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TITLE: The Role of MEKK3 Signaling Pathway in the Resistance of Breast Cancer Cells to TNF-Alpha-Mediated Apoptosis

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14. ABSTRACT

To test the hypothesis that the MEKK3 regulated NF-κB and MAPK pathways may play a critical role in breast cancer growth and survival, we altered the regulation of MEKK3 pathway using dominant MEKK3 mutants and small interference RNA strategy. We used MEKK3 specific antibodies determining MEKK3 expression and activation in normal mammary gland cells and in breast cancer cells. We also developed an anti-active MEKK3 antibody that detects activated form of MEKK3. Using these reagents and assay systems, we found that although breast cancer cells express only slightly more MEKK3 than that in normal cells, the MEKK3 in the breast cancer cells appeared to be activated. In collaboration with Dr. J Yang's group in Baylor College of Medicine, we found the mekk3 locus was amplified in breast cancer cell line MCF7. In addition, we found that over expression of the exogenous MEKK3 in breast cancer cells induced cell apoptosis. In contrast, knock down mekk3expression led to a slower growth of breast cancer cells. However, stably knock down MEKK3 causing MCF7 cells to grow as a multiple layers and migrate slower that that of control cells. Finally, we used Maldi-MS spectrometry technique and revealed a critical regulatory modification site in MEKK3 that was crucial for MEKK3 activation and function.

15. SUBJECT TERMS
breast cancer, MEKK3, MAPK, NF-κB, siRNA, apoptosis, and cell migration

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Role of MEKK3 signaling pathway in the resistance of breast cancer cells to TNFα-mediated apoptosis

Introduction

Breast cancer is the most commonly diagnosed malignancy and one of leading causes of death in American women (1). Elevated NF-κB activity was found in breast cancer cell lines and in primary breast cancer tissues (2-3). Activated signal transduction pathways including the mitogen-activated protein kinase (MAPK) pathways are required for mammary carcinogenesis in cooperation with breast cancer related oncogenes (4). Activation of the NF-κB and MAPK pathways prevents cancer cells from apoptosis, up-regulates the expression of growth factors and inflammatory cytokine genes, such as VEGF, IL-1, IL-6 and IL-8 (5-6). To date, the chemotherapy and radiotherapy are still common treatments for breast cancer. However, the efficiency of the treatment usually has been limited because breast cancer develops resistance to chemotherapeutic drugs, ionizing radiation, and tumor necrosis factor (TNF). TNFα is one of the most pleiotropic cytokine acting as a cytotoxic agent against a variety of tumor cell lines and also play a role in tumor regression mediated by cytotoxic T cells (7). TNFα is released by cytotoxic T cells and significantly contributes to the local immune response to tumors. Tumor cells including breast cancer cells were naturally or acquire resistance to TNF-mediated apoptosis yet the mechanism is still not fully understood (8-9). We recently created MEKK3 knockout mice to investigate its in vivo function (10). We found that MEKK3 play a crucial role in TNF induced NF-κB activation and apoptosis (11). NF-κB activity induced by TNF was severely impaired in MEKK3-disrupted MEF cells but UV-induced NF-κB response was normal (11). Interesting, $MEKK3^{-/-}$ MEF cells are sensitive to TNF-induced apoptosis. Being a key activator of the MAPK and the IKK-NF-κB pathways in various cell type, our studies suggest that MEKK3 may be involved in breast cancer cells' resistance to TNF-mediated apoptosis. We hypothesize that the NF-κB activation and MAPK activation plays a critical role in breast cancer growth and survival in response to anti-cancer drugs and to cytokine treatments, and MEKK3 is the key player in this process.

Body

To understand the role of MEKK3 and its regulated signaling cascades in breast cancer growth and survival, we have been taking different approaches to alter MEKK3 activity in normal and in breast cancer cells and then determine how breast cancer cell growth, survival and response to cytokine will be affected. Two different strategies are being used, one is to use dominant interfering mutants to alter MEKK3 in breast cancer cells, and the second is to develop siRNA technique, a highly specific and efficient way to knock-down endogenous gene expression. During the funding period, we have generated different MEKK3 mutants and characterized these mutants. The expression of these mutants was examined by western blot analysis using an anti-HA antibody. As shown in Figure 1, all the mutants expressed similarly as that of wild type MEKK3. To address if MEKK3 is involved in the NF-κB pathway regulation, we expressed a dominant negative MEKK3 (DN-MEKK3) and a dominant active MEKK3 mutant with a NF-κB reporter plasmid. As shown in Figure 2, we found that DN-MEKK3 blocked the NF-κB reporter expression (Figure 2A) while the active MEKK3 mutant activated NF-κB (Figure 2B).

To understand further how endogenous MEKK3 affect cellular growth and function, we constructed *mekk3* specific siRNA expression vectors and tested its efficiency and specificity. We have tested four anti-mouse *mekk3* siRNAs and six anti-human *mekk3* siRNAs. As shown in Figure 3, two *mekk3* specific siRNAs that specifically knockdown *mekk3* but not MEKK2 expression as tested by cotransfection with an GFP-MEKK2 or an GFP-MEKK3 expression vector as indicated in the Figure. These tested siRNA cassettes will be cloned into lentiviral (12) or adenoviral vectors to optimize the delivery efficiency especially for primary cells. To study the role of endogenous MEKK3 and to determine the mekk3 gene expression before and after siRNA transduction, we generated and characterized MEKK3 specific antibodies. As shown in Figure 4A, these antibodies worked well for immunoprecipitation and western blotting.

