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Complexes

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FOREWORD

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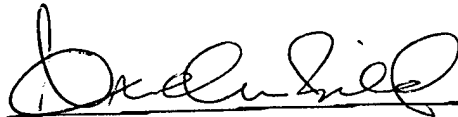
  
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## INTRODUCTION:

Chemotherapeutic drugs are used in the treatment of breast cancer because of their ability to induce apoptosis or inhibit proliferation. The MAPK JNK and MAPK p38 pathways regulate these growth and death responses. MEKK1 and MEKK4, which were cloned in our lab, are implicated in breast cancer signal transduction pathways due to their ability to activate specific MAPK pathways, and thus crucial downstream events such as apoptosis or cell growth regulation. My work focuses on the deviation of specific signal transduction pathways, their subsequent effects on potential cancer treatment, and on determining the underlying mechanisms of aberrant breast cancer cell proliferation and apoptosis. Specifically, I am studying the effects of MEKK1 and MEKK4 signal transduction pathways on breast cancer cell proliferation, survival, and apoptosis.

## BODY:

My original grant focused on the role of the ErbB receptor localization and downstream signal transduction pathways in breast cancer. This grant was subsequently altered and approved (via telephone conversation) by Dr. Patricia Modrow to investigate the role of MAPK signal transduction pathways in breast cancer due to the expertise in Dr. Gary L. Johnson's lab on MAPKs.

The uncontrolled growth of cancer cells can arise from either a loss of apoptotic signaling or proliferative signaling. With cancer chemotherapeutic and anti-angiogenic drugs, the shrinkage of tumors was found to be the result of increased apoptotic index. Thus, pathways influencing proliferation and apoptosis will complement the action of cancer therapies. Combined modality therapy that promotes pro-apoptotic signaling or inhibits proliferation in addition to cancer chemotherapeutic and anti-angiogenic drugs would form an efficacious strategy for breast cancer therapy.

The mitogen-activated protein kinase pathway (MAPK) is involved in crucial effects in breast cancer such as proliferation, cell survival, and cell death. Pathways involving MAPKs are activated by a highly diverse array of stimuli, including ErbB receptor ligation, irradiation, cancer chemotherapies, and others. The basic MAPK kinase pathways consist of three kinases in a sequential kinase cascade: MAPKKK→MAPKK→MAPK (Figure 1).

The three well defined MAPK families are ERK, JNK and p38, and their activation leads to phosphorylation of many substrates including other protein kinases, cytoskeleton associated proteins as well as transcription factors. Effects of the JNK, p38, and ERK pathways are differentiation, cell survival, proliferation, and apoptosis. Our lab has cloned many members of the MAPKKK family, including MEKK1 and MEKK4 (MTK1). MEKK1 and MEKK4 are 55% homologous in the kinase domain, making them the closest related MEKKs.

