ser 118 pre- and post-treatment was observed. Eleven patients showed an increase in Ser 118 phosphorylation post-treatment. *In vitro* studies, however, have shown increased phosphorylation of Ser 118 and ERK activity in tamoxifen resistant MCF7 cells, derived by long term culture in the presence of tamoxifen, compared to their tamoxifen sensitive MCF7 counterparts. The authors suggest that these results show that Ser 118 phosphorylation in breast tumors is not predictive of treatment failure as might be inferred from their *in vitro* results. They do, however, acknowledge that the change in Ser 118 phosphorylation post-treatment might indicate a role in the emergence of resistance. It has also been demonstrated that ERK phosphorylation and its increased activity are associated with endocrine therapy resistance and decreased survival in breast cancer patients. Taken together these data seem to suggest that phosphorylation of Ser 118 in the estrogen receptor is associated with favorable prognosis prior to tamoxifen treatment, but unfavorable prognosis after tamoxifen resistance is acquired. The other phosphorylation sites shown to be associated with tamoxifen response and targeted by ERK are Ser 104/106 and Ser 167. Phosphorylation of Ser 104/106 leads to ligand independent activation of the receptor and agonistic activity of tamoxifen. Phosphorylation of Ser 167 increases binding of the ER to DNA and enhances co-activator binding in the presence of estrogen. *In vitro* it has been shown that Ser 167 phosphorylation reduces sensitivity to tamoxifen. However, similar to Ser 118 phosphorylation, conflicting clinical data make it difficult to assess what the true contribution of this modification is to tamoxifen resistance. In addition to
phosphorylation by ERK, Ser 167 is phosphorylated by Akt. Akt activity has been associated with decreased overall survival in breast cancer. In metastatic breast cancer, however, phospho-Ser 167 is associated with longer survival following relapse.\(^27\)

Overexpression of HER2, which has been shown to lead to the activation of ERK in breast tumor cell lines, has also been suggested as a mechanism of tamoxifen resistance in breast cancer. It has been observed that tamoxifen treatment slightly increases the expression of both EGFR and HER2 and that the expression of these molecules was greatly elevated in resistant tumors.\(^36\) Clinical studies showed that patients whose tumors overexpress these two molecules were less likely to benefit from tamoxifen treatment. A few different underlying mechanisms have been suggested for the contribution of EGFR and/or HER2 overexpression to tamoxifen resistance.\(^36\) In a study done by Massarweh et al, elevated levels of EGFR in MCF7 xenografts were associated with acquired tamoxifen resistance, but it was shown that genes classically regulated by estrogen were repressed in the presence of tamoxifen, suggesting that tumor growth in this model was through non-genomic ER-mediated activation of EGFR. Similar results were seen in tumors engineered to overexpress HER2.\(^36\) It is thought that this non-genomic activation might be carried out by a small fraction of ER that remains near the plasma membrane.\(^36\) Strong correlations amongst HER2, ERK and MKP-1 protein expression have also been demonstrated. Following activation of the MAPK pathway, it is thought that strong, sustained ERK activity leads to cell cycle arrest via senescence or differentiation.
Based on the hyperactivation of MAPK signaling in tamoxifen resistant tumors, it has been suggested that the inhibition of this pathway might restore tamoxifen sensitivity in breast cancer cells. To this end, it has been shown that exogenous inhibitors of HER2 signaling are able to partially restore sensitivity to anti-estrogens. MCF7 cells engineered to overexpress HER2 were treated with AG1478. This treatment abolished HER2 and MAPK phosphorylation, reduced ERE driven luciferase reporter activity in the presence of tamoxifen and showed inhibition of colony formation at levels similar to vector control treated cells, which was 80% compared to untreated controls. Another similar study done by Ghayad et al, showed that inhibition of the ERK pathway using the MEK inhibitor PD98059 and the PI3K/Akt pathway with the inhibitor LY294002 was able to restore tamoxifen sensitivity in cell line models of acquired tamoxifen resistance. In their model of endocrine resistance, which was also initially derived from MCF7 cells, two independently selected clones developed concomitant activation of both the MAPK and PI3K/Akt pathways. These clones also showed increased phosphorylation of Ser 118 and Ser 167 of the ER. A combination treatment of tamoxifen and PD98059 completely reversed the tamoxifen resistant phenotype seen in these clones, shown by a reduction in BrdU incorporation and an increase in annexin V staining, which is an indicator of apoptosis, in comparison to vehicle treated control cells. ER Ser 118 phosphorylation was also reduced. Similar results were seen with the combination of tamoxifen and LY294002. When the clones were subjected to treatment with combinations of PD98059, LY294002 and tamoxifen, interestingly, one clone was most sensitive to inhibition by tamoxifen and PD98059 and the other was most sensitive to tamoxifen and
LY294002. The combination of all three drugs inhibited cell proliferation in a manner similar to the optimal inhibitor in each case. Since these clones were selected through the same process, the authors suggest that this model reflects the heterogeneity observed in breast tumors and conclude that inhibiting both the MAP Kinase and PI3K/Akt pathways would be the most effective treatment strategy in a clinical setting.\(^\text{38}\)

The previous data show that an increase in ERK signaling is a common event in breast cancer tamoxifen resistance and that inhibition of this pathway using small molecule inhibitors might be an effective approach to restore sensitivity. This begs the question as to what role MAP Kinase phosphatases, the endogenous negative regulators of MAPKs, play in this process. While very little is currently known about the connection between MKPs and tamoxifen response, MKP expression has been shown to be altered in a number of cancers and MKP-1 has been linked to resistance to a number of chemotherapeutic agents.

**MAP Kinase Phosphatases and Cancer**

To date, six of the eleven MKP family members, MKP-1, MKP-2, MKP-3, MKP-4, MKP-7 and PAC-1, have been shown to exhibit altered expression in a variety of human malignancies (Table 2). Most of what is known pertains to MKP-1, since it is the founding member and best characterized of the MKP family, but to gain perspective on what is known about the overall picture of MKP signaling in cancer cells, it is important to briefly survey the contribution of the other MKPs to cancer development and progression.
<table>
<thead>
<tr>
<th>Phosphatase</th>
<th>Cancer Type</th>
<th>Change in Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKP-1</td>
<td>Bladder, Breast, Colon, Lung, Ovarian, Prostate</td>
<td>Downregulated in Bladder (M) Upregulated in Breast (M, P) Downregulated in Colon (M) Upregulated in Lung (M, P) Upregulated in Ovarian (M, P) Downregulated in Prostate (M, P)</td>
</tr>
<tr>
<td>MKP-2</td>
<td>Breast, Liver, Ovarian, Pancreatic, Glioma</td>
<td>Upregulated in Breast (P) Upregulated in Liver (M) Downregulated in Ovarian (M) Upregulated in Pancreatic (P) Downregulated in Glioma (M)</td>
</tr>
<tr>
<td>MKP-3</td>
<td>Breast, Ovarian, Pancreatic</td>
<td>Upregulated in Breast (M) Downregulated in Ovarian (M, P) Downregulated in Pancreatic (M, P)</td>
</tr>
<tr>
<td>MKP-4</td>
<td>Skin</td>
<td>Downregulated (M, P)</td>
</tr>
<tr>
<td>MKP-7</td>
<td>Leukemia</td>
<td>Downregulated (M)</td>
</tr>
<tr>
<td>PAC-1</td>
<td>Ovarian</td>
<td>Upregulated (M)</td>
</tr>
</tbody>
</table>

**Table 2. Alterations of MKP Expression in Human Cancer.**  
M=change in mRNA expression.  P=change in protein expression.  References: MKP-1,3,4,8,9,14,24,39-42, MKP-2,9,24,43-45, MKP-3,9,46-49, MKP-4,50, MKP-7,51,52, PAC-1,53
**PAC-1, MKP-3, MKP-4, and MKP-7 and Cancer**

PAC-1, MKP-3, MKP-4 and MKP-7 have been shown to play a role in various cancer types. PAC-1, a nuclear phosphatase with activity directed toward ERK and p38, has been shown to be associated with overall survival in ovarian cancer. In a study of thirty-nine patient samples from serous ovarian tumors, high PAC-1 mRNA expression levels correlated with worse overall survival compared with those patients whose tumors expressed low PAC-1 levels.\(^5,\)\(^53\) MKP-3, a cytoplasmic, ERK specific phosphatase, is connected to pancreatic, ovarian and breast cancer. The loss of MKP-3 expression in pancreatic cancer is associated with disease progression. Compared to *in situ* carcinomas, a decrease in expression was observed in primary invasive pancreatic tumor tissues\(^46\). Another study revealed that many precursor lesions lacking MKP-3 expression harbored K-ras mutations.\(^5,\)\(^47\) Similar losses of expression have been observed in ovarian cancer cell lines. MKP-3 protein expression was shown to be much lower in these cell lines compared to normal samples and immortalized cell lines.\(^5,\)\(^49\) MKP-4 is an ERK and p38 specific phosphatase located in the cytoplasm. Loss of MKP-4 expression has been connected to skin cancer development. Reintroduction of MKP-4 in malignant cells led to microtubule disruption and *in vivo* tumor suppression.\(^5,\)\(^50\) MKP-7, a JNK and p38 phosphatase found in both the nucleus and the cytoplasm, has been associated with leukemia. Overexpression of MKP-7 in Rat-1 fibroblasts transformed with BCR-ABL showed a reduction in JNK activation and a decreased ability to be transformed both *in vitro* and *in vivo*.\(^5,\)\(^52\) Additionally, downregulation of MKP-7 by miR-24, whose expression is induced by both AML-1 and AML1-ETO, has been linked to development
of acute myeloid leukemia. Decreased MKP-7 led to increased phosphorylation of JNK and p38, which stimulated myeloid cell growth and inhibited differentiation.\textsuperscript{5,51}

\textbf{MKP-2 and Cancer}

MKP-2, which is a nuclear phosphatase that primarily targets ERK and JNK, has shown altered expression in liver, pancreatic, ovarian and breast cancers. MKP-2 was first identified by Misra-Press \textit{et al} in 1994.\textsuperscript{54} The authors of this study demonstrated that MKP-2 is expressed in a wide variety of rat tissues in a profile that is overlapping yet distinct from MKP-1. They also suggested that this differential expression combined with the unique N-terminal sequences of the two phosphatases indicate distinct functional roles for each protein.\textsuperscript{54} In a study that investigated hepatocarcinogenesis and hepatoma, no expression of MKP-2 could be detected in normal liver, but was present in three out of five primary hepatomas studied.\textsuperscript{5,43} MKP-2 mRNA levels were also elevated in ascites hepatoma cell lines compared to normal liver.\textsuperscript{5,43} The authors of this study suggest that MKP-2 expression might be used as a tumor marker in the liver.\textsuperscript{5,43} MKP-2 has also been linked to the suppression of ERK activity in pancreatic cancer cells harboring K-ras mutations.\textsuperscript{44} The expression of MKP-2 in pancreatic tumor cell lines correlated with MEK expression. When BxPC-3 and Capan-1 cells were treated with MEK inhibitor PD98059, MKP-2 expression was markedly decreased.\textsuperscript{5,44} Subsequent work examining post-translational modifications of MKP-2 showed that ERK is able to phosphorylate MKP-2 on Ser 386 and Ser 391, which stabilizes the protein and forms a feedback loop for the regulation of ERK signaling. Inhibition of ERK activity with MEK inhibitor U0126 abolished MKP-2 phosphorylation and co-treatment with the proteasome inhibitor
MG132 showed that in the absence of this phosphorylation, MKP-2 protein is degraded by the proteasome. Additionally, MKP-2 has been shown to be overexpressed in serous borderline tumors of the ovary, but expression was lost in serous carcinomas. MKP-2 is co-expressed with MKP-1 in breast cancer.