To investigate the role of MEKK3 in breast cancer cells, we first examined the expression level of MEKK3 in a breast cancer cell line MCF7. As shown in Figure 4B, we observed only a slight increase of EMKK3 expression in breast cancer cell line similarly to a previously reported study. Interestingly, in collaboration with Dr. Yang's group at Baylor college of Medicine, we found that the *mekk3* locus was amplified in MCF-7 cells. As shown in Figure 5, using a fluorescent in situ hybridization (FISH) assay to label mekk3 genomic sequence, we found that the *mekk3* locus (labeled green) was amplified in breast cell line MFC-7. We only observed a slight increase of *mekk3* gene transcription that only gives to a modest increase of MEKK3 protein level. We speculate that overexpression of MEKK3 may be toxic to cells due to its strong induction of the downstream MAPK cascades. Indeed, our recently published work indicating that overexpression of MEKK2 and MEKK3 led to their self-dimer formation and activation (13). In this regard, it is interesting to further examine if MEKK3 protein stability is altered in breast cancer cells.

Although the increase of MEKK3 expression in cancer cells was modest, interestingly, we found that the MEKK3 in the breast cancer cells appeared to be activated as shown by its up mobility shift as compared to that in control cells (Figure 4B). These results suggest that there maybe an abnormal regulation of the MEKK3 pathway. In this regard, we developed an anti-active MEKK3 antibody that detects activated form of MEKK3. Characterization of this active MEKK3 antibody was shown in Figure 6 and was recently published. We also tried to use this anti-p-MEKK2/3 antibody to assess if the MEKK3 in MCF7 cell was hyper-phosphorylated but the results were unclear. This may be due to the insolubility of the activated form of MEKK3 in cancer cells that was difficulty to immunoprecipitate. We are in the process of optimizing the conditions for this assay.

To understand the mechanism for MEKK3 regulated MAPK and NF-κB activation, we studied MEKK3-deficient mouse fibroblasts. In this study, we found that MEKK3 as a specific activator of NF-κB, JNK, and p38 MAPK but not the ERK1/2 MAPK since in the absence of MEKK3, activation of NF-κB, JNK, and p38 but not the ERK1/2 was severely compromised (Figure 7) (14).

We also investigated the role of MEKK3 in breast cancer cells. We expressed exogenous MEKK3 in MCF-7 cells and determined how it may affect MCF-7 survival. As shown in Figure 8, over expression of MEKK3 but not a MEKK3 mutant induced cancer cell apoptosis as compared to control mock transfected cells. Thus this result may partially explain why MEKK3 protein level is not highly increased although its gene locus was amplified in breast cancer cell MCF-7. The MEKK3 over expression induced cell apoptosis seems also requiring MEKK3 enzymatic activity since expression of a kinase inactive MEKK3 (MEKK3(mut)) had only marginal effect as shown in Figure 8. Furthermore, in addition to induce cell death, over-

expression of MEKK3 in breast cancer cells appeared to cause G1 cell cycle arrest as examined by propidium iodide (PI) staining (Figure 9).

To further determine the role of MEKK3 in breast cancer cells, we selected breast cancer cells that stably express control or MEKK3 specific siRNA to knock down MEKK3 expression. In this study, we found that the control siRNA expressing breast cancer cells grow as a single layer as it normally grow while the MEKK3 siRNA expressing cells grow as a multiple layer cluster as shown in Figure 10. Although the underlying molecular mechanism for this change is unclear, this result may suggest that MEKK3 control tumor cell growth conditions. We also determined if MEKK3 is required for breast cancer cell migration since this property of cancer cells may associate with the potential of cancer cell metastasis. We compared the migration potential of the control siRNA expression MCF-7 cells with that of *mekk3* specific siRNA expressing cells. In these experiments, we found that knock down MEKK3 expression caused a slow down of tumor cell migration (Figure 11).

Finally, we carried out experiments that were proposed in Task 3 to determine MEKK3 regulatory mechanisms. Two major goals were proposed originally. The first goal is to access if Akt regulates MEKK3 in breast cancer cells. Since we generated a unique anti-p-MEKK2/3 antibody against active MEKK3, we compared if Akt expression would inhibit MEKK3 phosphorylation on its activation site at Ser526 as we published recently (15). We did not detect any significant change in MEKK3 activation indicating that Akt had no effect on MEKK3 activation. Our result was consistent with a previous report showing that also MEKK3 could be phosphorylated on serine-166 on certain conditions, this phosphorylation had no effect on overall MEKK3 activity (16).

The second goal in Task 3 is to identify MEKK3-associated proteins by chromatography. We used two different strategies: 1) Use an anti-MEKK3 specific antibody to pull-down endogenous MEKK3-associated proteins; 2) use an anti-HA antibody to immunoprecipitate either control cells or cells stably expressing HA-MEKK3. Unfortunately, the proteins isolated from these approaches were complicated and majority of them were none-specific which include heat shock proteins and cytoskeletal proteins as identified by MALDI ion trap Mass Spectrometry in our collaborator, Dr. Qin Jun's laboratory at Baylor College of Medicine. For example, as shown in Figure 12, we prepared cell lysates from ten 10-cm dishes of control or HA-MEKK3 expressing cells for immunoprecipitation with anti-HA antibody. Most protein bands identified by CB staining were similar in control and HA-MEKK3 expressing cells and Mass Spectrometry analysis of many of them only reveled non-specific protwins. We are still working to improve the conditions to isolate and identify specific interacting proteins. One approach we are using currently is to generate stable cell lines that express MEKK3 at a level similar to endogenous level that are fused with two epitope tags: an HA- and Flag-tag at its N- and C-terminal respectively. Sequential immunoprecipitation will be carried out to identify the associated proteins. While we are still pursuing for the novel MEKK3 associated proteins, in collaboration with Dr. Jun Qin's group, it allowed us to use Maldi-MS technology to identify the crucial regulatory phosphorylation site, Ser526 in MEKK3. As shown in Figure 5B and Figure 11, we generated antibody specific to this site and demonstrated that its phosphorylation is essential for MEKK3 function. Most importantly, this phospho-specific antibody may allow us to develop a strategy to screen various cancer samples including but not limited to breast cancer for MEKK3 activity.