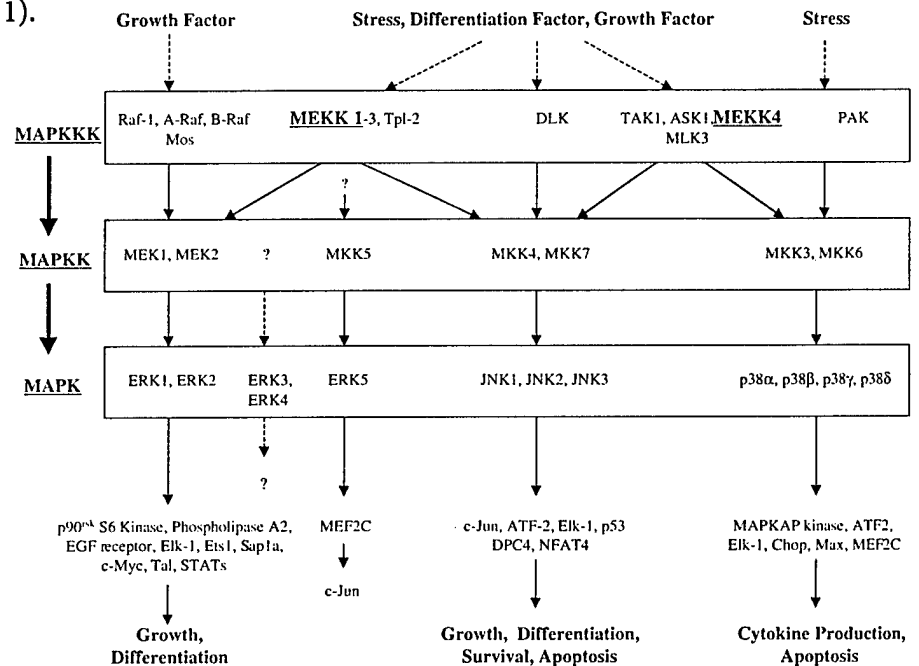


Figure 1: MAPK module

MEKK1 is the MAPKKK that specifically regulates the JNK pathway and promotes cell survival during changes in the microtubule integrity. In our lab, we found that the loss of MEKK1 expression by targeted disruption of the gene causes complete loss of JNK activation in response to microtubule disruption due to the cancer chemotherapeutic drugs used in breast cancer treatment such as taxol. Further, while full length MEKK1 is found at the cell membrane and is involved in cell survival, when full length MEKK1 is cleaved by caspases, a 91 kDa activated kinase domain is released into the cytoplasm where it serves to amplify the activation of caspases and lead the cell to apoptosis. Thus, MEKK1 serves as a switch inside the cell where on one hand the full-length protein will protect the cell in response to microtubule disruptors. On the other hand, when specific caspases are activated

in the cell due to stresses such as genotoxic agents and anoikis (release of the cell from the extracellular matrix), the pro-apoptotic response of MEKK1 is lost by its cleavage into a 91 kDa form that will then act in the cytoplasm of the cell to amplify caspase activity and thus lead to apoptosis. A dominant negative form of MEKK1 blocks the actions of apoptotic genotoxins used in the treatment of breast cancer such as etoposide and cisplatin. To better understand MEKK1's role in cancer cell apoptosis, I examined its ability to activate apoptosis in the presence of specific oncogenes that have been implicated in breast cancer. Akt is a serine threonine protein kinase implicated in mediating inhibition of apoptosis and stimulation of cellular growth. Akt is upregulated in certain breast cancers, as well as potentially contributing to a more aggressive type of breast cancers. Our lab has shown Akt can inhibit apoptosis induced by cancer chemotherapeutics. Akt has also been found to inhibit apoptosis in cells after they detach from the extracellular matrix (anoikis), potentially promoting the ability of cancer cells to metastasize. Contrarily, MEKK1 cleavage and activation causes anoikis based apoptosis. When a cleavage mutant form of MEKK1 (form that cannot be cleaved by caspases into its 91 kDa form) is expressed in cells undergoing anoikis, the cells expressing this mutant underwent anoikis about one-fourth as frequently as control cells, indicating a loss of the apoptotic effect due to inhibition of cleavage of MEKK1. Understanding the relation between MEKK1 and Akt could further our knowledge of the pathways in cell death and survival in response to stresses such as cancer chemotherapeutics and anoikis, with ultimate effects on breast tumor growth and metastasis.

Using confocal microscopy to determine apoptosis by Tdt staining, I show that expression of the oncogene Akt (myristoylated is an activated form of Akt) specifically blocks MEKK1-induced apoptosis by 90% (Figure 2). The caspase inhibitor p35 was used as a control in my experiments. In addition, MEKK1-induced caspase activity is strongly inhibited by the expression of Akt to levels that are below basal (Figure 3). This inhibition of the caspases by Akt protects cells from cleavage of endogenous MEKK1 to its active 91 kDa form that causes apoptosis (Figure 4). I also show that stimulation of another downstream effector of MEKK1, JNK, is not affected by Akt expression (Figure 5). Because expression of Akt does not affect MEKK1 induced JNK activity and does inhibit MEKK1 induced apoptosis, JNK activation is not sufficient for apoptosis, as had previously been suggested. Together, my results suggest that Akt blocks MEKK1 cleavage and subsequent apoptosis through inhibition of caspase activity.