**MKP-1 and Cancer**

MKP-1 is a nuclear phosphatase that is able to dephosphorylate all three MAPKs, with preference for JNK and p38 as substrates compared to ERK. It is unclear what the precise physiological function of MKP-1 is in normal tissues, as MKP-1 knockout mice show no obvious phenotype. Other studies seem to indicate, however, that MKP-1 may be involved in the inhibition of pro-inflammatory signaling. MKP-1 has also been identified as a transcriptional target of p53, suggesting it may play a role in cell cycle control. The second intron of MKP-1 contains a p53 binding site. This study also showed that when MKP-1 phosphatase activity was inhibited using vanadate, a general phosphatase inhibitor, induction of p53-mediated G1 arrest in response to growth factor stimuli was compromised. p53 also regulates MKP-1 protein expression in response to oxidative stress in colon cancer cells. In addition to colon cancer, MKP-1 expression has been shown to be altered in prostate, bladder, ovarian, non-small cell lung and breast cancers. In colon, bladder and prostate cancer, MKP-1 is overexpressed in the early stages of disease, but expression seems to be lost as the disease progresses. Microarray analysis of colorectal tumor samples from nine patients participating in a Phase I/II clinical trial examining a treatment regimen consisting of bevacizumab, a VEGF inhibitor, and radiation therapy revealed that MKP-1 mRNA expression was significantly
downregulated in all samples after treatment. The size of decrease, however, varied from patient to patient.\textsuperscript{5,40} The authors suggest that this decrease is due to the reduction in tumor proliferation following treatment.\textsuperscript{40} MKP-1 expression has been linked to clinical outcome in ovarian cancer, where increased expression correlated with shorter progression-free survival.\textsuperscript{5,9} In a separate study, moderate to strong MKP-1 expression was seen in 57.6\% of invasive primary ovarian tumors (n=66).\textsuperscript{5,41} In lung cancer, when compared to normal tissue samples, MKP-1 expression was increased in non-small cell lung cancer tissue samples. In this study, high MKP-1 expression levels independently predicted better survival outcomes.\textsuperscript{8} In a small clinical study, tumor samples were obtained from 14 breast cancer patients and examined for expression of the three MAPKs. This study showed that ERK, JNK and p38 were all upregulated in malignant versus non-malignant tissue samples. The study also looked at JNK activity and found that it was 30\% lower in malignant tissue than in normal tissue. Further investigation into the disparity between higher protein expression level and reduced activity level of JNK revealed that MKP-1, along with MKP-2, displayed increased expression in the malignant tissue. The authors suggest this increase in MKP expression as a possible mechanism for the decrease in JNK activity.\textsuperscript{5,24} Since a reduction in JNK activity may play a role in reducing the effectiveness of chemotherapy drugs, it is possible that downregulating MKP-1 expression might be a novel way to combat chemotherapy resistance.\textsuperscript{5}
MKP-3, MKP-1 and Breast Cancer Treatment Resistance

MKP-3 and Tamoxifen Resistance

MKP-3 is the only MKP so far to be directly studied in relation to tamoxifen resistance in breast cancer. A study done by Cui et al analyzed MKP-3 mRNA expression in nine tumors, four of which were tamoxifen sensitive and five that were resistant to tamoxifen treatment. Their results showed that MKP-3 mRNA expression was increased 2.5 times in resistant tumors compared to sensitive ones. They then used an in vitro model of tamoxifen resistance. MCF7 and T47D cells were cultured long term (over six months) in the presence of tamoxifen and used to confirm that MKP-3 mRNA was increased in these cells compared to their tamoxifen sensitive counterparts. Using MCF7 cells engineered to overexpress MKP-3, they next tested the effect of this overexpression on tamoxifen sensitivity. They showed that MCF7 vector control cells exhibited increased colony forming ability in the presence of estrogen and that this increase was blocked following tamoxifen treatment. MKP-3 overexpression cells treated with estrogen exhibited a similar colony forming ability to vector control cells treated with estrogen, but when these cells were treated with tamoxifen they showed a ten-fold increase in colony formation compared to vector control cells under the same conditions. Western blot analysis showed that MKP-3 overexpression cells treated with tamoxifen had the highest level of activated ERK compared to vehicle or estrogen treated MKP-3 overexpressing cells. To explain these seemingly paradoxical results, the authors suggested that breast tumors might upregulate phosphatase expression to compensate for chronic activation of ERK signaling. They hypothesize that tamoxifen resistance
emerges due to inactivation of MKP-3 phosphatase activity by ROS and that this loss of activity disrupts the compensatory MAPK regulatory loop seen in breast tumors.\textsuperscript{48}

In addition to tamoxifen resistance, MKP-3 has also been implicated in cisplatin resistance in ovarian cancer. As mentioned previously, MKP-3 protein expression was decreased in tumor tissue samples and ovarian cancer cell lines compared to normal samples and immortalized, non-tumorigenic cell lines. Exogenous expression of MKP-3 in A2780cp cells, which are cisplatin resistant, increased their sensitivity to cisplatin treatment up to 2.5-fold when compared to vector control cells.\textsuperscript{5,49}

**MKP-1 and Chemotherapy Resistance**

Growing evidence suggests that MKP-1 may play a role in chemotherapy resistance (Fig. 5).\textsuperscript{6} In human lung cancer cell lines, overexpression of MKP-1 protected them from cisplatin-induced death.\textsuperscript{6,39} It was shown that MKP-1 targets JNK in response to cisplatin, leading to increased c-Jun activity and that MKP-1\textsuperscript{-/-} mouse embryo fibroblasts (MEFs) were more sensitive to cisplatin and etoposide than MKP-1\textsuperscript{+/+} MEF cells. This study also demonstrated that activation of JNK is required for sensitizing cells to cisplatin.\textsuperscript{6,39} It has also been shown that induction of MKP-1 after treatment with cisplatin is a general event in ovarian cancer cell lines, with knockdown of MKP-1 by siRNA increasing cisplatin induced, JNK-mediated cell death.\textsuperscript{3,6}

While MKP-1 has not yet been linked to tamoxifen resistance, it has been well-studied in connection with resistance to many other chemotherapeutic agents in breast cancer. Overexpression of MKP-1 was able to protect breast cancer cells from chemotherapy-mediated apoptosis when they were treated with doxorubicin,
mechlorethamine and paclitaxel. This is significant because many chemotherapy drugs, including anthracyclines, alkylating agents and taxanes, use JNK activation to carry out their anticancer activity. A study in NSCLC looked at the effect of the EGFR tyrosine kinase inhibitor AG1478 on JNK signaling. The results showed that following treatment of PC-9 cells, MKP-1 expression was reduced. Ectopic overexpression of MKP-1 reduced JNK activation and decreased AG1478-induced apoptosis. These results indicate that AG1478 also uses JNK activation as a mechanism for apoptosis induction. Treatment of breast cancer cell lines with anthracyclines resulted in the repression of MKP-1 and increased phosphorylation of ERK. Further silencing of MKP-1 with siRNA resulted in decreased ERK activation, but the mechanism for this remains unclear. A similar study looked at MKP-1 overexpression following treatment with doxorubicin in a panel of breast cell lines. Results showed that doxorubicin treatment decreased MKP-1 protein expression and that this decrease preceded an increase in active ERK and JNK. MKP-1 staining was done in 30 complete tissue sections that included histologic normal breast (n=30), as well as hyperplastic (n=11), in situ (n=18) or infiltrating carcinomas (n=30) within the same tissue section. MKP-1 was overexpressed in all in situ carcinomas and in 50% of infiltrating carcinomas. This study also further characterized the effect of doxorubicin treatment ex vivo in 50 patient samples, 27 of which did not show overexpression of MKP-1 and 23 of which did. MKP-1 expression was decreased following doxorubicin in 39 samples, while the other 11 samples showed a modest increase in MKP-1 levels. ERK and JNK
Figure 5. MKP-1 and chemoresistance. Overexpression of MKP-1 plays a role in the development of resistance to chemotherapy in breast, lung and ovarian cancers. In breast cancer, decreased JNK and p38 activity contributes to resistance to oxidative stress induced death. Treatment with proteasome inhibitors increases ERK and decreases JNK activity, leading to proteasome inhibitor resistance because of decreased levels of apoptosis. Activation of the glucocorticoid receptor increases MKP-1 mRNA and leads to decreased JNK and ERK activity, which factors in to paclitaxel resistance. Cisplatin resistance in lung and ovarian cancer is caused in part by decreased JNK activity. (Figure reproduced from Haagenson, K. K. & Wu, G. S. The role of MAP kinases and MAP kinase phosphatase-1 in resistance to breast cancer treatment. Cancer Metastasis Rev 29, 143-149, (2010).)
activation increased following the downregulation of MKP-1, mirroring the results seen in tumor cells. Additionally, in a series of 96 patients, it was shown that following surgery and systemic treatment with chemotherapy, anti-hormonal therapy or both, those who harbored MKP-1 overexpression were more likely to experience relapse than those who did not, suggesting that MKP-1 might be a novel drug target in breast cancer.\textsuperscript{60}

Proteasome inhibition has been linked to the induction of MKP-1 expression.\textsuperscript{6,61} This resulted in a decrease in ERK signaling and further blockade of ERK led to an increase in proteasome inhibitor mediated apoptosis.\textsuperscript{6,61} This induction of MKP-1, however, is thought to also limit the efficacy of proteasome inhibitors because of a subsequent decrease in JNK activity, resulting in decreased levels of apoptosis.\textsuperscript{6,62} Knockdown of MKP-1 resulted in increased proteasome inhibitor sensitivity.\textsuperscript{6,62} Interestingly, evaluation of a combination treatment with proteasome inhibitors and doxorubicin, which is known to decrease MKP-1 levels in breast cancer, showed increased apoptosis, decreased MKP-1 levels and increased JNK phosphorylation \textit{in vitro} and resulting in delayed tumor growth in an \textit{in vivo} xenograft model.\textsuperscript{6,62} Similar to the combination of proteasome inhibitor treatment and doxorubicin, a combination of proteasome inhibitor treatment and p38 blockade also inhibited MKP-1 expression, increased JNK activity and increased apoptosis in the A1N4-\textit{myc} and BT474 breast cancer cell lines.\textsuperscript{6,63}

Activation of the glucocorticoid receptor following treatment with dexamethasone can also inhibit paclitaxel induced apoptosis by preventing the induction of ERK and JNK activation.\textsuperscript{6,64} Activation of the glucocorticoid receptor has been shown to lead to
an increase in MKP-1 mRNA, which could be an underlying mechanism for the decrease in ERK and JNK activation. Using siRNA directed at MKP-1 decreased the anti-apoptotic activity of glucocorticoids.\textsuperscript{6,64}

Inhibition of JNK and p38 signaling by overexpression of MKP-1 also increased resistance to H\textsubscript{2}O\textsubscript{2}-induced death in MCF7 breast cancer cells, with a correlation between MKP-1 induction and the disappearance of phosphorylated MAPKs, suggesting that MKP-1 might play a physiologic role in the inactivation of oxidative damage induced MAPK activities. Furthermore, loss of MKP-1 sensitized cells to oxidative damage induced death.\textsuperscript{4,6}

