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ABSTRACT

With the support from the DOD Breast Cancer Research Program, the research conducted in the past three years has tested the hypothesis that the NF-κB activation and MAPK activation by MEKK3 plays a critical role in breast cancer growth and survival in response to anti-cancer drugs and to cytokine treatment. We used dominant interfering forms of MEKK3 mutant to examine how perturbation of the MEKK3 pathway may affect breast cancer growth, survival and responses to cytokines. We found that MEKK3 is essential for cytokine induced NF-κB and MAPK activation in fibroblasts. We also developed small interference (si) RNA strategy to inhibit MEKK3 expression in breast cancer cells and used MEKK3 specific antibodies to examine the MEKK3 expression and activation in normal mammary gland cells and in breast cancer cells. Our studies revealed that MEKK3 signaling pathway may be a key regulator for breast cancer cell growth, survival and migration. 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. These studies will allow us to reveal novel targets and to develop new strategies to treat and prevent breast cancer.

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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-kB 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-kB 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 test this hypothesis, we have been working on the conditions to altering the MEKK3 activities 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 various MEKK3 mutants and characterized these mutants by western blot (fig 1). We showed that the dominant negative MEKK3 (DN-MEKK3) blocked the NF-κB reporter activation, while a dominant active MEKK3 mutant activated NF-kB pathway (fig 2). We constructed siRNA expression vectors and tested its efficiency and specificity (fig 3). Up to data, we have tested four anti-mouse Mekk3 siRNAs and six anti-human Mekk3 siRNAs. Two of the three anti-mouse siRNAs works specifically while two of six anti-human Mekk3 siRNAs worked for the human Mekk3 gene. Eventually, we will use either lentiviral (12) or adenoviral vectors to deliver the best tested anti-Mekk3 siRNAs. To determine the mekk3 gene expression before and after siRNA transduction, we generated and characterized MEKK3 specific antibodies to examine the MEKK3 expression and activation in normal mammary gland cells and in breast cancer cells for immunoprecipitation and western blotting (fig 4). Using these reagents, we found that although both the normal mammary gland

and breast cancer cells express MEKK3, there was a clear increase of MEKK3 expression in the cancer cells. Interestingly, the MEKK3 in the breast cancer cells appeared to be activated as shown by its up mobility shift. These results suggest that there maybe an abnormal regulation of the MEKK3 pathway (fig 4B). We also developed an anti-active MEKK3 antibody that detects activated form of MEKK3 (fig 5). To understand the mechanism for MEKK3 regulated MAPK and NF-κB activation, we studied MEKK3-deficient mouse fibroblasts in the lab and found that MEKK3 is a specific activator of NF-κB, JNK, and p38 MAPK but not the ERK1/2 MAPK (fig 6, ref.13). Interestingly, we found that expression of the exogenous MEKK3 in breast cancer cells induced cell apoptosis (fig 7). This MEKK3 induced cell apoptosis seems requiring MEKK3 enzymatic activity since expression of a kinase inactive MEKK3 had only marginal effect (fig 7). Expression of MEKK3 in breast cancer cells appeared to induce G1 arrest (fig 8). However, when stable siRNA expressing cells were selected, we found 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 (fig 9). This result indicates that MEKK3 may control tumor cell growth conditions. We also determined if MEKK3 is required for breast cancer cell migration that this property may associate with the potential of cancer cell metastasis. We compared the migration of breast cancer cells with that transduced with anti-human Mekk3 specific siRNAs, and found knock down MEKK3 appeared to slow down cell migration (fig 10).

Key research accomplishment

The key accomplishments are listed above. In brief, we have generated many valuable reagents that would enable us to dissect the role of MEKK3 in breast cancer cell growth and survival during normal growth condition and in response to anti-cancer drugs or apoptosis inducing cytokines. Overall, our studies revealed that MEKK3 signaling pathway might be a key regulator controlling breast cancer cell growth, survival and migration. 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

In the studies carried out in the funding period, we discovered that the breast cancer cells have relatively low transfection efficiency using standard procedures. In order to modulate the activity of MEKK in normal mammary gland cells and in breast cancer cells, we have taken various strategies such as using dominant interfering MEKK3 mutants and Mekk3 specific siRNA interference. During the course of the study, we have established many useful reagents such as specific siRNAs, retroviral delivery system, and peptide-antibodies against normal and activated form of MEKK3. In addition, we also used Mekk3 deficient cells to characterize the molecular mechanisms of MEKK3 activation of the MAPK and NF-kB pathways.