Key research accomplishment

1) Generation and characterization of MEKK3 mutants with mutations at the sub-kinase domain VII-III.
2) Construction of retroviral expression vectors for MEKK3 mutants;
3) Determination of the role of MEKK3 in NF-κB activation by TNF and other stimuli by IκBα western blot, by NF-κB reporter assay, and by gel-mobility shift assay;
4) Construction and characterization of *mekk3* specific siRNA vectors and tested its efficiency in cell lines;
5) Generation and characterization of peptide antibodies against MEKK3 and optimizing conditions for immunoprecipitation and western blotting with these antibodies;
6) Determination of MEKK3 dimer formation is crucial for its activation;
7) Identification of MEKK3 Ser 526 as the key regulatory phosphorylation site;
8) Generation and characterization of an active MEKK3 specific antibody;
9) Revealed that the MEKK3 signaling pathway might be a key regulator of breast cancer cell growth, survival and migration;
10) Our studies also suggest that too little MEKK3 activity may cause cancer growth retardation and affect its migration potential, while too much activity may lead to cell death.

Reportable outcomes

Manuscrips:

1. Qiaojia Hunag, Jianhua Yang, Jinke Cheng, CM Walker, Z.G. Liu, and Su B. Differential regulation of interleukin-1 receptor and toll like receptor (TLR) signaling by MEKK3. Nature Immunology, 5:95-103, 2004.
2. Jinke Cheng, Ling Yu, Dongyu Zhang, Qiaojia Huang, David Spencer, and Su B. Dimerization through the catalytic domain is essential for MEKK2 activation. J. Biol. Chem. 280:13477-82, 2005.
3. Dongyu Zhang, Valeria Fachinnetti, Xiaofang Wang, Qiaojia Huang, Jun Qin and Su B. Identification of MEKK2/3 serine phosphorylation site targeted by the Toll-like receptor- and stress-pathways. EMBO J. 25, 97-107, 2006.

Abstract:

Ling Yu, Huang Q., Kim K., Wang X. and Su, B. Role of MEKK3 signaling in the resistance of breast cancer cells to TNFa-mediated apoptosis. Breast Cancer Research Program meeting, DOD, Philadelphia, PA. June 6-12, 2005.

Presentation: Poster presentation:

Ling Yu, Juang,Q., Kim K., Wang X. and Su, B. Role of MEKK3 signaling in the resistance of breast cancer cells to TNFa-mediated apoptosis. Breast Cancer Research Program meeting, DOD, Philadelphia, PA. June 6-12, 2005.

Patents/licenses: None
Degree obtained: None
Cell lines/other reagents:MCF7(luci-siRNA), MCF7(mekk3-siRNA); anti-p-MEKK2/3-p-antibody and anti-MEKK3 antibody (1415).

Conclusions

With the support from the DOD Breast Cancer Research Program, we tested the hypothesis that the MEKK3 regulated NF-κB and MAPK pathways may play a critical role in breast cancer growth and survival. We altered the regulation of MEKK3 pathway to assess how it affected breast cancer growth, survival. Using small interference RNA strategy, we knock down MEKK3 expression in breast cancer cells, and used MEKK3 specific antibodies determining MEKK3 expression and activation in normal mammary gland cells and in breast cancer cells. We also developed an anti-active MEKK3 antibody that detects activated form of MEKK3. Using these reagents, we found that although breast cancer cells express only slightly more MEKK3 than that in normal cells, the MEKK3 in the breast cancer cells appeared to be activated as shown by its up mobility shift. Interestingly, in collaboration with Dr. J Yang's group in Baylor College of Medicine, we found the mekk3 locus was amplified in breast cancer cell line MCF7. In addition, we found that expression of the exogenous MEKK3 in breast cancer cells induced cell apoptosis. This MEKK3 induced cell apoptosis required MEKK3 enzymatic activity since expression of a kinase inactive MEKK3 had only marginal effect. Using mekk3 specific siRNA to transduce human breast cancer cell MCF7 led to slow down the growth of these cells. However, when stable siRNA expressing cells were selected, we found that the control siRNA expressing breast cancer cells grow as a single layer as it normally grow while the MEKK3 siRNA expressing cells grow as a multiple layers. This result indicates that MEKK3 may control tumor cell growth conditions. We further found that MEKK3 may be a critical regulator for cancer migration. Finally, we used biochemical approaches and the Maldi-MS spectrometry technique to identify MEKK3 regulators and MEKK3 modification. Although we haven't succeeded in isolating breast cancer cell specific regulators for MEKK3, we did reveal a critical regulatory modification site in MEKK3 that was crucial for MEKK3 activation and function. Part of this study was published recently (see ref. 15). In the future, we will build mouse breast cancer models using mice with conditional deletion of MEKK3 in the mammary gland tissues to examine the potential roles of MEKK3 in breast cancer development, growth and survival. These studies will allow us to reveal novel targets and to develop new strategies and screen for new drugs to treat and perhaps prevent breast cancer.