**Conclusion**

These results show that, in general, overexpression of MKP-1 seems to contribute to chemotherapy resistance in breast cancer, but MKP-1 involvement in tamoxifen sensitivity has yet to be characterized. Taking into consideration that MKP-1 phosphatase activity is preferentially directed toward JNK and p38 rather than ERK, this suggests that increases in MKP-1 expression might contribute to changes in tamoxifen sensitivity via the inhibition of JNK-mediated apoptosis. MKP-2, which can dephosphorylate all three MAPKs, but preferentially inactivates ERK and JNK, has also been shown to be overexpressed in breast cancers. Both MKP-1 and MKP-2 proteins have been shown to be stabilized following phosphorylation by ERK, forming a feedback loop. The activation of the ERK pathway in tamoxifen resistant breast cancers is well documented and has been connected to poor prognosis in patients. Taken together, these data suggest that further investigation of the connection among MKPs, MAPK signaling
and tamoxifen resistance is warranted, leading to the hypothesis that increases in MKP expression are associated with changes in tamoxifen sensitivity. Investigating MKPs and their regulation of MAP Kinases in breast cancer is important because tamoxifen resistance is a major clinical problem and MAPK signaling has been implicated in its development. In the research presented in this dissertation, the characterization of MKP-1 and MKP-2 expression in breast cancer cells will form the knowledge base necessary to begin to dissect their contributions to tamoxifen sensitivity. Investigating changes in MAP Kinase activation and correlating them to MKP expression in both tamoxifen sensitive and tamoxifen resistant cells will clarify the overall picture of MAPK signaling in tamoxifen sensitivity and finally, examining the promoter sequence features of MKPs will provide insight into their regulation in the context of breast cancer.
CHAPTER 2
CHARACTERIZATION OF MKP-1 IN BREAST CANCER TAMOXIFEN SENSITIVITY

Introduction

MKP-1 is the founding member of the MAP Kinase Phosphatase family. It has the ability to dephosphorylate all three MAPKs, however at physiological levels seems to preferentially inactivate JNK and p38. It is unclear what the precise physiological function of MKP-1 is in normal tissues, as MKP-1 knockout mice show no obvious phenotype. Other studies seem to indicate, however, that MKP-1 may be involved in the inhibition of pro-inflammatory signaling. In vascular smooth muscle cells, MKP-1 is activated by mechanical stress and seems to inhibit the proliferation of these cells through MAPK dephosphorylation. MKP-1 has also been identified as a transcriptional target of p53, suggesting it may play a role in cell cycle control. The second intron of MKP-1 contains a p53 binding site. This study also showed that when MKP-1 phosphatase activity was inhibited using vanadate, a general phosphatase inhibitor, induction of p53-mediated G1 arrest in response to growth factor stimuli was compromised. p53 also regulates MKP-1 protein expression in response to oxidative stress in colon cancer cells.

Changes in MKP-1 expression in several cancer types have been shown to lead to changes in metastatic potential and time to progression. In non-small cell lung cancer (NSCLC), knockdown of MKP-1 by siRNA reduced the invasive ability and angiogenic
potential of H460 cells and decreased tumorigenicity and metastasis in *in vivo* mouse models. In colorectal cancer, MKP-1 expression in human tumor samples from patients treated with cetuximab, a monoclonal antibody toward EGFR, was assessed in conjunction with KRAS and BRAF mutational status. The results showed that patients with wild-type KRAS and BRAF that overexpressed MKP-1 had a significantly shorter time to progression than those that did not overexpress MKP-1.

Particularly relevant to the studies presented here, MKP-1 has been shown to be overexpressed in breast cancer and overexpression in other cancer types has been linked to tumor progression, which suggests that inhibiting MKP-1 activity might be a strategy to employ in the treatment of breast cancer. To this end, studies have been done to try to identify small molecule inhibitors of MKP-1 from a pyrrole carboxamide library of compounds. Structural analogs of the lead compound SID 3717140 were developed in an attempt to identify MKP-1 inhibitors with improved potency, activity and selectivity. A separate study using a pyrrole carboxamide library identified two compounds, PSI2106 and MDF2085, which were determined to be potent and selective in chemical screens, but their biological activity was not as good as expected. Subsequent work in this area identified a cell active compound, NSC 95397, using a fluorescence based chemical complementation assay. This compound was able to reverse dexamethasone protection in paclitaxel treated cells. Combination treatment of NSC 95397 and paclitaxel in the absence of dexamethasone proved to be antagonistic, but in the presence of dexamethasone, the effect of their combination was synergistic. These results suggest that inhibition of MKP-1 occurred only in conditions of MKP-1
upregulation. These data indicate that a considerable amount of effort is still needed to discover small molecules capable of effectively inhibiting MKP-1. Success in this area would be beneficial to dissecting the precise role of MKP-1 in the inhibition of inflammatory signaling as well as its connection to tumor progression and chemotherapy resistance.

In breast cancer, women who present with estrogen receptor positive disease are treated with tamoxifen and it is well known that the development of recurrent, tamoxifen resistant disease is a major problem in the clinical setting. MKP-1 has been connected to chemotherapy resistance in lung, ovarian and breast cancers. Thus far, no connection has been made between MKP-1 and response to tamoxifen, but its history of being involved in chemotherapy resistance suggests that the potential for a link between the two be investigated further. In order to fully explore this possibility, this work will set out to identify a cell line model appropriate to study the role of MKP-1 in tamoxifen sensitivity, examine the response of MKP-1 protein expression following treatment with estrogen and tamoxifen and determine if overexpression of MKP-1 contributes to changes in tamoxifen sensitivity in breast cancer cells. These studies will form the foundation of investigating the hypothesis that increases in MKP-1 expression are associated with changes in tamoxifen sensitivity in breast cancer.

**Materials and Methods**

**Cell Lines and Culture Conditions**

MDA-MB-231, T47D, CAOV3-pLKO.1 and CAOV3-shMKP1 cells were cultured in Dulbecco’s Minimal Essential Medium (DMEM) supplemented with 10%
MCF10A cells were maintained in DMEM/F12 (1:1) medium supplemented with 5% FBS, 10 µg/mL insulin, 20 ng/mL epidermal growth factor, 100 ng/mL cholera enterotoxin and 0.5 µg/mL hydrocortisone. MCF7 cells were maintained in DMEM/F12 (1:1) medium supplemented with 5% FBS, 10 µg/mL insulin and 0.5 nM estrogen. MCF7 tamoxifen resistant cells (MCF7-TAMR) cells were generated in the lab of Dr. Malathy Shekhar by gradual exposure to increasing concentrations of 4-OH-tamoxifen over a period of six months. (Gerard and Shekhar, manuscript in preparation.) MCF7-TAMR cells are able to tolerate exposure of up to 10 µM TAM. These cells were routinely maintained in DMEM/F12 (1:1) medium supplemented with 5% FBS, 10 µg/mL insulin and 1 µM tamoxifen. MCF7 cells stably expressing empty vector (pEGFP or pCDNA3.1) and MKP-1 or MKP-2 were maintained in the same medium as MCF7 cells and stable clones were selected with 500 µg/mL G418. All cell lines were maintained in a 37°C incubator with a humidified atmosphere consisting of 5% CO2. For experiments involving treatment with estrogen, tamoxifen (TAM) or ICI 182,780 (ICI), cells were depleted of hormones by culturing in phenol red free media supplemented with charcoal-stripped FBS and 10 µg/mL insulin.

Reagents

17-β-estradiol, G418 antibiotic and 4-OH tamoxifen were purchased from Sigma. ICI 182,170 was purchased from Tocris. Human recombinant insulin was purchased from GIBCO. Anisomycin (Sigma) was a gift from Dr. Raymond Mattingly.
Whole Cell Lysates

To prepare whole cell lysates, cells were trypsinized with .05% Trypsin-EDTA (GIBCO) and counted using a hemocytometer. 3 x 10^5 to 5 x 10^5 cells were plated in 60 mm dishes and allowed to adhere overnight. Treatments were added the following morning. At the end of the treatment period, medium was removed and cells were washed with ice cold phosphate-buffered saline (PBS). Following the removal of PBS, cells were lysed with NP-40 lysis buffer (150mM NaCl, 1% NP40, 50 mM Tris-Cl) containing a Complete Mini Protease Inhibitor Cocktail tablet (Roche Diagnostics). Cells were detached by scraping and incubated on ice for one hour to ensure complete lysis, then centrifuged at 14,000 rpm for ten minutes at 4°C to clear cell debris. Protein concentration was determined using the Bio-Rad protein assay and absorbance was measured at 595nm with a Genesys 10UV spectrophotometer (Spectronic Unicam). 2X Laemmli’s sample buffer was added in equal volume to the lysates which were then boiled for five minutes.

Western Blot Analysis

Steady state levels of protein expression were measured by western blot analysis. Whole cell lysates were electrophoresed through a 12% polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) using a semi-dry transfer method. Membranes were blocked for thirty minutes in either 10% milk (ECL Plus detection), 5% bovine serum albumin (ECL Plus detection) or 0.1% casein in 0.2X PBS (Odyssey detection) according to antibody specifications. Membranes were incubated in primary antibody overnight at 4°C, washed for ten minutes three times in
either TBST (Tris-buffered saline with Tween 20; for detection with ECL Plus) or PBST (Phosphate-buffered saline with Tween 20; for detection with Odyssey) and then incubated with secondary antibody for one to two hours. Proteins were detected using either the Amersham ECL Plus Western Blotting Detection Reagents Kit (GE Healthcare) or the Odyssey Infrared imaging system according to the manufacturer’s protocol.

**Antibodies**

MKP-1 (C-19) antibody and Estrogen Receptor α HC-20 antibody were purchased from Santa Cruz Biotechnology. PhosphoPlus SAPK/JNK (Thr183/Tyr185) Antibody Kit, PhosphoPlus p44/p42 MAPK (ERK1/2) (Thr202/Tyr204) Antibody Kit, Phospho-Estrogen Receptor α (Ser118) (16J4) antibody, GAPDH (D16H11) XP monoclonal antibody, goat-anti-mouse-HRP and goat-anti-rabbit-HRP secondary antibodies were purchased from Cell Signaling Technology. Anti-V5 antibody was purchased from Invitrogen. Anti-β-actin antibody was purchased from Sigma Aldrich. When using the Odyssey scanner, Alexa-Fluor 680 conjugated to goat-anti-rabbit or goat-anti-mouse (Invitrogen) or IRDye 800 conjugated to goat-anti-rabbit or goat-anti-mouse (Li-Cor) were used as secondary antibodies.

**RNA Isolation and cDNA Synthesis**

1.0x10⁶ cells were seeded in 10 cm dishes. Cells were lysed using TRIzol Reagent (Life Technologies) according to the manufacturer’s protocol to isolate total RNA. RNA was quantified using the Genesys UV10 spectrophotometer (Spectronic Unicam). Absorbance was measured at 260 and 280 nm and RNA concentration in µg/ul
was calculated using the following formula: \( \frac{\text{OD}_{260} \times 100 \times 40}{1000} \). cDNA was generated using the SuperScript III Reverse Transcriptase kit (Invitrogen) using 5 \( \mu \)g total RNA as a template and random primers. All other steps were carried out according to the manufacturer’s protocol. Samples were incubated in the PTC-200 Peltier thermal cycler.