Conclusions

With the support from the DOD Breast Cancer Research Program, the research conducted in the past three years has tested the hypothesis 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 treatment, and MEKK3 is the key player in this process. We used dominant negative forms and active MEKK3 to examine how perturbation of the MEKK3 pathway may affect breast cancer growth, survival and response to cytokines. We found that MEKK3 is essential for TNFa and IL-1 mediated MAPK activation in fibroblasts. To investigate if the MEKK3 activity also plays a critical role in breast cancer cells, we have developed small interference RNA strategy to inhibit the MEKK3 expression in breast cancer cells. Up to data, we have tested four anti-mouse Mekk3 siRNAs and six anti-human Mekk3 siRNAs. Two of the three anti-mouse siRNAs works

specifically while two of six anti-human Mekk3 siRNAs worked for the human Mekk3 gene. To determine the mekk3 gene expression before and after siRNA transduction, we generated and characterized MEKK3 specific antibodies to examine the 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 both the normal mammary gland and breast cancer cells express MEKK3, there was a clear increase of MEKK3 expression in the cancer cells. Interestingly, the MEKK3 in the breast cancer cells appeared to be activated as shown by its up mobility shift. These results suggest that there maybe an abnormal regulation of the MEKK3 pathway. In addition, we found that expression of the exogenous MEKK3 in breast cancer cells induced cell apoptosis. This MEKK3 induced cell apoptosis seems require MEKK3 enzymatic activity since expression of a kinase inactive MEKK3 had only marginal effect. Furthermore, we found that anti-mouse Mekk3 siRNAs were very effective in knock down mouse MEKK3 expression. Human specific siRNA to transduce human breast cancer cell MCF7 appeared to slow down the growth of these cells. However, when stable siRNA expressing cells were selected, we found 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. To determine if MEKK3 is required for breast cancer cell migration, we used the anti-human Mekk3 specific siRNA transduced breast cancer cells. We found that MEKK3 may be a critical regulator for cancer migration. 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.

Reference

1. Stewart, S. L., King, J. B., Thompson, T. D., Friedman, C. & Wingo, P. A. Cancer mortality surveillance--United States, 1990-2000. MMWR Surveill Summ 53, 1-108 (2004).
2. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr. & Sledge, G. W., Jr. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Mol Cell Biol 17, 3629-39 (1997).
3. Cogswell, P. C., Guttridge, D. C., Funkhouser, W. K. & Baldwin, A. S., Jr. Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. Oncogene 19, 1123-31 (2000).
4. Amundadottir, L. T. & Leder, P. Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. Oncogene 16, 737-46 (1998).
5. Luo, J. L., Maeda, S., Hsu, L. C., Yagita, H. & Karin, M. Inhibition of NF-kappaB in cancer cells converts inflammation- induced tumor growth mediated by TNFalpha to TRAIL-mediated tumor regression. Cancer Cell 6, 297-305 (2004).
6. Li, J. J., Oberley, L. W., Fan, M. & Colburn, N. H. Inhibition of AP-1 and NF-kappaB by manganese-containing superoxide dismutase in human breast cancer cells. Faseb J 12, 1713-23 (1998).

7. Aggarwal, B. B. 2000. Tumour necrosis factors receptor associated signalling molecules and their role in activation of apoptosis, JNK and NF-kappaB. Annals of the Rheumatic Diseases 59 Suppl 1:i6-16.
8. Ameyar, M., A. Atfi, Z. Cai, R. Stancou, V. Shatrov, A. Bettaieb, and S. Chouaib. 1998. Analysis of human breast adenocarcinoma MCF7 resistance to tumor necrosis factor-induced cell death. Lack of correlation between JNK activation and ceramide pathway. Journal of Biological Chemistry 273:29002-8.
9. Cai, Z., C. Capoulade, C. Moyret-Lalle, M. Amor-Gueret, J. Feunteun, A. K. Larsen, B. B. Paillerets, and S. Chouaib. 1997. Resistance of MCF7 human breast carcinoma cells to TNF-induced cell death is associated with loss of p53 function. Oncogene 15:2817-26.
10. Yang, J., M. Boerm, M. McCarty, C. Bucana, I. J. Fidler, Y. Zhuang, and B. Su. 2000. Mekk3 is essential for early embryonic cardiovascular development. Nature Genetics 24:309-13.
11. Yang, J., Y. Lin, Z. Guo, J. Cheng, J. Huang, L. Deng, W. Liao, Z. Chen, Z. Liu, and B. Su. 2001. The essential role of MEKK3 in TNF-induced NF-kappaB activation. *Nat Immunol* 2: 620-4.
12. Qin, X.F., D.S. An, I.S. Chen, and D. Baltimore. 2003. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci U S A* 100: 183-8.
13. Huang, Q. Jianhua Yang, Jinke Cheng, Y Ling, Z-G Liu, B Su. 2004. Differential regulation of interleukin 1 receptor and Toll-like receptor signaling by MEKK3. Nat Immunol 5, 98-103.