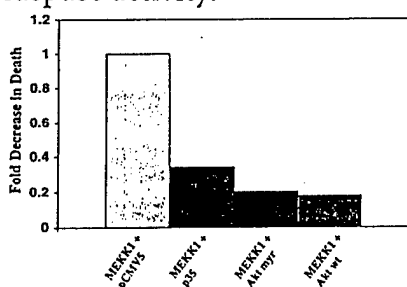


Figure 2: Cell Death Assay

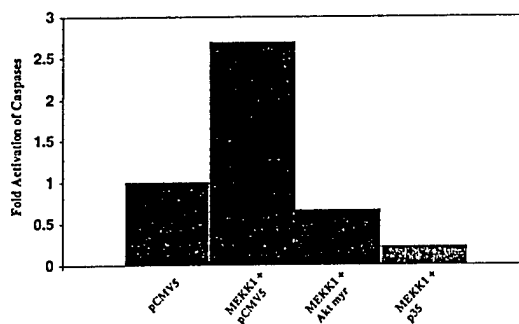


Figure 3: Caspase Activity

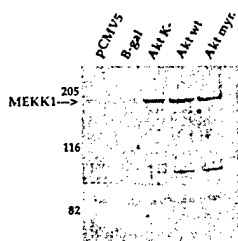


Figure 4: Endogenous MEKK1 Cleavage Assay

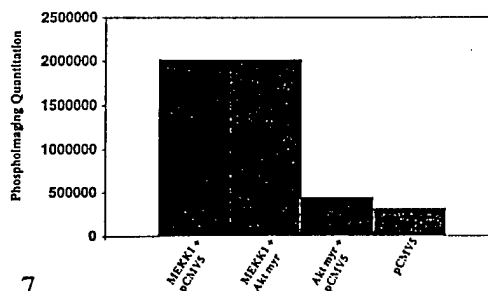


Figure 5: c-Jun Kinase Activity

Another MAPKKK family member implicated in breast cancer signal transduction pathways is MEKK4. MEKK4 activates both the JNK and the p38 MAPK signal transduction pathways via SEK1/MKK4 and MKK3 or MKK6, respectively. These pathways contribute to the regulation of cell survival and cell cycle regulation. Our lab has shown that MEKK4 interacts with Cdc42 and Rac, which are GTP-binding proteins of the Rho superfamily characterized for their ability to regulate cytoskeletal function, as well as cell contact and malignant transformation. Analysis of breast tumors showed high increases in the levels of Rac, RhoA, and Cdc42, whereas in the corresponding normal tissues these Rho family proteins were hardly or not detectable. Upon interaction between Cdc42 and Rac, MEKK4 is phosphorylated and leads to JNK activation via SEK1. Therefore, upregulation of Cdc42 may lead to increased activation of MEKK4 and subsequent pathways. Other studies have shown that a downstream target of MEKK4, SEK1 carries somatic missense mutations in 14% of breast carcinomas (all reported mutations). These findings suggest that MKK4 could also potentially act as a tumor suppressor gene. MEKK4 was also shown by one lab to bind to and become activated by GADD45. GADD45 is a growth arrest and DNA damage-inducible gene which is rapidly induced by DNA damaging agents (such as genotoxic cancer chemotherapeutics) and coordinately induced in growth-arrested cells. GADD45 was also found to be the gene most upregulated in response to expression of BRCA1, a tumor suppressor gene that is mutated in the germline of women with a genetic predisposition to breast and ovarian cancers. BRCA1 induction also led to increased JNK dependent apoptosis in breast cancer cell lines. Potentially, due to the published role of GADD45 in activating MEKK4, the upregulation of GADD45 by BRCA1 could lead to increased activation of MEKK4 and the JNK pathway, causing apoptosis. However, JNK activity was found to precede GADD45 expression in response to acute stresses, and GADD45 null fibroblasts show no decrease in JNK activity in response to cell stresses. Thus, due to discrepancies between labs on MEKK4's role in signal transduction pathways and downstream effects, my goal is to delineate MEKK4's role in the JNK and p38 pathways, and the downstream effects on cell cycle regulation and cell growth.