**Real Time RT-PCR**

Samples were tested in either duplicate or triplicate. Reactions were carried out using the Choice-Taq DNA polymerase (Denville) in the PTC-200 Peltier thermal cycler (MJ Research) under the following conditions: 95°C for 2 minutes, followed by thirty-five cycles of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 60 seconds. The final cycle was followed by a 5 minute extension step at 72°C. Primer sequences used for both semi-quantitative and Real Time RT-PCR were as follows: GAPDH forward (5’-ATC AAG AAG GTG GTG AAG CAG-3’, +946 to 966; NM_002046.4), GAPDH reverse (5’-TGT CGC TGT TGA AGT CAG AGG-3’, +1042 to 1022; NM_002046.4), MKP-1 forward (5’-GAA GTG GGC ACC CTG GAC GC-3’, +258 to 277; NM_004417.3), MKP-1 reverse (5’-TGG CCG GCG TTG AAA GCG AA-3’, +364 to 345; NM_004417.3), MKP-2 forward (5’-GAG TCC GCG TGT GAC GC-3’, +494 to 515; NM_001394.6) and MKP-2 reverse (5’-CCT CGC GGT CAC ATA GCA GTC G-3’, +642 to 623; NM_001394.6). Primers for MKP-1 and MKP-2 were generated using the Primer BLAST software from NCBI. GAPDH primer sequences were used previously in the lab of Dr. Michael Tainsky.\(^{73}\)
Real-Time PCR was carried out using the SYBR Green PCR core reagents kit (Applied Biosystems). Each 20 µl reaction contained 2 mM MgCl$_2$, 0.2 mM dNTPs, 0.1 mM forward and reverse primer mixture, 2 µl cDNA, 2 µl 10X SYBR Green, 0.5 U AmpliTaq Gold Polymerase and sterile DNase-RNase free water (GIBCO). Samples were run in 96 well plates using the Step One Plus Real-Time PCR System and Step One Plus Software version 2.1 (Applied Biosystems). Thermal cycling was conducted according to the following protocol: 95°C for 10 minutes followed by forty cycles of 95°C for 15 seconds and 60°C for 60 seconds. Samples were then subjected to melt-curve analysis to make sure the products were free of primer dimers. Relative mRNA quantitation was calculated using the ΔΔCt method with GAPDH used as the internal control.

**shRNA**

CAOV3-pLKO.1 and CAOV3-shMKP-1 cells were generated in the Wu lab by Dr. Juan Wang. Bacterial stocks of Mission shRNA (Sigma) directed against MKP-1 (5'-'CGGCAAGAAGGATACGAAGCGTTCTCGAGAAGCTTCGTATCCTCCTTTGTTTTT-3') were obtained and pLKO.1 vector DNA was stably transfected into CAOV3 ovarian cancer cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Positive clones were obtained after selection with puromycin.

Four MKP-2 shRNAs subcloned into the pGIPZ vector (Open Biosystems) were tested. The MKP-2 targeting sequences were as follows: shRNA 1 (RHS4430-101067857): CCCCAGTGGAGATAACCACAA; shRNA 2 (RHS4430-101069298): ATTCGGTCAACGTGCCTGTAA; shRNA 3 (RHS4430-101073316):
ACTGGTTCCATGGAAGCCATAGA; shRNA 4 (RHS4430-98713911): AGCCTACCTGATGATGAAGAAA. Constructs were transiently transfected into MCF7-MKP-2 cells using Lipofectamine 2000 according to the manufacturer’s protocol. Non-silencing shRNA was used as a negative control.

Sequence Alignment

Protein and DNA sequence alignments were performed with the ClustalW2 software program from the European Bioinformatics Institute. Default parameters were used. Sequences were obtained from GenBank. The following accession numbers were used: MKP-1 mRNA (NM_004417.3), MKP-1 protein (NP_004408.1), MKP-2 mRNA (NM_001394.6) and MKP-2 protein (NP_001385.1).

Generation of MKP-1 and MKP-2 Overexpressing Cell Lines

Pooled populations of MCF7 cells overexpressing either MKP-1 [GenBank: BC022463] or MKP-2 [GenBank: BC002671.2] and subcloned into the pCDNA3.1 expression vector were generated by stable transfection. Cells were transfected using the Metafectine Easy transfection reagent (Biontex). Briefly, 3 µl of metafectine was incubated in 100 µl phenol red free, serum free DMEM/F12 (1:1) medium for twenty minutes. Five micrograms of the empty pCDNA3.1 vector, pCDNA3.1-MKP-1 construct or pCDNA3-MKP-2 construct DNA in 15 µl of DNAse-RNAse free water (GIBCO) were added to the metafectine and incubated at room temperature for twenty minutes to allow complexes to form. The mixture was added drop-wise to the cells. Stable clones expressing V5-His tagged MKP-1 or MKP-2 were selected with G418 antibiotic (500 µg/mL) and pooled to minimize clonal bias. Stable pools of MCF7 cells expressing
empty vector, MKP-1, or MKP-2 were maintained in DMEM/F12 (1:1) medium supplemented with 5% FBS, 10 µg/mL insulin, 0.5 nM 17-β-estradiol and 500 µg/mL G418.

**MTT Assay**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) assay was used to measure cell viability following drug treatment. MCF7, MCF7-TAMR, MCF7-pCDNA3.1-Pool, MCF7-MKP-1-Pool or MCF7-MKP-2-Pool (1x10³ cells per well) were seeded in triplicate in 96 well plates (day 0) and treated with vehicle (EtOH, 0.01% v/v), 10 nM E₂, 100 nM TAM, 1000 nM TAM or a combination of 10 nM E₂ and 1-, 10- or 100-fold molar excess TAM (day 1). Treatments were refreshed on day 4. Assays were terminated on day 7 or when control wells reached 85% confluency, whichever was longer. Cell viability was assessed by MTT assay (Sigma) and measured on a Synergy 2 plate reader (BioTek) with Gen5 1.10 software.

**Results**

Before beginning to study the relationship between MKP-1 and tamoxifen sensitivity, it was necessary to characterize its expression in breast cancer cell lines. In order to select a cell line model to focus this study, a panel of breast cell lines was screened for MKP-1 protein expression. This panel included the immortalized, non-tumorigenic MCF10A cell line, the ER-negative MDA-MB-231 cells and ER-positive MCF7 cells. MKP-1, a 39 kDa protein, was shown to be expressed in MDA-MB-231 cells (Fig 6A, Lane 2). MKP-1 protein was not detected in MCF10A or MCF7 cells. A 43 kDa band was shown to be highly expressed in MCF10A and MDA-MB-231 cells, but
was only weakly detected in MCF7 cells (Fig. 6A). Similar results were seen in ER-positive T47D cells (data not shown). Real-time RT-PCR analysis showed that MCF10A and MDA-MB-231 cells express similar levels of both MKP-1 and MKP-2 mRNA, whereas MCF7 cells expressed low levels of both MKP-1 and MKP-2 mRNAs (Fig. 6B). The undetectable level of MKP-1 protein in MCF7 cells, as well as the low expression of the 43 kDa band, made it a good model to use for investigating the impact of MKP-1 overexpression.

Because the antibody used recognizes two bands, the ovarian cancer cell line CAOV3, which is known to highly express MKP-1, was used to confirm which band actually represents the protein. Compared to cells transfected with a non-target shRNA (CAOV3-pLKO.1), cells transfected with MKP-1 shRNA (CAOV3-shMKP-1) showed a decrease in expression in the 39 kDa band, confirming that the 39 kDa band is MKP-1 (Fig. 6C).

The antibody being used for these studies is a polyclonal MKP-1 antibody from Santa Cruz. The antibody epitope is located within the last 50 amino acids of the MKP-1 C-terminus. The C-terminal region of MKP-1 contains the catalytic site, which is highly conserved among MKP family members. MKP-2 is a 43 kDa protein and among the MKP family members it is most similar to MKP-1 in that it is also a nuclear phosphatase and is able to dephosphorylate all three MAPKs. To investigate whether the 43 kDa protein could be MKP-2, the amino acid sequences of both MKP-1 and MKP-2 were aligned using ClustalW2 software. This analysis showed that 29 of the 50 amino acids in the MKP-1 antibody epitope are identical to those present in MKP-2 (Fig. 7). To
confirm further that the 43 kDa band observed in the previous study is indeed MKP-2, four MKP-2 shRNA constructs from Open Biosystems were transiently transfected into MCF7 cells engineered to overexpress MKP-2 with a V5-His tag (MCF7-MKP-2 cells) and whole cell lysates prepared from these cells were analyzed by Western blot with MKP-1 antibody (Fig. 8). It was found that shRNA constructs 3 and 4 greatly reduced expression of both the 43 kDa band and the V5-His tagged band compared to the non-target control. From this analysis it can be concluded that the 43 kDa band is MKP-2 and that the MKP-1 antibody recognizes both MKP-1 and MKP-2 proteins.

Previous work in the field has demonstrated that MKP-1 expression can be altered following drug treatment.\textsuperscript{58,60,61} After determining that MCF7 is an appropriate cell line model, cells that were engineered to overexpress MKP-1 (MCF7-MKP-1 cells) were used to investigate whether MKP-1 protein expression is altered following treatment with $E_2$, $E_2$ plus 100-fold molar excess TAM or TAM alone (Fig. 9). Both the MCF7-MKP-1 and MCF7-MKP-2 cells were derived from single clonal populations following positive selection with G418 antibiotic. Cells were plated in phenol red free media and treated with 1 or 10 nM $E_2$, 100 nM or 1 $\mu$M TAM or the combination of $E_2$ and TAM for 24 hr. In both the vector control cells and MCF7-MKP-1 cells, no obvious change in MKP-1 protein expression was observed.

Interestingly, a small but noticeable increase in the expression of MKP-2 was observed in both the vector control and MCF7-MKP-1 cell lines following tamoxifen treatment when compared with the vehicle control (Fig. 9, Vector Control Lanes 5-7, MCF7-MKP-1 Lanes 7-9). Since endogenous MKP-2 is increased by tamoxifen
Figure 6. MKP-1 and MKP-2 protein and mRNA expression in breast cell lines. A. Western blot showing the protein expression levels of MKP-1 (39 kDa) and MKP-2 (43 kDa) in a panel of breast cell lines. Whole cell lysates were probed with anti-MKP-1 antibody. Actin was used as a loading control. B. Real-Time RT-PCR analysis was used to measure mRNA expression levels of MKP-1 and MKP-2 in the same panel of breast cell lines. Relative mRNA levels were determined using the ΔΔCt method. GAPDH was used as the internal control. Results are representative of at least three independent experiments with each sample in triplicate. Error bars represent the standard error of the mean. C. CAOV3 ovarian cancer cells were stably transfected with either a non-target shRNA or shRNA toward MKP-1. Whole cell lysates were probed with anti-MKP-1 antibody. Actin was used as a loading control.
Figure 7. MKP-1 and MKP-2 Amino Acid Sequence Alignment. The amino acid sequences of MKP-1 and MKP-2 were aligned using the ClustalW2 software in order to determine if the epitope of the MKP-1 antibody used in these studies could also recognize MKP-2. Boxed area indicates the sequence containing the epitope of the MKP-1 antibody. Identical residues are shown in white.
Figure 8. The 43 kDa protein recognized by the MKP-1 antibody is MKP-2. MCF7 cells engineered to overexpress MKP-2 with a V5-His tag were transiently transfected with either a non-target control shRNA or one of four shRNA constructs targeting MKP-2. Constructs 3 and 4 showed the ability to decrease MKP-2 and MKP-2-V5-His protein expression. Whole cell lysates were probed with anti-MKP-1 antibody. Actin was used as a loading control.
Figure 9. MKP-1 protein expression is unchanged, but MKP-2 protein expression increases following tamoxifen treatment.  A.  MCF7 vector control cells were treated for 24 hrs. with either vehicle, 1 nM E2, 10 nM E2, 1 nM E2 and 100 nM TAM, 10 nM E2 and 1000 nM TAM, 100 nM TAM or 1000 nM TAM.  Whole cell lysates were analyzed by Western blot and membrane was probed with anti-MKP-1 antibody.  Actin was used as a loading control.  B.  MCF7-MKP-1 cells were treated as described in panel A.  Whole cell lysates were analyzed by Western blot and membrane was probed with anti-MKP-1 antibody.  Actin was used as a loading control.  VC = MCF7 vector control cells.
treatment, it suggests that MKP-2 expression may be regulated in an ER-mediated manner. This possibility will be investigated in greater depth in subsequent chapters.