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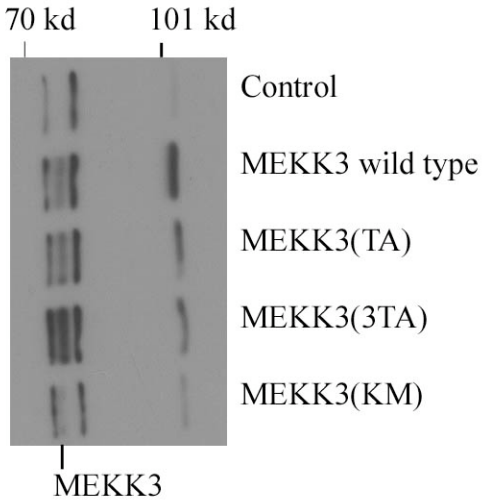

Figure 1 Expression HA-tagged wild type and mutant MEKK3. One microgram of empty vector of SRaHA-MEKK3 expression vectors were transfected into 293T cells and cell lysates prepared 36 h later for western blot analysis with anti-HA antibody as indicated.

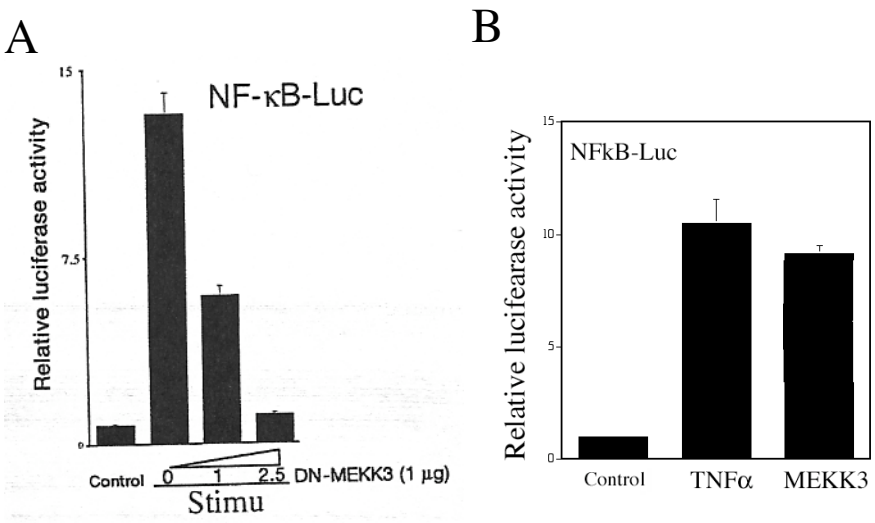

Figure 2 A. Dominant negative MEKK3 (DN-MEKK3) inhibits NF-kB reporter gene expression. One microgram of NF-kB-Luc reporter plasmid was transfected with either empty vector or with increasing amounts of DN-MEKK3. Transfected cells were either unstimulated (control) or stimulated (stimu) with Il-1 for 24 hr before being assayed for the luciferase activity. DN-MEKK3 inhibits the reporter gene expression significantly. B. One microgram of NF-κB-Luc reporter plasmid was transfected with either empty vector or with expression vector for MEKK3. Transfected cells were either unstimulated or stimulated with TNF for 12 h before being assayed for the luciferase activity.