Appendice fig1-fig10.

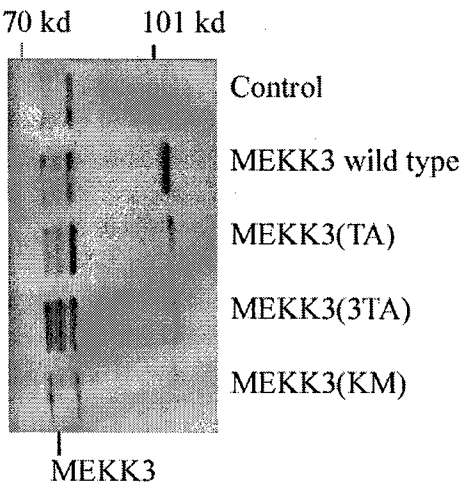

Figure 1 Expression HA-tagged wild type and mutant MEKK3. One microgram of empty vector of SRaHA-MEKK3 expression vectors were transfected into 293T cells and cell lysates prepared 36 h later for western blot analysis with anti-HA antibody as indicated.

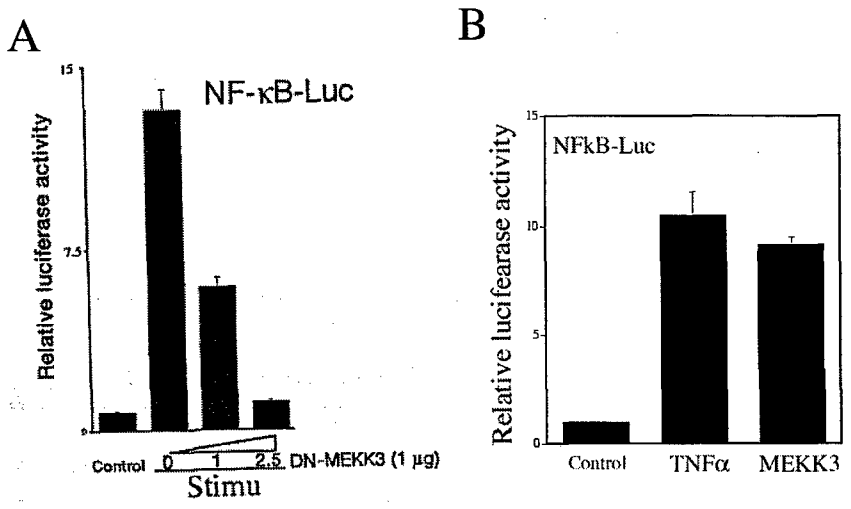

Figure 2 A. Dominant negative MEKK3 (DN-MEKK3) inhibits NF-kB reporter gene expression. One microgram of NF-kB-Luc reporter plasmid was transfected with either empty vector or with increasing amounts of DN-MEKK3. Transfected cells were either unstimulated (control) or stimulated (stimu) with Il-1 for 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.