Next, it was necessary to investigate whether MKP-1 overexpression contributes to changes in tamoxifen sensitivity. MTT assays were used to assess the effects of MKP-1 overexpression on tamoxifen sensitivity. The assay was first conducted with parental MCF7 cells, which are tamoxifen sensitive. These cells were plated in phenol red free medium supplemented with charcoal stripped serum to minimize any baseline estrogenic effects. On the following day, cells were treated with vehicle (EtOH, 0.01% v/v), 10 nM E2, 1000 nM TAM or a combination of 10 nM E2 and 100 or 1000 nM TAM. Treatments were refreshed on Day 4 and the assay was allowed to continue for seven days. Treatment with E2 showed approximately a 2.5 fold increase in cell proliferation (p<0.01) and this increase was blocked by the addition of tamoxifen (Fig. 10A). These results demonstrate that the MCF7 cells used in this study are indeed E2 responsive and tamoxifen sensitive.

Before looking at the effect of MKP overexpression on TAM sensitivity, pooled populations of MCF7 cells engineered to stably overexpress either MKP-1 or MKP-2 were generated to better reflect the heterogeneity known to be present in human tumors. These cell lines express MKP-1 or MKP-2 with a V5-His tag. Western blot analysis was conducted to confirm the presence of the V5 tag, which was expressed at the expected size of MKP-1 at 43 kDa and MKP-2 at 48 kDa. The results of this analysis showed that the V5 tag is present in the MCF7-MKP-1-Pool and MCF7-MKP-2-Pool cells, but not in MCF7-pCDNA3.1-Pool cells (Fig. 10B). MCF7-pCDNA3.1-Pool cells and MCF7-MKP-
1-Pool cells were then subjected to MTT analysis (Fig. 10C). This assay was carried out in regular medium because the cells were very sensitive to E₂ withdrawal and showed poor viability in phenol red-free medium. Treatment with E₂ doubled proliferation (p<0.01) of MCF7-pCDNA3.1-Pool cells compared to vehicle control. Treatment with TAM in the presence of E₂ blocked this increase and cell proliferation was further decreased with TAM alone. In the MCF7-MKP-1-Pool cells, overexpression of MKP-1 almost completely eliminated the E₂-induced increase in cell proliferation. Treatment with E₂ plus TAM or TAM alone reduced cell proliferation by similar amounts compared to vehicle control. The results of this assay suggest that overexpression of MKP-1 does not increase the sensitivity of MCF7 cells to tamoxifen, but that it does decrease cell proliferation in the presence of estrogen.

The decrease in cell proliferation in the presence of estrogen following MKP-1 overexpression suggests that the activity of MKP-1 is directed toward the MAPK that drives the proliferation of these cells. To further investigate this, activation of two MAPKs closely associated with cell growth, ERK1/2 and JNK1/2, was examined in MCF7-pCDNA3.1-Pool and MCF7-MKP-1-Pool cells. Preliminary experiments failed to detect p38 protein expression (data not shown), so it was not included in any further experiments. MCF7-pCDNA3.1-Pool and MCF7-MKP-1 Pool cells were plated in phenol red-free medium and treated with either vehicle (EtOH, 0.01% v/v), E₂, the combination of E₂ plus TAM, TAM alone, the combination of E₂ and ICI 182,170 (ICI) or ICI alone for four hours. This time point was chosen based on previous time course experiments (data not shown). MCF7 cells that were serum-starved overnight and then
treated with serum for 10 minutes were used as the positive control for ERK1/2 phosphorylation. Basal levels of phospho-ERK1/2 were observed in vector controls that were not affected by treatment. This activity was completely eliminated following MKP-1 overexpression (Fig. 11A). MCF7 cells treated with 10 ng/mL anisomycin for 30 minutes served as the positive control for JNK1/2 phosphorylation. JNK1/2 activation was not detected in either cell line (Fig. 11B), suggesting that ERK1/2 signaling is a major driver of MCF7 cell proliferation.

**Conclusion**

The MAP Kinase phosphatase MKP-1 has been linked to chemotherapy resistance and is known to exhibit altered expression in many different cancer types, including breast. This previous work led to the formulation of the hypothesis that an increase in MKP-1 expression is associated with changes in breast cancer tamoxifen sensitivity. In order to study this association, breast cell lines were screened for MKP expression. The low expression of MKP-1 and MKP-2 in MCF7 cells made this cell line an ideal candidate to study the effects of the overexpression of MKP-1 in breast cancer. The first step was to examine the effect of E2 and TAM treatment on MKP-1 protein expression. Treatment with E2, TAM or their combination revealed no obvious change in MKP-1 protein expression. E2 is known to increase cell proliferation by upregulating growth factor pathways. TAM treatment blocks this activation. MKP-1 overexpression failed to change tamoxifen sensitivity, exhibiting a similar cell viability profile to tamoxifen-sensitive MCF7 vector control cells. MKP-1 overexpression did decrease cell proliferation in the presence of E2 and examination of ERK1/2 and JNK1/2 activation in
those cells showed that, compared to vector control cells, ERK1/2 phosphorylation was completely eliminated and that JNK1/2 activation was not present in either cell line. These data indicate that, contrary to the study hypothesis, MKP-1 does not play a role in contributing to changes in breast cancer tamoxifen sensitivity. However, MKP-2 protein expression was increased following tamoxifen treatment. This result suggests that MKP-2 expression might be regulated via an ER mediated mechanism and that changes in MKP-2 expression could play a role in contributing to changes in tamoxifen sensitivity.
Figure 10. MKP-1 Overexpression Does Not Alter Tamoxifen Sensitivity. A. MCF7 cells were treated with Vehicle, E$_2$, E$_2$+TAM or TAM alone and subjected to MTT analysis. Cells were treated on days 1 and 4. Absorbance was read on day 7. Results are representative of at least three independent experiments with samples plated in triplicate. Error bars represent the standard error of the mean. Statistical analysis was done using Student’s t-test. ** = p<0.01  
B. Western blot depicting the expression of the V5 tag in MCF7-MKP-1-Pool and MCF7-MKP-2-Pool cells. Population was selected with G418 antibiotic for one month and whole cell lysates were probed with V5 antibody. GAPDH was used as a loading control.  
C. MCF7-pCDNA3.1-Pool and MCF7-MKP-1-Pool cells were treated with Vehicle, E$_2$, E$_2$+TAM or TAM alone and subjected to MTT analysis. Cells were treated on days 1 and 4. Absorbance was read on day 7. Results are representative of at least three independent experiments with samples plated in triplicate. Error bars represent the standard error of the mean. Statistical analysis was done using Student’s t-test. ** = p <0.01
Figure 11. MKP-1 Overexpression Eliminates ERK1/2 activation. A. MCF7-pCDNA3.1-Pool cells and MCF7-MKP-1-Pool cells were treated with vehicle, E2, the combination of E2+TAM, TAM alone, the combination of E2+ICI or ICI alone for four hours. Serum-starved MCF7 cells were treated for 10 min. with serum and used as a positive control for ERK1/2 phosphorylation. Whole cell lysates were analyzed by western blot and membranes were probed with either phospho-ERK1/2 (P-ERK1/2) or Total ERK1/2 antibody. GAPDH was used as a loading control. B. MCF7-pCDNA3.1-Pool and MCF7-MKP-1-Pool cells were treated as described in panel A. MCF7 cells treated with 10 ng/mL anisomycin for 30 minutes were used as a positive control for JNK1/2 phosphorylation. Whole cell lysates were analyzed by western blot. Membranes were probed with either phospho-JNK1/2 (P-JNK1/2) or Total JNK1/2 antibody. GAPDH was used as a loading control.
CHAPTER 3
CHARACTERIZATION OF MKP-2 IN BREAST CANCER TAMOXIFEN SENSITIVITY

Introduction
The data presented thus far show that MKP-1 overexpression does not change tamoxifen sensitivity of MCF7 cells, that MKP-1 protein expression is not obviously altered after treatment with estrogen, tamoxifen or their combination and that MKP-2 protein expression increases under these conditions. These data suggest MKP-2, rather than MKP-1, might be a player in tamoxifen sensitivity of MCF7 cells.

Next to MKP-1, MKP-2 is the best characterized MKP, although considerably less is known about its role in cancer and chemotherapy resistance. MKP-2 was first identified by Misra-Press et al in 1994. The authors of this study demonstrated that MKP-2 is expressed in a wide variety of rat tissues in a profile that is overlapping yet distinct from MKP-1. They also suggested that this differential expression combined with the unique N-terminal sequences of the two phosphatases indicate distinct functional roles for each protein.

Subsequent work by this group investigated the ability of MKP-1 and MKP-2 to dephosphorylate the stress-activated MAPK, JNK. Their analysis revealed that MKP-1 was better able to dephosphorylate JNK-1 than MKP-2 and that both phosphatases showed similar abilities to dephosphorylate JNK-2. Further analysis of MKP-2 substrate specificity showed that it is also able to dephosphorylate ERK1/2, but had little effect on p38. These analyses of substrate specificity were conducted using
exogenously expressed proteins. Additionally, MKP-2 substrate specificity has not been examined in the context of tamoxifen sensitivity. Recently, MKP-2 knockout mice were created to study MKP-2 physiological function. This study showed that deletion of MKP-2 decreased cell proliferation and cell cycle analysis showed that MKP-2/− MEFS accumulated in the G2/M phase, suggesting that MKP-2 might play a role in cell cycle progression.76

Studying the transcriptional regulation of MKP-2 has provided insight into its possible mechanistic role in cancer. Evidence has been put forth that MKP-2 is a transcriptional target of p53 in response to oxidative stress. This would seem to be consistent with the idea that MKP-2 might play a role in cell cycle regulation.77 The authors of this study suggested that MKP-2 might contribute to tumor suppression77, which is certainly contrary to the notion that it would play a role in cell cycle progression. MKP-2 has also been identified as a transcriptional target of E2F-1. E2F-1/− MEFs showed decreased levels of H2O2-induced MKP-2 protein compared to E2F-1+/+ MEFs and also displayed resistance to oxidative stress induced cell death.78 When MKP-2 was overexpressed in MCF7 cells, these cells were more sensitive to H2O2 treatment than vector control cells. MCF7 cells overexpressing MKP-2 also showed decreased colony formation in soft agar compared to vector control.78 When injected into female athymic nude mice, MCF7-MKP-2 cells formed tumors in 2 of 12 mice, whereas all mice injected with vector control cells formed tumors.78

The exploration of the expression of MKP-2 in cancer has been limited thus far. As discussed in Chapter 1, its expression levels have been examined in pancreatic44,
liver\textsuperscript{43}, ovarian\textsuperscript{9} and breast cancers\textsuperscript{24}, as well as in glioblastomas\textsuperscript{45}. MKP-2 expression has been shown to be upregulated in all of these cancer types, with the exception of ovarian cancer and glioblastoma. In primary tumors as well as in glioblastoma cell lines, methylation of a CpG island in the MKP-2 promoter was detected.\textsuperscript{45} MKP-2 promoter methylation was found to occur more frequently in tumors with p53 mutation, but in tumors with amplified EGFR it was virtually absent. Additionally, the overexpression of MKP-2 in glioma cell lines showed a decrease in cell proliferation, furthering the idea that MKP-2 plays a role in growth suppression.\textsuperscript{45} Another study that supports this idea showed that the MKP-2 promoter was more highly methylated in basal-like breast tumors resected following neo-adjuvant chemotherapy compared to other breast cancer subtypes and overexpression of MKP-2 in these tumors increased chemotherapy-induced apoptosis.\textsuperscript{79} These results suggest that loss of MKP-2 expression may contribute to chemotherapy resistance.

Prior to the work presented in this chapter, no connections had been made between MKP-2 expression and the role it plays in regard to tamoxifen sensitivity. To investigate this, MKP-2 gene and protein expression were characterized in tamoxifen sensitive and tamoxifen resistant cells, along with the effect of MKP-2 overexpression on cell proliferation following tamoxifen treatment. The putative promoter of MKP-2 has also been identified in order to study the regulation of MKP-2 gene expression in response to estrogen and tamoxifen treatments. The studies presented here collectively show that MKP-2 is expressed in tamoxifen resistant cells, that overexpression of MKP-2 decreases cell proliferation in response to estrogen yet maintains tamoxifen sensitivity,
and that MKP-2 promoter activity is not changed following E₂ or TAM treatment. These data suggest that in the context of tamoxifen resistance, MKP-2 may be upregulated to return the cell to a tamoxifen sensitive phenotype.