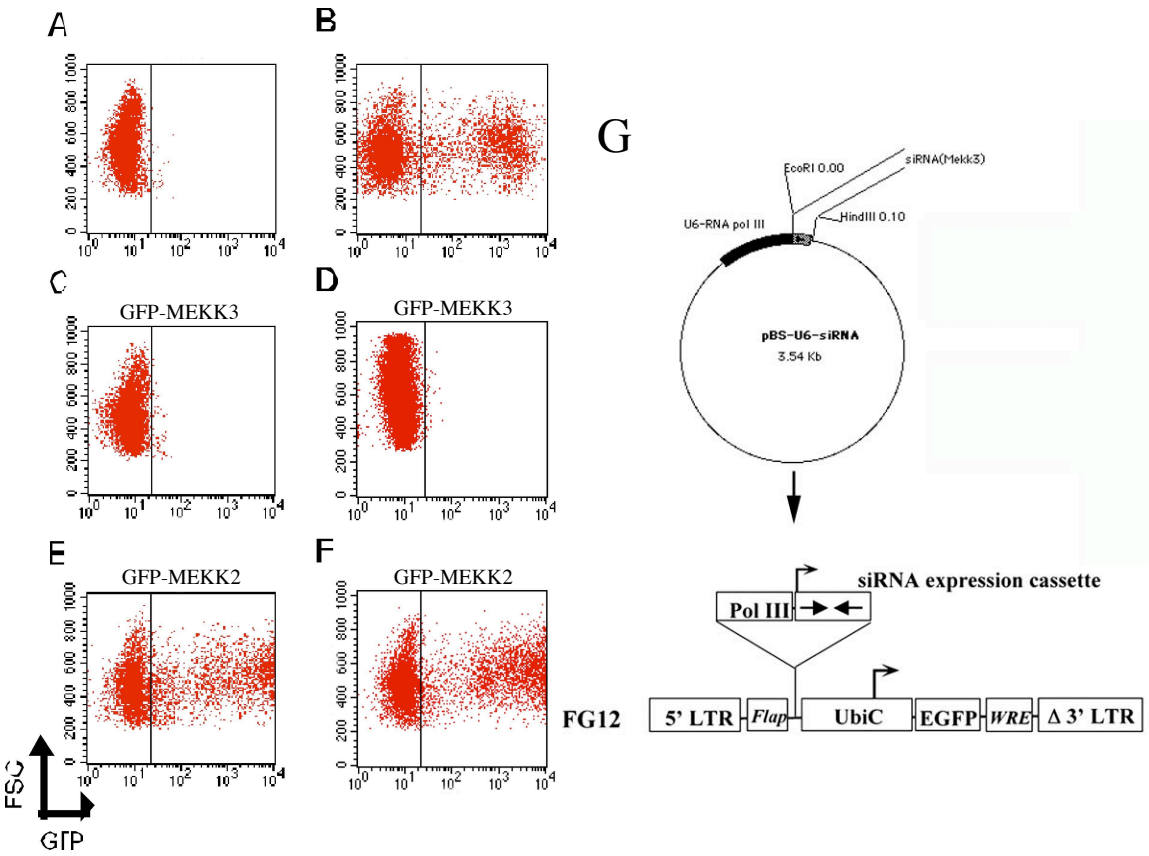

Figure 3 Anti-Mekk3 siRNA1 and siRNA2 specifically knock down MEKK3-GFP expression. A. Control cell analyzed by flow cytometry. B-F. Control Luci siRNA (B), or Mekk3 specific siRNA1 (C, E), siRNA2 (D, F) expression vectors were co-transfected with a MEKK3-GFP (C, D) or MEKK2-GFP (E, F) expression vector s into BOSC cells and the GFP fusion proteins were analyzed 36 h later by flow cytometry. G. Diagram for the construction of lentiviral expression vectors for the MEKK3 siRNA.

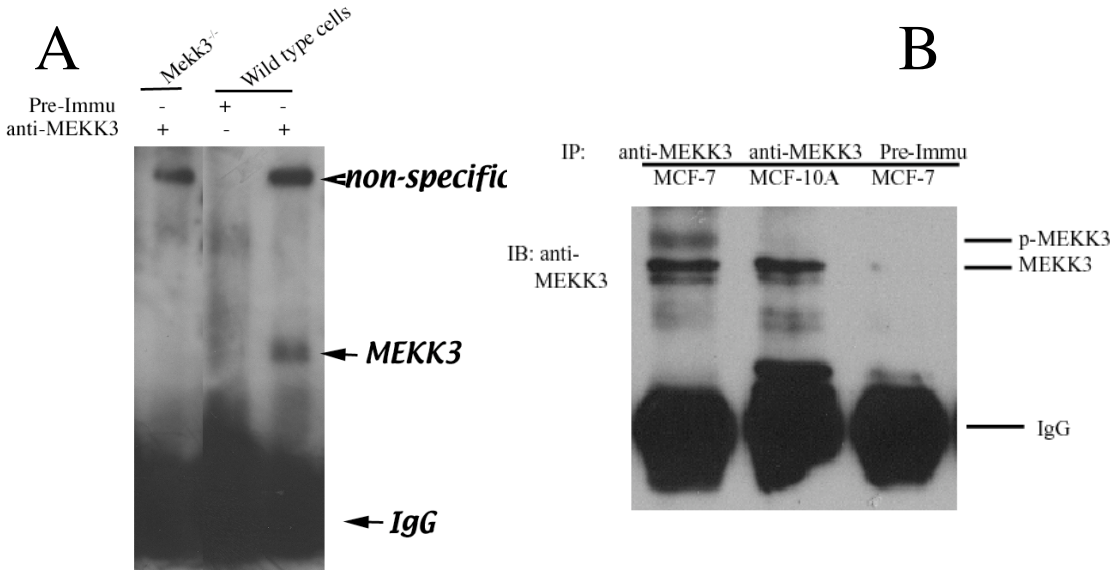

Figure 4 A. Immunoprecipitation-western blotting (IP-western) of endogenous MEKK3 with MEKK3-peptide specific antibody. Five million of 293T cells were lysed in 600 ul lysis buffer and subjected immunoprecipitation with 1 ug of purified anti-MEKK3 peptide antibody. The immunocomplex was separated by a SDS-PAGE and subjected to western blotting with the same antibody at a concentration of 1 ug/ml. A pre-immune serum was used as a control for immunoprecipitation. Endogenous MEKK3 was indicated. Same amount of lysate from a MEKK3$^{-/-}$ fibroblast cells were used as a negative control. B. MEKK3 expression in normal and breast cancer cell lines. Two millions of breast cancer cells MCF7 and normal breast cell line MCF-10A were subject to immunoprecipitation with the indicated antibodies and analyzed by immunoblotting using anti-MEKK3 antibody. Activated MEKK3 was indicated.