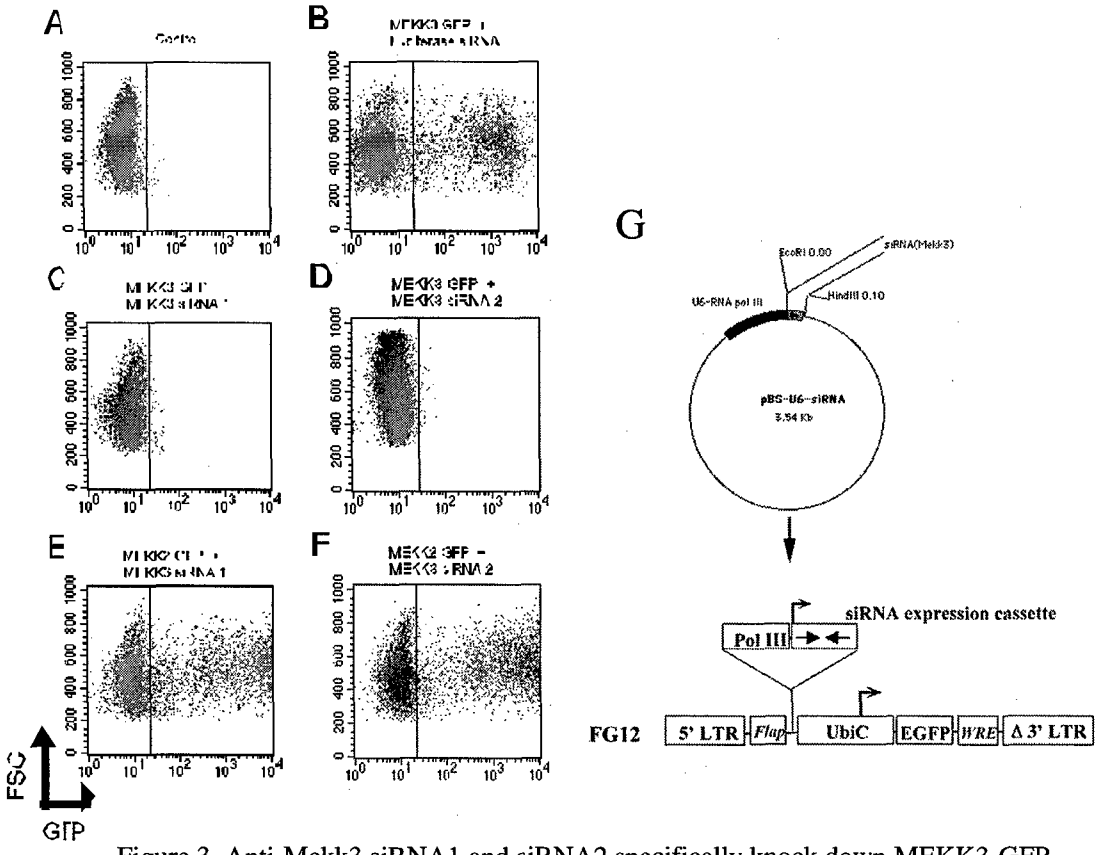

Figure 3 Anti-Mekk3 siRNA1 and siRNA2 specifically knock down MEKK3-GFP expression. Control Luci siRNA (B), or Mekk3 specific siRNA1 (C, E), siRNA2 (D, F) expression vectors were co-transfected with a MEKK3-GFP (C, D) or MEKK2-GFP (E, F) expression vector s into BOSC cells and the GFP fusion proteins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