**Materials and Methods**

Cell culture, generation of whole cell lysates, Western blot analyses, MTT assays, RNA isolation and real-time RT-PCR were conducted as described in the Materials and Methods section of Chapter 2.

**Identification of the Putative MKP-2 Promoter**

The putative promoter of MKP-2 was identified using the Genomatix software suite by entering DUSP4 in the Gene2Promoter database and aligning the resulting sequences with the gene sequence of MKP-2 (gene id 1846). The promoter sequence that was determined to be the best match was GXP_35747, a 1207 bp sequence on the negative strand of chromosome 8 from bp 29,208,769 to 29,207,563. Transcription factor binding sites were identified using the MatInspector program.

**Amplification of the MKP-2 Promoter**

The promoter of MKP-2 was amplified from human placental genomic DNA using primers containing XhoI (forward primer) and HindIII (reverse primer) restriction sites to facilitate the cloning of the promoter into the appropriate vectors. The primer sequences were as follows: MKP-2-pro-XhoI (5’-CCG CTC GAG GGC TGT CAC GCG GGG AAG CG-3’; +1 to 20; Genomatix accession id: GXP_35747) and MKP-2-pro-HindIII (5’-CCC AAG CTT TTA GCC CGC CGC CGC ACG AT-3’; +1207 to 1188; Genomatix accession id: GXP_35747). The MKP-2 promoter was amplified using
Choice Taq DNA polymerase (Denville) in a PTC-200 Peltier thermal cycler under the following conditions: 95°C for 5 min, followed by 35 cycles of a 1 min, 95°C denaturing step, 2 min, 55°C annealing step and 3 min, 72°C extension step. Upon the completion of 35 cycles, a 10 minute final extension step was performed at 72°C.

**Cloning of the MKP-2 Promoter**

The putative MKP-2 promoter was cloned into the pCR-TOPO4 TA cloning vector (Invitrogen). The fragment containing the putative MKP-2 promoter was released from the TA cloning vector using the EcoRI (Invitrogen) restriction sites according to the manufacturer’s protocol. The promoter fragment was subsequently digested with XhoI and HindIII and subcloned into the pGL3-Basic Luciferase Reporter vector (Promega) using the XhoI and HindIII (New England Biolabs) restriction sites according to the manufacturer’s protocol.

**Sequencing**

Sequencing of the pCR4-MKP-2 promoter construct was carried out by Genewiz using the M13 forward (5’-ACT GGC CGT CGT TTT AC-3’) and reverse (5’-ACA GGA AAC AGC TAT GA-3’) priming sites found on the pCR4-TOPO vector. After subcloning the MKP-2 promoter into the pGL3-Basic vector, the construct was sequenced by Genewiz using the MKP-2-pro-XhoI (forward) and MKP-2-pro-HindIII (reverse) primers (see Cloning section above for sequences) as well as the GLprimer2 primer (5’-TGG AAG ACG CCA AAA ACA TAA AG-3’), which recognizes the luciferase transcription start site present in the pGL3-Basic vector.
Transfection and Luciferase Reporter Assay

Transfection of the empty pGL3 and pGL3-MKP-2-pro constructs into MCF7, MCF7-TAMR and MDA-MB-231 cells (2.5 x 10^5 cells per well in 6 well plates) was carried out using the Metafectine Easy transfection reagent (Biontex). Briefly, 5 µl of metafectine was incubated in 100 µl phenol red free, serum free DMEM/F12 (1:1) medium for twenty minutes. Five micrograms of either empty pGL3 or pGL3-MKP-2-pro DNA and 0.5 ng of pRLTK (expressing Renilla luciferase and serving as an internal control) in 15 µl of DNAse-RNAse free water (GIBCO) was added to the metafectine and incubated at room temperature for twenty minutes to allow complexes to form. The mixture was added drop-wise to the cells. Vehicle, E2, TAM or ICI treatments were added 24 hrs post-transfection. Luciferase reporter activity was measured 48 hrs post-transfection using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. The TD-20/20 luminometer (Turner Designs) was used to read the luciferase activity of each sample.

Results

The data presented thus far show that overexpression of MKP-1 does not change tamoxifen sensitivity in MCF7 cells and that MKP-2 protein was increased with tamoxifen treatment. It was important to verify a potential role for MKP-2 in tamoxifen sensitivity under physiological conditions. This was done with an isogenic model of acquired tamoxifen resistance (MCF7 and MCF7-TAMR cells). MCF7 cells that had been selected with increasing amounts of tamoxifen for over six months (MCF7-TAMR cells) were obtained from the lab of Dr. Malathy Shekhar. MKP protein expression in
these cells was determined by western blot analysis and it was shown that as compared to parental MCF7 cells, MKP-2 protein expression was 3.5–fold higher and that MKP-1 protein expression was not detectable (Fig. 12A). This was supported by real-time PCR analysis, which showed that MKP-2 mRNA was increased 12-fold in tamoxifen resistant cells when compared to parental MCF7 cells and that MKP-1 mRNA expression was negligible (Figure 12B).

To further characterize the MCF7-TAMR cells, MTT analysis was carried out in phenol red free medium as described previously. It was found that these cells showed a modest increase in proliferation when treated with 10 nM E₂ and that tamoxifen treatment did not block this increase, but in fact further stimulated cell proliferation. This indicates that these cells are not sensitive to estrogen and are indeed tamoxifen resistant (Fig 12C). Since it has been shown that phosphorylation of Ser118 in the estrogen receptor leads to its ligand-independent activation and it has been suggested that this site is associated with tamoxifen resistance, phosphorylation of this site in MCF7 and MCF7-TAMR cells was examined (Fig. 13). Cells were plated in phenol red-free medium and treated with vehicle (EtOH, 0.01% v/v), 10 nM E₂, 1 µM tamoxifen, the combination of E₂ and 100-fold molar excess tamoxifen, the combination of 10 nM E₂ and 100-fold molar excess ICI or 1 µM ICI for 4 hours. In both cell lines, estrogen treatment increased Ser118 phosphorylation. This increase was reduced by treatment with anti-estrogens. Additionally, MKP-2 protein expression in MCF7 (Fig. 13A) and MCF7-TAMR (Fig. 13B) cells under these conditions was examined. In MCF7 cells, MKP-2 levels were
Figure 12. Characterization of MCF7-TAMR Cells. A. MKP-2 protein expression is increased in MCF7-TAMR cells compared to MCF7 parental cells. Whole cell lysates were analyzed by western blot and probed with anti-MKP-1 antibody. MCF7-MKP-1 cells were included as a positive control for MKP-1 expression. B. MKP-2 mRNA expression is increased in MCF7-TAMR cells compared to MCF7 parental cells. Gene expression was detected by qRT-PCR. GAPDH was used as an internal control. Results are representative of two independent experiments. Samples were plated in triplicate. Error bars represent the standard error of the mean. C. MCF7 and MCF7-TAMR cells were treated with vehicle, E2, E2+TAM or TAM alone and subjected to MTT analysis. Cells were treated on days 1 and 4. Absorbance was read on day 7. Results are representative of at least three independent experiments with samples plated in triplicate. Statistical analysis was done with Student’s t-test. ** = p<0.01
Figure 13. MKP-2 protein expression is increased following anti-estrogen treatment in MCF7 cells and constitutively expressed in MCF7-TAMR cells. A. MCF7 cells were treated with vehicle, E2, E2+TAM, TAM alone, E2+ICI or ICI alone for 4 hours in phenol red free medium. Whole cell lysates were subjected to western blot analysis and probed with antibody toward P-S118, total ERα, or MKP-2. Actin was used as a loading control. B. MCF7-TAMR cells were treated with vehicle, E2, E2+TAM, TAM alone, E2+ICI or ICI alone for 4 hrs in phenol red free medium. Whole cell lysates were subjected to western blot analysis and probed with antibody toward P-S118, total ERα or MKP-2. Actin was used as a loading control.
slightly increased with anti-estrogen treatment, which is similar to what was observed in vector control and MKP-1 overexpression cells previously (Fig. 9). In MCF7-TAMR cells, however, MKP-2 was shown to be constitutively expressed, regardless of treatment condition. MKP-1 expression was not detected in either cell line.

To study MAP Kinase activation in these cells, levels of phosphorylated ERK1/2 in both MCF7 and MCF7-TAMR cells following 4 hour treatment with 10 nM E2, 1 µM tamoxifen, 1 µM ICI or the combination of estrogen and tamoxifen or estrogen and ICI were examined (Fig. 14). MCF7 cells that were serum-starved overnight and subsequently treated with serum for 10 minutes served as a positive control for ERK1/2 phosphorylation. Following treatment with E2, basal ERK1/2 phosphorylation in MCF7 cells was observed at a similar level as vehicle control and this phosphorylation was decreased following treatment with E2 plus TAM, TAM alone, E2 plus ICI and ICI alone, suggesting that levels of phosphorylated ERK1/2 in MCF7 cells are responsive to anti-estrogen treatment (Fig. 14A). Previous work in the field has shown that treatment of MCF7 cells with E2 induces ERK1/2 phosphorylation to a much greater extent than shown here.\textsuperscript{80} This is possibly due to the absence of phosphatase inhibitors in the lysis buffer used to generate whole cell lysates. In MCF7-TAMR cells, ERK1/2 activation was present regardless of treatment condition (Fig. 14B). JNK1/2 activation was also examined in both cell lines, with MCF7 cells treated with anisomycin for 30 minutes serving as a positive control. JNK1/2 phosphorylation could not be detected in either MCF7 (Fig. 14A) or MCF7-TAMR cells (Fig. 14B).
As mentioned in Chapter 2, in order to study the role of MKP-2 in tamoxifen sensitivity, a pooled population of MCF7 cells engineered to overexpress MKP-2 was generated. MKP-2 protein expression was measured in the MCF7-pCDNA3.1-Pool cells and the MCF7-MKP-2-Pool cells (Fig. 15A). MKP-2 expression was low in MCF7-pCDNA3.1-Pool cells. In the MCF7-MKP-2-Pool cells, endogenous MKP-2 expression was present at low levels, while exogenous MKP-2 expression was present at higher levels. These cells were then used to perform an MTT assay under the same treatment conditions described previously (Fig. 15B). The vector control cells used in this experiment were the same as those used in Fig. 10. MCF7-MKP-2-Pool cells showed a decreased response to estrogen, but maintained tamoxifen sensitivity. These data suggest that MKP-2 overexpression inhibits cell proliferation in response to estrogen, but retains sensitivity to tamoxifen.