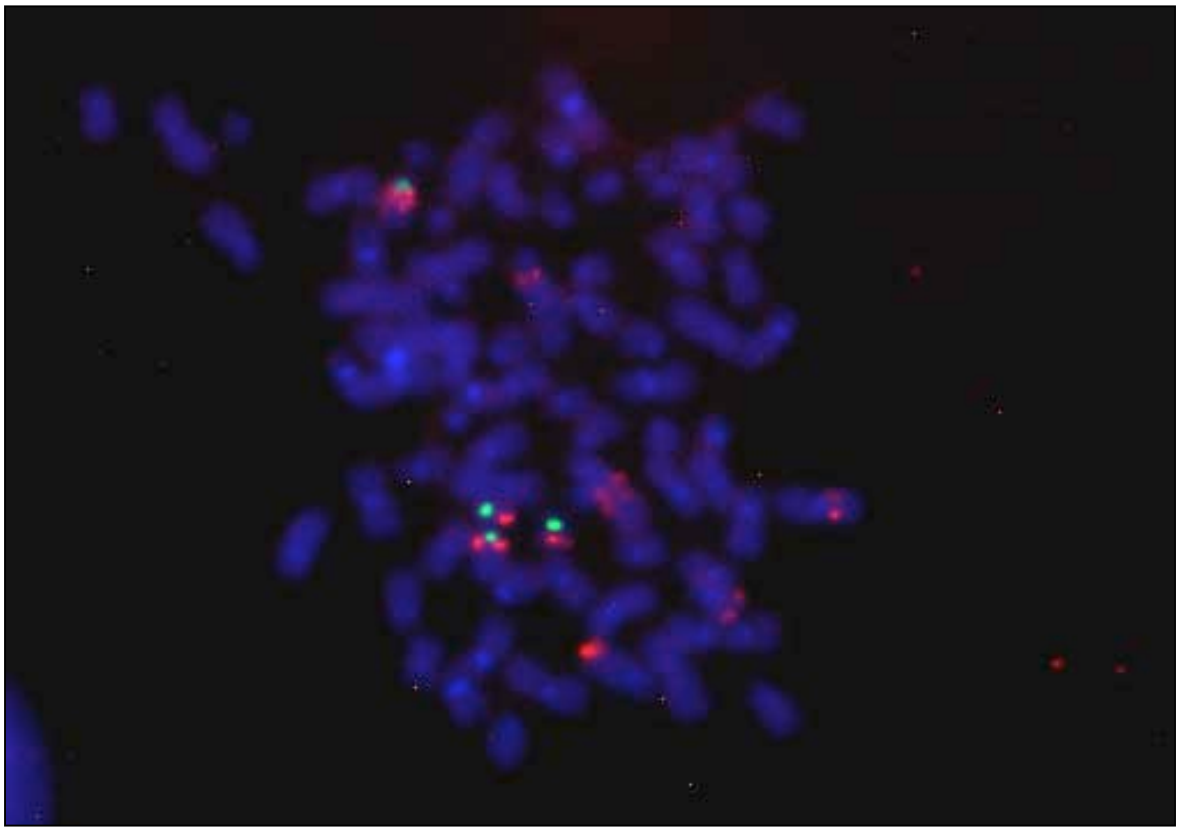

Figure 5 The *mekk3* locus is amplified in MCF-7 cells. Metaphase chromosomes of MCF-7 cells were analyzed by fluorescent in situ hybridization (FISH) for the *mekk3* locus (green). Multiple Mekk3 locuses were identified.

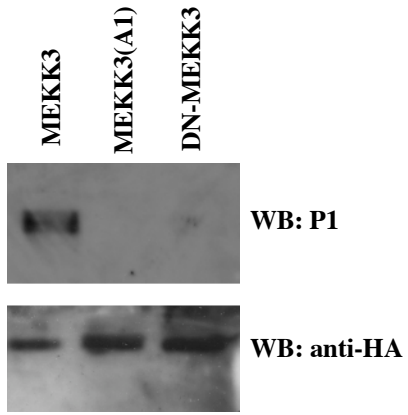

Figure 6 Characterization of anti-phosph-MEKK3 antibody P1. COS-1 cells were transfected with HA-tagged MEKK3, MEKK3 phosphorylation site mutant MEKK3(A1), and DN-MEKK3 as indicated. Cell lysates were prepared 36 hr later and analyzed by immunoblotting with the P1 antibody (top panel) and an anti-HA antibody (bottom panel).

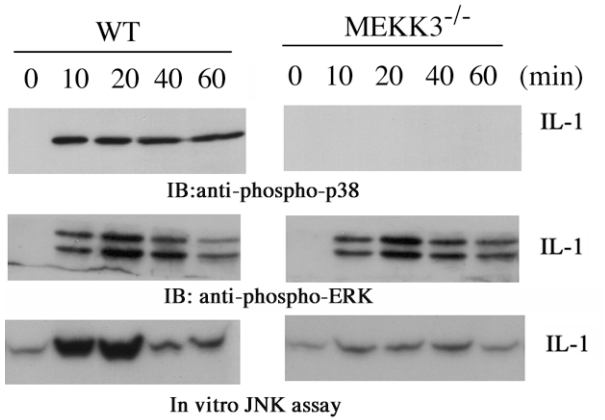

Figure 7 Activation of JNK, ERK and p38 MAPK in wildtype and Mekk3$^{-/-}$ MEFs. Wild type and Mekk3$^{-/-}$ MEFs either untreated or stimulated with IL-1 were harvested at the indicated time points. JNK activation was determined by an in vitro kinase assay. ERK and p38 MAPK activation was measured by immunoblotting and anti-phospho-p38 and –ERK1/2 antibodies.

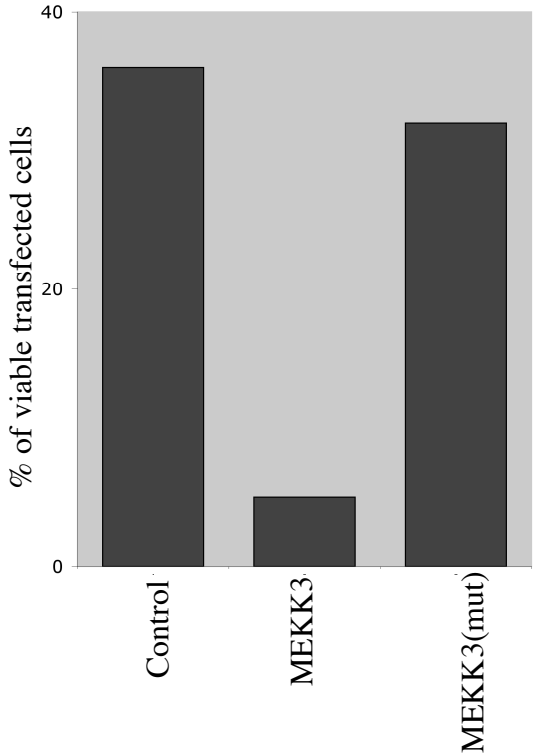

Figure 8 Transient expression of active MEKK3 but not inactive MEKK3 causes cell apoptosis. Empty vector or expression vectors for GFP tagged MEKK3 or MEKK3 mutant were transfected into tumor cells. Viable transfected cells were scored under the microscope.