To determine MEKK4's role in cell growth, we transfected empty pCEP4 vector and the pCEP4 MEKK4 kinase domain (activated form of MEKK4) into an epithelial transformed cell line (HEK293). Since pCEP4 encodes the gene for hygromycin resistance, transfected cells should give rise to colonies in the presence of the drug, unless the coexpressed kinase blocks growth or causes cell death. Table 1 shows that transfection of the kinase domain of MEKK4 gave a similar number of colonies as the control transfection with pCEP4 vector alone, but the colonies were 4-5 times smaller. This experiment implies that MEKK4 is growth inhibitory to cells. Our hypothesis was then confirmed by measuring the growth rate of cells expressing MEKK4. As shown in Table 2, the doubling time of MEKK4 expressing cells was increased compared to pCEP4 expressing cells. These experiments propose that MEKK4 plays a

PCEP4 (empty vector)	MEKK1 (kinase domains)	MEKK4
$2.2 \pm 0.2$ (n=6)	$(<0.2)^*$ *due to apoptosis	$0.4 \pm 0.1$ (n=4)

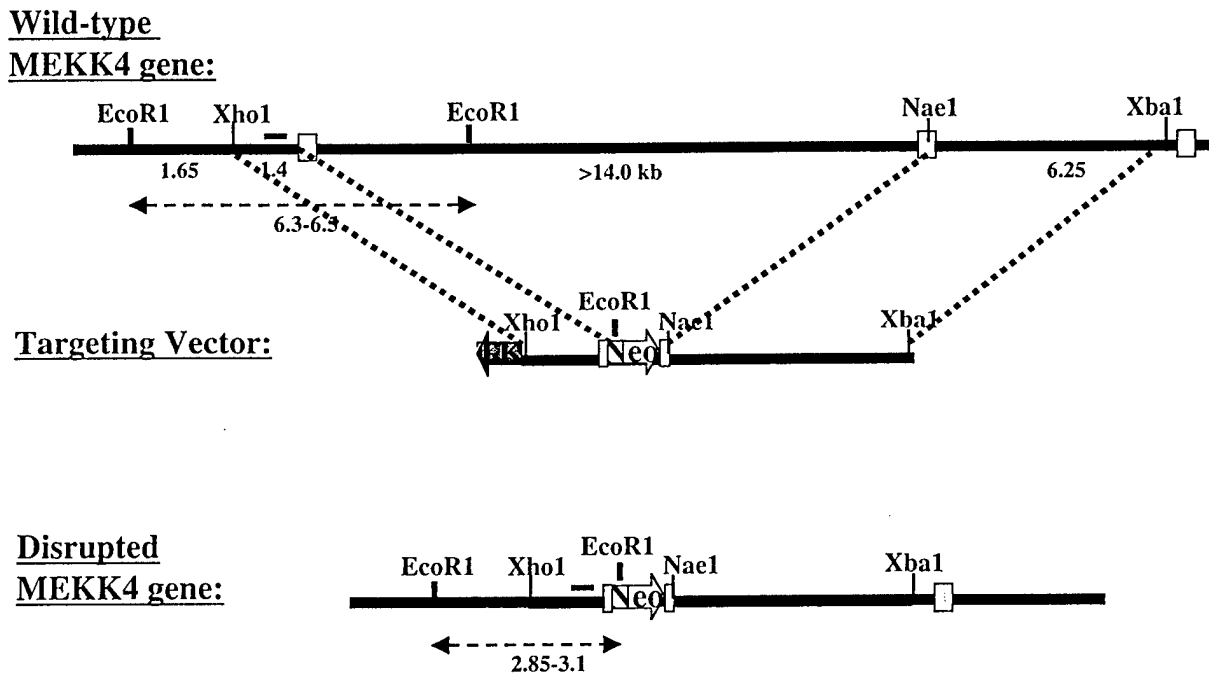
**Table 1:** Colony diameter (in mm) 12 days after transfection with pCEP4 alone or MEKK constructs.