As noted earlier, in MCF7-TAMR cells, MKP-2 protein expression is increased compared to MCF7 cells and is constitutively expressed regardless of treatment condition. To further assess the role of MKP-2 activity in the context of tamoxifen response, MCF7-pCDNA3.1-Pool, MCF7-MKP-1-Pool and MCF7-MKP-2-Pool cells were treated with vehicle, E₂ or the combination of E₂ and one of three increasing concentrations of TAM and cell proliferation was analyzed using an MTT assay (Fig. 15C). Compared to the vector control cells, overexpression of MKP-2 caused a significant (p<0.05) decrease in cell proliferation following E₂ treatment. Treatment with a combination of E₂ with 1, 10 or 100-fold molar excess TAM resulted in dose-dependent inhibition of MCF7-MKP-1-Pool and MCF7-MKP-2-Pool cell proliferation.
Figure 14. Phosphorylation of ERK1/2 in MCF7 cells is responsive to anti-hormone treatment. A. MCF7 cells were treated with vehicle, E$_2$, E$_2$+TAM, TAM alone, E$_2$+ICI or ICI alone for 4 hours in phenol red free medium. Serum starved MCF7 cells were treated with serum for 10 minutes and were included as a positive control for ERK1/2 phosphorylation. MCF7 cells treated with 10 ng/mL anisomycin for 30 minutes were included as a positive control for JNK1/2 phosphorylation. Whole cell lysates were subjected to western blot analysis and probed with antibody toward P-ERK1/2, total ERK1/2, P-JNK1/2 or total JNK1/2. GAPDH was used as a loading control. B. MCF7-TAMR cells were treated as described in Panel A.
Figure 15. Overexpression of MKP-2 affects cell proliferation in response to E2, and increases TAM sensitivity compared to vector control and MKP-1 overexpressing cells. A. Whole cell lysates of MCF7-pCDNA3.1-Pool and MCF7-MKP-2-Pool were treated with vehicle, E2, E2+TAM, TAM, E2+ICI or ICI for 4 hours, then subjected to western blot analysis and probed with antibody toward MKP-1. GAPDH was used as a loading control. B. MCF7-pCDNA3.1-Pool cells and MCF7-MKP-2-Pool cells were treated with vehicle, 10 nM E2, E2+TAM, 100 nM TAM or 1000 nM TAM and MTT analysis was performed. Cells were treated on days 1 and 4. Absorbance was read on day 7. Results are representative of at least three independent experiments with samples plated in triplicate. Statistical analysis was done using Student’s t-test. ** = p<0.01 C. MCF7-pCDNA3.1-Pool cells, MCF7-MKP-1-Pool cells and MCF7-MKP-2-Pool cells were treated with vehicle, E2 or E2 plus one of three increasing concentrations of TAM and and MTT assay was performed. Cells were treated on days 1 and 4 and absorbance was read on day 7. Results are representative of two independent experiments with samples plated in triplicate. Statistical analysis was done using Student’s t-test. * = p<0.05, ** = p <0.01, *** = p<0.001
Compared to vector control or MKP-1 overexpressing cells, MKP-2 overexpressing cells were associated with greater tamoxifen sensitivity as significant (p<0.01) decreases were observed at all three doses of tamoxifen.

To identify the MAPK that MKP-2 was inactivating, ERK1/2 and JNK1/2 phosphorylation were measured by western blot (Fig. 16). The vector control cells shown here are the same as those in Fig. 11. As discussed before, basal ERK1/2 activation was observed and JNK1/2 activation was not present in these cells (Fig. 16A). MKP-2 overexpression completely abolished ERK1/2 activity. As expected, no JNK1/2 phosphorylation was detected (Fig. 16B), suggesting that ERK1/2 is a major driver of proliferation in these cells.

The increase in MKP-2 protein expression following treatment with tamoxifen suggested that the MKP-2 promoter might be regulated via an ER-mediated mechanism. In order to investigate this, the Genomatix software suite was used to identify the putative promoter for MKP-2, and MatInspector was used to locate putative transcription factor binding sites. This analysis revealed that there are three estrogen response element (ERE) half sites within the putative promoter sequence, two on the negative strand, which is the DNA strand containing the MKP-2 gene, and one on the positive strand. Fig. 17 shows the MKP-2 gene with the promoter region, and the putative ERE half sites. Although these are not canonical EREs in the sense that a full response element is not present, the presence of a single ERE half site has been shown to be sufficient to
Figure 16. Overexpression of MKP-2 eliminates ERK1/2 phosphorylation. A. MCF7-pCDNA3.1-Pool and MCF7-MKP-2-Pool cells were plated in phenol red-free medium and treated with vehicle, E₂, E₂+TAM, TAM, E₂+ICI or ICI for 4 hours. Whole cell lysates were analyzed by western blot and probed with either P-ERK1/2 or total ERK1/2 antibody. Serum starved MCF7 cells treated with serum for 10 minutes were used as positive control for ERK1/2 phosphorylation. GAPDH was used as a loading control. B. MCF7-pCDNA3.1-Pool and MCF7-MKP-2-Pool cells were treated as described in Panel A. Whole cell lysates were analyzed by western blot and probed with either P-JNK1/2 or total JNK1/2 antibody. MCF7 cells treated with 10 ng/mL anisomycin for 30 minutes were included as a positive control for JNK1/2 phosphorylation. GAPDH was used as a loading control.
produce a response to estrogen if stabilized through interactions with another transcription factor.\textsuperscript{30} Interestingly, these ERE half sites in the putative MKP-2 promoter are located in very close proximity to canonical AP-2 response elements.

To study MKP-2 promoter regulation, the MKP-2 promoter was amplified from human placental genomic DNA. The promoter fragment was subcloned into the pCR4-TOPO TA cloning vector. After obtaining a clone positive for the MKP-2 promoter, it was verified by sequencing using M13 primers that recognize the pCR4 vector sequence. Sequence analysis confirmed that the insert was the MKP-2 promoter and that no mutations had been introduced during the amplification and cloning process. The next step was to subclone the promoter into the pGL3-Basic vector. This was done using the XhoI and HindIII restriction sites. Restriction digest analysis was performed with the XhoI and HindIII enzymes and showed that the insert was the same size obtained from the TA cloning vector (Fig. 18). The presence of the MKP-2 promoter was verified by sequencing with the primers initially used to isolate it, as well as a primer that recognized the transcription start site of the luciferase reporter gene present in the pGL3-Basic vector. The results of both of these sequencing reactions confirmed the presence of the MKP-2 promoter and that no mutations were introduced during cloning.

Once the MKP-2 promoter-driven luciferase reporter construct was created, its activity was tested in MDA-MB-231 cells, as MDA-MB-231 cells express high levels of MKP-2 mRNA (Fig. 6), indicative of elevated MKP-2 promoter activity. The cells were transfected with either the empty pGL3, or 2, 3, 5 or 10 µg of the pGL3-MKP-2-pro
Figure 17. The putative MKP-2 promoter contains ERE half sites. Schematic depicting the sequence features of the MKP-2 promoter, including estrogen response element half sites and AP-2 transcription factor binding sites.
Figure 18. The putative MKP-2 promoter is subcloned into the pGL3-Basic Luciferase Reporter vector. Following TA cloning and subcloning into pGL3-Basic vector, constructs underwent restriction digest analysis with XhoI and HindIII to confirm presence of MKP-2 promoter insert. Products were run on a 1% agarose gel.
construct with *Renilla* luciferase used as an internal control. MKP-2 promoter-driven luciferase was expressed robustly in MDA-MB-231 cells and increased with increasing amounts of MKP-2 promoter DNA transfection (Fig. 19A). These data suggest that the putative MKP-2 promoter sequence possesses promoter activity. Next, the pGL3-MKP-2-pro construct was transfected into MCF7 and MCF7-TAMR cells. Cells were plated in phenol red free medium and transfected with 5 µg of either the pGL3-Basic vector or the pGL3-MKP-2-pro construct along with pRLTK. On the day following transfection, cells were treated with vehicle (EtOH, 0.01% v/v), 10 nM E2, 1 µM TAM, 1 µM ICI or the combination of E2 plus TAM or E2 plus ICI. Levels of basal reporter gene activation in MCF7 and MCF7-TAMR cells were substantially lower than in the ER-negative MDA-MB-231 cells, and treatment with E2, TAM or ICI did not alter the basal activity in MCF-7 and MCF-7-TAMR cells. Since promoter activity was seen in MDA-MB-231 cells, this suggests the presence of functional transcription regulatory elements in the 1207 bp fragment used for MKP-2 promoter analysis. The weak activity exhibited in MCF7 and MCF7-TAMR cells may be due to low transfection efficiency, as *Renilla* luciferase levels were much lower in MCF7 and MCF7-TAMR cells as compared to MDA-MB-231 cells. Since the basal activities of the promoter construct were unaffected by hormones, it is possible that the hormonal response elements capable of regulating MKP-2 promoter activity reside outside the selected DNA fragment, or that MKP-2 gene expression is not controlled by classical ER regulated mechanisms. However, since MCF7-TAMR cells express higher levels of MKP-2 mRNA compared to parental MCF7 cells, this suggests that a more detailed analysis of MKP-2 promoter regulation is warranted. Another
Figure 19. MKP-2 promoter driven luciferase activity. A. Increasing amounts of the pGL3-MKP-2-pro construct were transfected into MDA-MB-231 cells to test construct activity. 4.0 x 10^5 cells were plated in 6-well plates and luciferase activity was measured 48 hr. post-transfection. B. The pGL3-MKP-2-pro construct was transfected into MCF7 cells to examine regulation of MKP-2 promoter following treatment with vehicle, 10 nM E_2, 10 nM E_2+1µM TAM, 1µM TAM, 10 nM E_2+1µM ICI or 1 µM ICI. 2.5 x 10^5 cells were plated in phenol red-free medium in 6-well plates. Treatments were administered 24 hrs post-transfection and luciferase activity was measured 48 hr. post-transfection. Results represent the average of three independent experiments. Error bars represent the standard error of the mean. C. MCF7-TAMR cells were transfected with the pGL3-MKP-2-pro construct and luciferase activity was measured as described in Panel B.
possible explanation for the observed accumulation of MKP-2 protein in MCF7-TAMR cells is its post-translational modification by ERK1/2 or another kinase. As noted previously, MKP-2 protein can be phosphorylated by active ERK1/2, which stabilizes the protein. This phosphorylation is required to prevent rapid proteasomal degradation and to allow MKP-2 to carry out the dephosphorylation of MAPKs. Taking these results together, it is clear that further investigation is necessary to identify the mechanisms responsible for regulating MKP-2 in response to tamoxifen treatment.

**Conclusion**

MKP-2, a nuclear MKP that preferentially activates ERK and JNK1/2 over p38, has been shown to be overexpressed in breast cancer tissues, but no connection had been made previously between MKP-2 expression and its role in tamoxifen sensitivity. The data presented here have shown that compared to parental MCF7 cells, MCF7-TAMR cells exhibit increased MKP-2 gene and protein expression and that MKP-1 gene and protein expression are negligible. MTT analysis revealed that MKP-2 overexpression diminishes cell proliferation in response to estrogen compared to vector control cells, but that tamoxifen sensitivity was maintained. MKP-2 expression was increased following tamoxifen treatment and a 1207 bp putative promoter was identified. Its regulation by E2 and anti-estrogens was examined in MCF7 and MCF7-TAMR cells. MKP-2 basal promoter activity was low and treatment with hormone had no influence on basal activity. However, this 1207 bp fragment showed robust luciferase reporter activity in MDA-MB-231 cells. These data suggest that elements regulating hormonal response
of MKP-2 probably lie outside the 1207 bp fragment used for the promoter assay or that MKP-2 gene expression is controlled by non-classical E₂ responsive mechanisms.