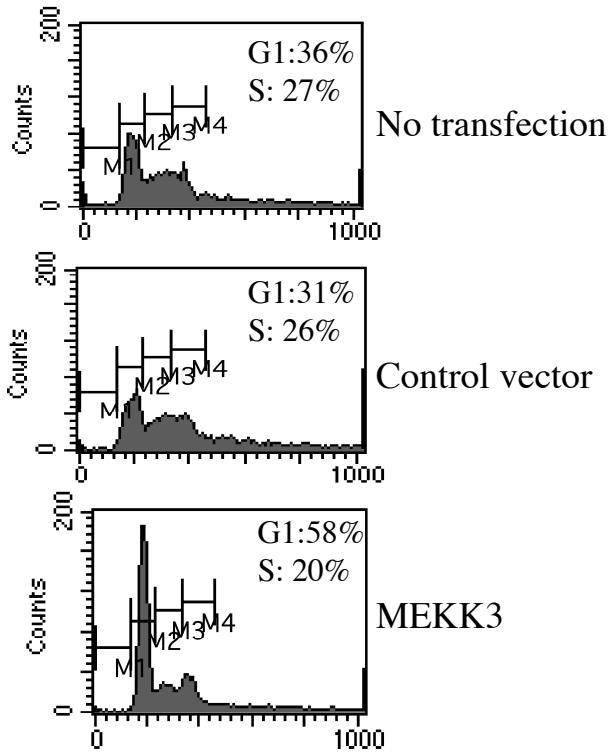

Figure 9 Expression of exogenous MEKK3 in breast cancer cells causes G1 cell cycle arrest. MCF cells were transfected with control vector or MEKK3 expression vector together with a GFP expression plasmid. Transfected cells were stained with propidium iodide and analyzed by a FACScaliber for cell cycle progression. Compared with the non-transfected or control vector transfected cell,MEKK3 expressing cells had significantly increased cells in the G1 phase (from around 30% to 58%).

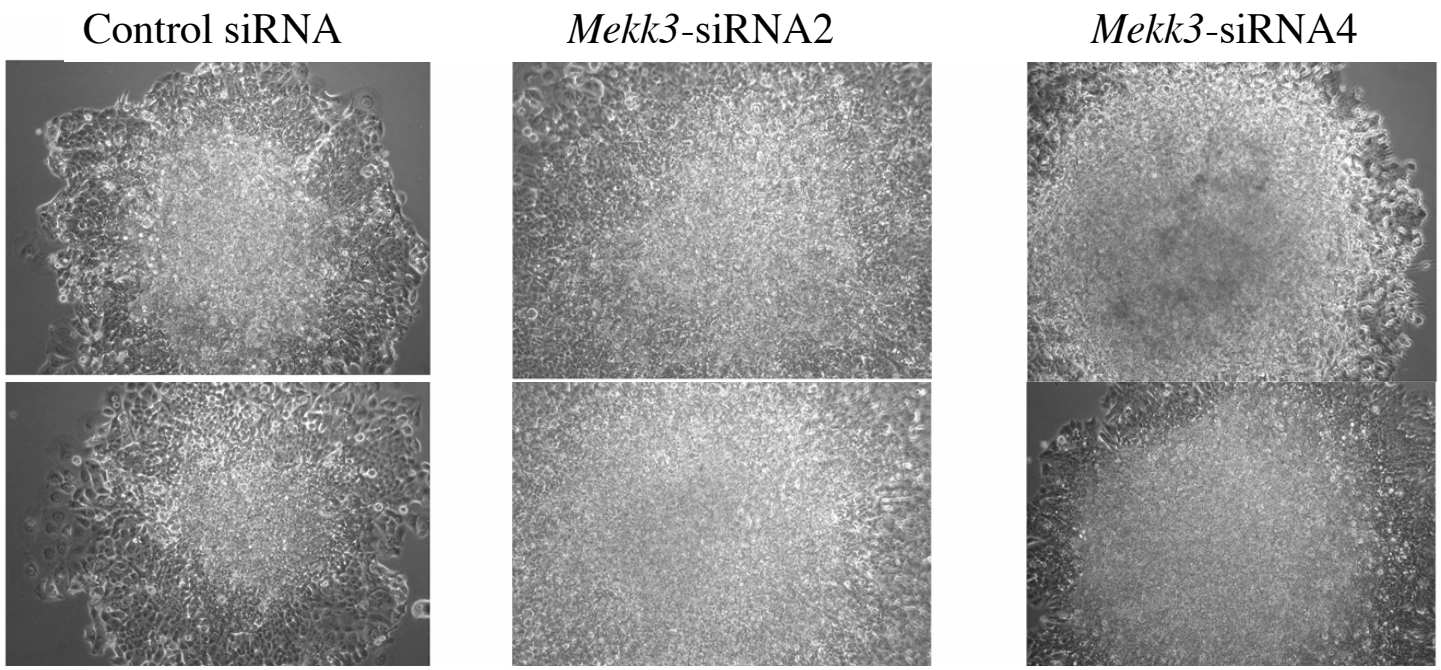

Figure 10 MCF7 cells transduced with control- or mekk3-specific siRNAs were selected with hygromycine 48 hr later as indicated. Colonies were visualized under microscope after one-week selection. Cells transduced with mekk3-specific siRNA have high potential to grow as multiple layers.