Empty vector Control	MEKK4a
$17.8 + 0.5$	$28.8 + 3.6$

**Table 2:** Doubling time (in hours: mean+ SE of three independent experiments) of vector or MEKK4 expressing cells.

role in inhibition of cell growth and thus could function as a tumor suppressor in breast cancer.

To unequivocally determine MEKK4's role in signal transduction pathways and physiological role in the cell, I have designed a targeting vector (Figure 6) for homologous recombination that will generate cells which are MEKK4 knockout cells.



**Figure 6: Strategy for MEKK4 Targeted Gene Disruption**

This MEKK4 targeting vector has been electroporated into embryonic stem cells, which have been selected for, and are now being screened for homologous recombination. Our lab has successfully designed MEKK1 and MEKK2 knockout cells and mice that are being used in further cancer studies. Also, we have novel technology that allows us to differentiate our knockout embryonic stem cells into epithelial cells to study breast cancer. With the MEKK4 knockout cells, I will be able to study MEKK4's role (in a null background) in cell survival, proliferation, and apoptosis in response to breast cancer chemotherapeutics and cell stresses. Further, I will be able to define MEKK4's role in the JNK and p38 MAPK pathways that is currently in dispute due to discrepancies between labs, as well as define novel pathways MEKK4 is involved in. In the future, MEKK4 knockout mice will be used to determine the physiological role of MEKK4 as well as breast tumor growth studies.

In delineating the roles of MEKK1 and MEKK4 in breast cancer signal transduction pathways and subsequent effects such as apoptosis, cell cycle regulation, and cell growth, I will identify new targets for development of novel anti-cancer therapies and strategies. Further, this work will increase our understanding of the pathways controlling the aberrant signaling that allows breast cancer tumors to grow and metastasize.

## Addendum:

### Key research accomplishments:

#### 1. Conclusions from work beginning September 15, 1998 and ending September 14, 1999:

- \*MEKK1 is a kinase implicated in having crucial roles in cancer due to its apoptotic effects. MEKK1 induced apoptosis is blocked 90% by expression of the Akt oncogene.
- \*MEKK1 induced caspase activity is inhibited to below basal levels with expression of Akt.
- \*Endogenous cleavage of MEKK1 into its pro-apoptotic kinase domain is inhibited by expression of Akt.
- \*MEKK1's stimulation of the JNK pathway is not inhibited by Akt expression. This result implies that JNK activation is not sufficient for apoptosis, as had previously been suggested. *Thus, MEKK1 cleavage and subsequent apoptosis is blocked by Akt's inhibition of caspase activity.*
- \*MEKK4, another MEKK implicated in aberrant breast cancer signaling, inhibits the growth rate of cells by 4-5 fold.
- \*MEKK4 expression increases the doubling time of cells, delineating MEKK4's role in inhibition of cell growth.
- \*Mapping of the exon/intron boundaries of MEKK4 was determined following screening and cloning from a lambda phage library.
- \*A targeting vector was designed to knockout the start site of MEKK4 (exon 1 and 2), thus knocking out expression of MEKK4.
- \*The targeting vector was electroporated into GK129 embryonic stem cells and selected for in G418 and ganciclovir.
- \*One hundred and twenty clones were picked and grown for screening for homologous recombination by Southern blotting.