Taken together, the results presented in this chapter suggest that MKP-2 overexpression sensitizes breast cancer cells to tamoxifen treatment and that tamoxifen resistant cells upregulate MKP-2 expression to potentially return them to a tamoxifen sensitive state. In a proposed working model (Fig. 20), treatment of MCF7 cells with tamoxifen increases MKP-2, which dephosphorylates ERK1/2. MCF7 cells are dependent on E₂/ERα for activation of ERK1/2, which is inhibited by tamoxifen. Dephosphorylation of ERK1/2 by tamoxifen-induced MKP-2 slows cell proliferation and induces cell death. In tamoxifen resistant MCF7 cells, phosphorylated ERK1/2 is present at high levels. These cells are insensitive to E₂ and ERK1/2 activation is driven by growth factor signaling pathways, such as EGFR and HER2, which are known to be overexpressed in tamoxifen resistant tumors.³⁶ MKP-2 levels are upregulated in TAMR cells, but are unable to inhibit ERK1/2 activation probably because of robust activation of ERK1/2 by growth factor pathways. Previous work in our lab has shown that activated ERK1/2 phosphorylates MKP-2 on Ser 386 and Ser 391, which stabilizes the MKP-2 protein and protects it against proteasomal degradation without affecting its catalytic activity.¹⁹
Figure 20. Working model depicting the role of MKP-2 in tamoxifen sensitivity. In tamoxifen sensitive cells, phosphorylated ERK1/2 is present, indicating that cell-growth signaling pathways are activated. Following treatment with anti-estrogens such as TAM, MKP-2 protein expression is increased, which leads to the dephosphorylation of active ERK1/2, slowing or eliminating cell proliferation. In tamoxifen resistant cells, phosphorylated ERK1/2 is constitutively present at higher levels than in tamoxifen sensitive cells. These cells are constantly upregulating MKP-2 protein expression in an attempt to return the level of active ERK1/2 to that of a tamoxifen sensitive cell. Continuous stimulation of ERK1/2 by growth factors, which are not sensitive to inhibition by tamoxifen, leads to the persistence of growth signals in the cell.
CHAPTER 4

SUMMARY AND DISCUSSION OF CONCLUSIONS

It is no secret that the emergence of resistance to currently available therapies is a major problem seen all too frequently in the clinical setting. Research aimed at discovering the mechanisms underlying drug resistance and finding biomarkers that would be able to classify tumors as either drug resistant or drug sensitive has been a major focus of the field in recent years. Cell signaling pathways have been central figures in many of these studies. One such family of signaling pathways that has emerged as a player in breast cancer progression and resistance is the MAP Kinase family.

In mammalian cells, the MAPK family is made up of three branches: ERK, JNK and the p38 MAPKs. These kinases are activated by a variety of cellular stimuli, including growth factors, cytokines and cellular stress. ERK1/2 in particular has been shown to have increased activity in breast tumors and to be connected to poor prognosis and endocrine therapy resistance in breast cancer patients. Subsequent research revealed that it was possible to pharmacologically inhibit ERK1/2 activity and restore TAM sensitivity. This begs the question: what role do MAP Kinase Phosphatases, the endogenous negative regulators of MAPKs, have in affecting the tamoxifen sensitivity of breast cancer cells?

The MKPs are a family of dual specificity phosphatases that attenuate the activity of the MAP Kinases by dephosphorylating the threonine and tyrosine residues found in a
TXY motif. MKP-1 and MKP-2 have shown the ability to dephosphorylate all three of the MAPKs, although MKP-1 prefers to act on JNK and p38 over ERK and MKP-2 acts primarily on ERK and JNK and not on p38. Both MKP-1 and MKP-2 are known to be overexpressed in breast cancer, but to date no connection between these proteins and breast cancer tamoxifen sensitivity has been established. MKP-1 has been previously linked to chemotherapy resistance in a variety of cancer types, with its overexpression leading to a decrease in JNK activity, which contributes to decreased apoptosis in cancer cells. The hypothesis for this project, which states that an increase in MKP-1 protein expression would contribute to changes in tamoxifen sensitivity via the inhibition of JNK-mediated apoptosis, was based on this evidence.

In the research presented in this dissertation, the objectives were to: (a) characterize MKP-1 and MKP-2 expression in breast cancer cells to begin to dissect their contributions to tamoxifen sensitivity; (b) investigate changes in MAP Kinase activation and correlate them to MKP expression in both tamoxifen sensitive and tamoxifen resistant cells in order to clarify the overall picture of MAPK signaling in tamoxifen sensitivity; and (c) to examine the promoter sequence of MKP-2 to provide insight into its regulation in breast cancer.

MKP-1 and MKP-2 expression were found to be low in MCF7 breast cancer cells, which are known to be tamoxifen sensitive. MCF7 cell lines overexpressing MKP-1 or MKP-2 were generated and treated with E2, E2+TAM, TAM alone, E2+ICI or ICI alone and it was demonstrated that MKP-1 protein expression was not altered following these treatments, but that MKP-2 protein expression increased in response to treatment with
anti-estrogens. Overexpressing MKP-1 decreased cell proliferation in response to E₂ treatment and did not change TAM sensitivity. Western blot analysis also showed that MKP-1 activity was directed toward ERK1/2, not JNK1/2, as JNK1/2 phosphorylation was not observed in vector control cells. MKP-1 overexpression eliminated ERK1/2 phosphorylation compared to vector control cells. These results taken together disproved the study hypothesis that MKP-1 contributes to changing tamoxifen sensitivity by inhibiting JNK1/2-mediated apoptosis. However, the observation that MKP-2 protein expression was increased following TAM treatment provided an interesting new focus for this project.

Before beginning to study the effect of MKP-2 overexpression on tamoxifen sensitivity, MKP expression in MCF7 and MCF7-TAMR cells was characterized. These experiments showed that MKP-2 protein was increased in MCF7 cells following anti-estrogen treatment and constitutively expressed in MCF7-TAMR cells. MKP-1 protein expression was not detected in either cell line. MKP-2 mRNA was also shown to be increased in MCF7-TAMR cells, while MKP-1 mRNA was undetectable. When MKP-2 was overexpressed in MCF7 cells, MTT analysis revealed that, similar to MKP-1, it decreased cell proliferation in the presence of estrogen when compared to vehicle control cells. MKP-2 overexpression cells were still sensitive to TAM treatment. When MKP-1 or MKP-2 protein was overexpressed in MCF7 cells and treated with E₂ or the combination of E₂ and one of three increasing concentrations of tamoxifen, MTT analysis showed that MKP-2 significantly decreased cell proliferation compared to vector control cells. When compared to MKP-1 overexpression, MKP-2 significantly decreased cell
proliferation across all treatment conditions, suggesting that MKP-2 overexpression actually sensitizes cells to tamoxifen treatment. Western blot analysis showed that MKP-2 was able to eliminate ERK1/2 phosphorylation.

MCF7-TAMR cells exhibited a high level of ERK1/2 activation and constitutive expression of MKP-2 protein. These two results seem at odds with each other given that MKP-2 overexpression seems to slow cell proliferation by dephosphorylating ERK1/2 and that these cells were sensitive to tamoxifen treatment. Previous work in our lab, however, has shown that activated ERK1/2 is able to phosphorylate MKP-2 on Ser 386 and Ser 391, forming a feedback loop that stabilizes the protein and protects it from proteasomal degradation. The working model proposes that MCF7-TAMR cells may upregulate and stabilize MKP-2 protein in an attempt to return activated ERK1/2 to normal cellular levels. Even when constitutively expressed, MKP-2 is unable to dephosphorylate all of the active ERK1/2 present in the tamoxifen resistant cells and the drug is rendered ineffective in halting cell proliferation.

Inhibiting Raf/MEK/ERK signaling has become an attractive therapeutic strategy in recent years and has been met with various degrees of success. Many drugs have been developed to target EGFR activity, which is the head of the pathway, but complex signaling networks and crosstalk between pathways also necessitate the development of inhibitors to target downstream components of this signaling axis. One class of agents particularly relevant to the studies presented here is MEK inhibitors. While finding a good deal of preclinical success, only a handful of drugs have progressed beyond Phase I clinical trials, often due to poor pharmacologic profiles and toxicity. The results
presented here demonstrate that increasing MKP-2 expression in hormone-responsive MCF7 cells reduced ERK1/2 activity and increased their sensitivity to tamoxifen treatment. While increasing the expression and activity of MKP-2 itself to sensitize cells to tamoxifen treatment is probably not a viable therapeutic strategy at this time, these results support the importance of continuing to develop clinically effective ways to reduce ERK1/2 activity in breast cancer cells. The idea that MKP-2 plays a role in chemotherapy response is also supported by a recent study by Balko et al, in which molecular profiling of basal-like breast cancer tissues revealed that loss of MKP-2 expression was associated with increased ERK1/2 pathway activation and reduced disease-free survival following neo-adjuvant chemotherapy. The authors also suggest that MKP-2 expression could be used as a biomarker for MEK inhibitor sensitivity in these patients. In light of these results, MKP-2 might also be useful as a marker for sensitivity in tumors that are candidates for tamoxifen treatment, although further development of reagents that can specifically differentiate the activity and expression of MKP-2 from MKP-1 are necessary for this strategy to be effective.

The regulation of MKP-2 gene expression in response to estrogen and tamoxifen treatment has proven to be complex and in need of further clarification. MKP-2 promoter analysis revealed the presence of estrogen response element half-sites, as well as response elements for other transcription factors, such as AP-2, that are known to be regulated by estrogen signaling. Measurement of MKP-2-driven luciferase reporter activity following treatment with estrogen, tamoxifen or ICI did not demonstrate any significant change compared to vehicle control treated cells in either MCF7 or MCF7-
TAMR cells. This suggests that further investigation is necessary to uncover the mechanisms responsible for regulating MKP-2 expression in response to tamoxifen treatment.

The increased ERK signaling present in breast cancer and its connection to tamoxifen resistance has been well documented. Much research has been done to try to discover underlying mechanisms responsible for this activation, but less attention has been paid to the molecules responsible for attenuating MAPK signaling, namely MKPs. The studies presented in this dissertation characterize for the first time the expression and activity of MKP-1 and MKP-2 in response to tamoxifen treatment and their effect on tamoxifen sensitivity. While this is an important advance in the MAP Kinase Phosphatase field, further examination of the connection between MKP-2 and response to tamoxifen is necessary to determine how this knowledge might be translated into clinical benefit for breast cancer patients.
REFERENCES


ABSTRACT

THE EXPRESSION AND REGULATION OF MKPS IN BREAST CANCER TAMOXIFEN SENSITIVITY

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The deregulation of cell signaling is a very important component in the development and progression of cancer. One group of signaling molecules that has been implicated in these processes is the Mitogen-Activated Protein Kinase (MAPK) family which consists of three major branches in mammalian cells: ERK, JNK and p38. The activity of these kinases has wide-ranging effects within the cell and must be tightly regulated. This is partially accomplished through the activity of Mitogen-Activated Protein Kinase Phosphatases (MKPs). The MKPs are a family of eleven dual-specificity threonine-tyrosine phosphatases that attenuate MAP Kinase signaling by dephosphorylating them.
Increased ERK signaling has been shown to correlate with poor prognosis in breast cancer patients and is commonly found in tumors that are resistant to tamoxifen treatment. JNK signaling has also been shown to be increased in breast cancer tissue samples. MKP-1 overexpression in breast cancer has been connected with resistance to a number of different chemotherapeutic agents with the underlying mechanism being a decrease in JNK-mediated apoptosis, but no association with tamoxifen response has been previously studied. These observations led to the hypothesis that MKP-1 overexpression contributes to changes in tamoxifen sensitivity via the inhibition of JNK-mediated apoptosis.

The characterization of MKP-1 following its overexpression in the MCF7 cell line revealed that its expression is not changed after tamoxifen treatment, but that the expression of MKP-2 was increased following treatment with anti-estrogens. Both MKP-1 and MKP-2 decreased cell proliferation in response to estrogen and maintained the tamoxifen sensitivity of MCF7 cells. This decrease in proliferation was attributed to the elimination of ERK phosphorylation, as no JNK activation was observed in these cells. MKP-2 protein expression was shown to be constitutive in MCF7 tamoxifen resistant cells, while MKP-1 expression was not detected. All of these results suggest that MKP-2 expression is upregulated in response to tamoxifen treatment in order to dephosphorylate ERK and slow cell proliferation. In tamoxifen resistant cells, upregulation of MKP-2 expression is most likely an attempt to bring the high levels of ERK activation back down to a more normal level. Its inability to do so is what allows the tamoxifen resistant phenotype to persist.
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