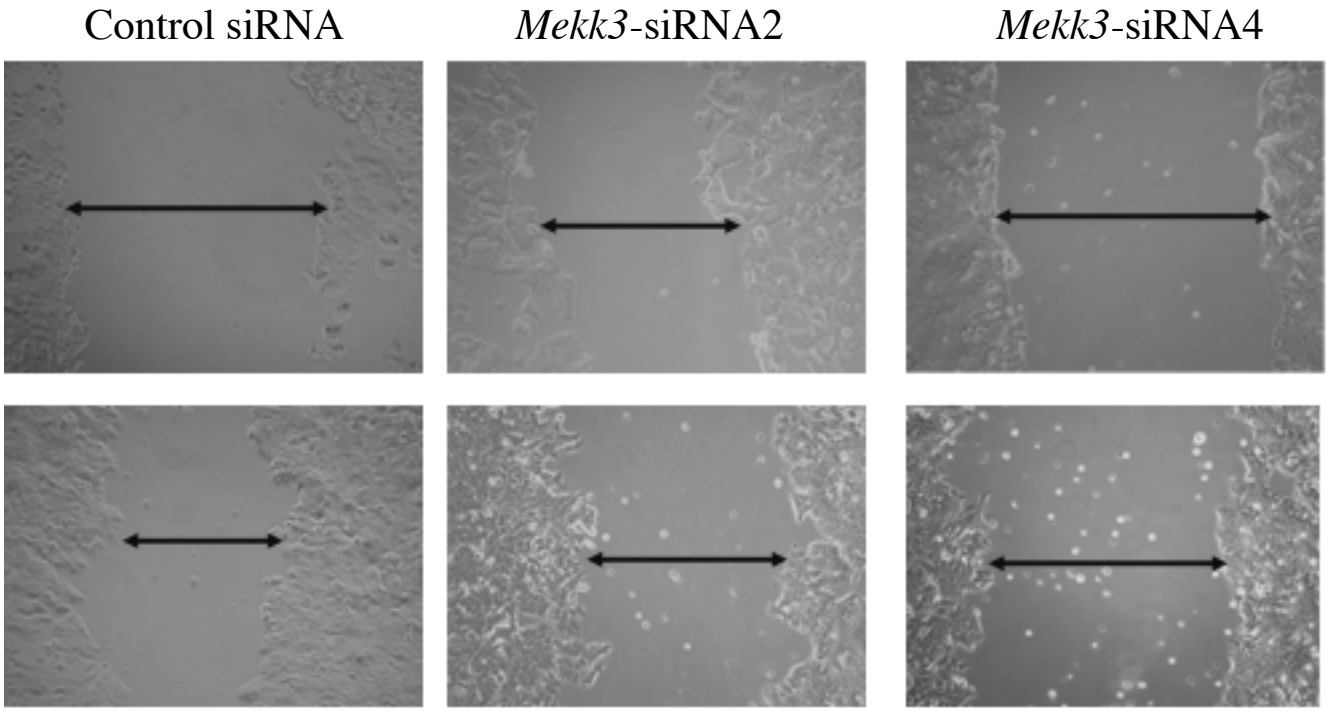

Figure 11 MCF7 cells transduced with control or MEKK3 siRNA were assayed for migration. Cells expressing control- or *mekk3* specific-siRNA2 and 4 as indicated were plated as single layer and subjected to an in vitro wound-healing assay. Photographs were taken at 1 hr (top panels) and 12 hr (bottom panels) after injury. Compared with the control siRNA transduced cells (left two panels) that had about 50% decrease in the wounding gap, the two *mekk3* siRNA transduced cells (middle and right panels) had lesser than 10% decrease in the closing of the gap.

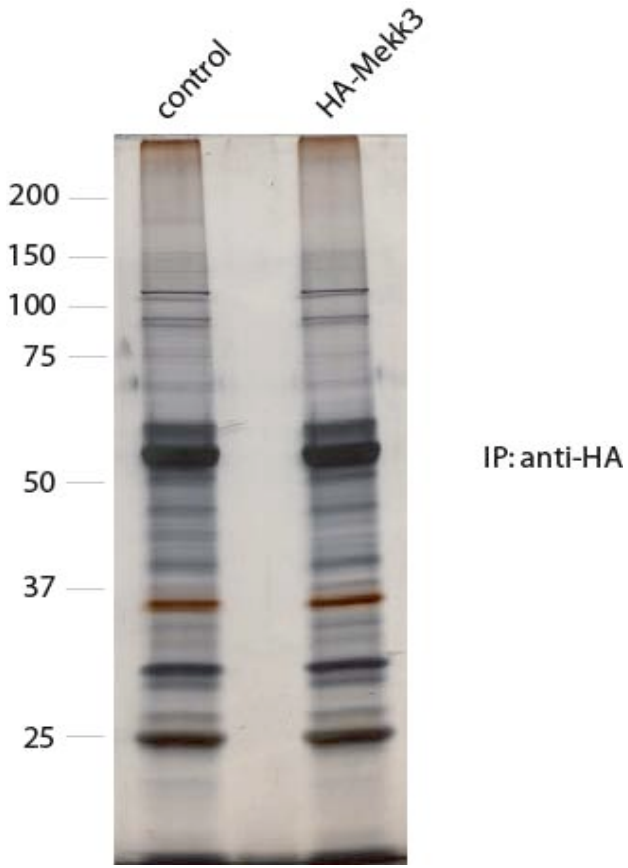

Figure 12 Ten-dishes of control or HA-MEKK3 expressing cells were immunoprecipitated with 5 microgram of anti-HA antibody as indicated. The immunoprecipitates were separated by a SDS-PAGE gel and several visible bands were further analyzed by MS-Spectrometry method.