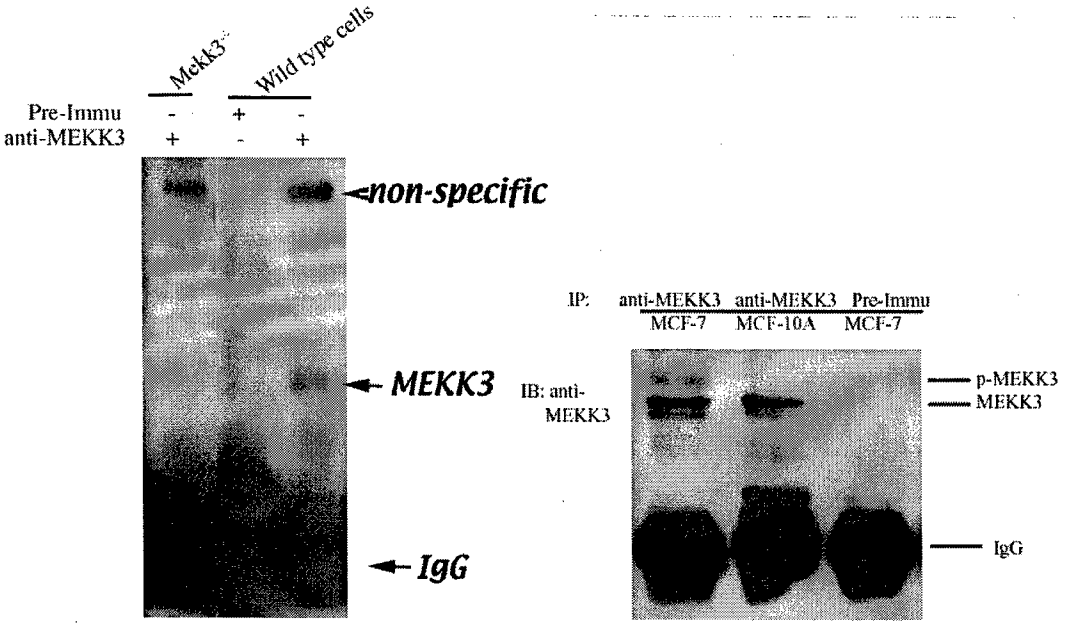

Figure 4 A. Immunoprecipitation-western blotting (IP-western) of endogenous MEKK3 with MEKK3-peptide specific antibody. Five million of 293T cells were lysed in 600 ul lysis buffer and subjected immunoprecipitation with 1 ug of purified anti-MEKK3 peptide antibody. The immunocomplex was separated by 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.

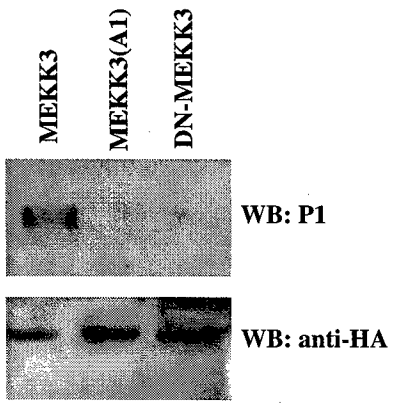

Figure 5 Characterization of anti-active MEKK3 antibody p1. COS-1 cells were transfected with HA-tagged MEKK3, MEKK3 phosphorylation site mutant MEKK3(A1), and DN-MEKK3 as indicated. Cell lysates were prepared 36 hr later and analyzed by immunoblotting with the P1 antibody (top panel) and an anti-HA antibody (bottom panel).

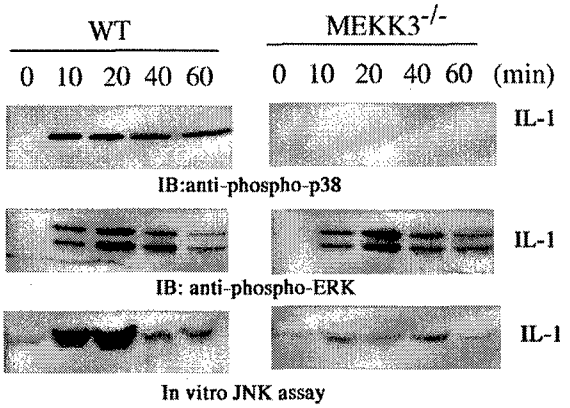

Figure 6 Activation of JNK, ERK and p38 MAPK in wildtype and Mekk3$^{-/-}$ MEFs. Wild type and Mekk3$^{-/-}$ MEFs either untreated or stimulated with IL-1 were harvested at the indicated time points. JNK activation was determined by an in vitro kinase assay. ERK and p38 MAPK activation was measured by immunoblotting and anti-phospho-p38 and –ERK1/2 antibodies.

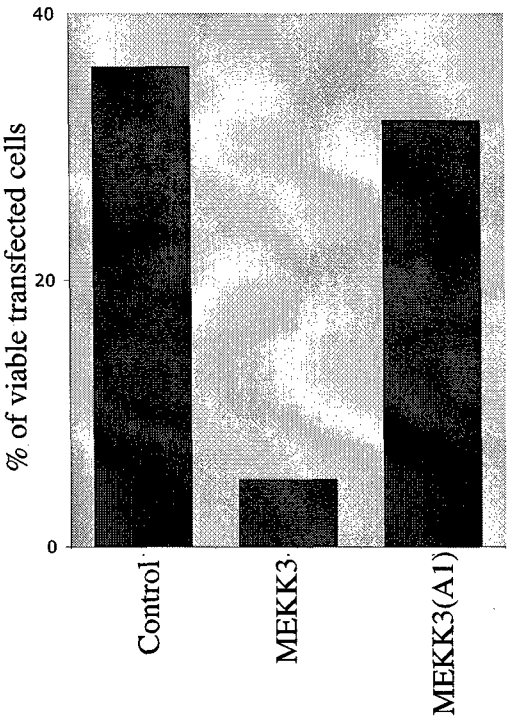

Figure 7 Transient expression of active MEKK3 but not inactive MEKK3 causes cell apoptosis. Empty vector or expression vectors for GFP tagged MEKK3 or MEKK3 mutant were transfected into tumor cells. Viable transfected cells were scored under the microscope.

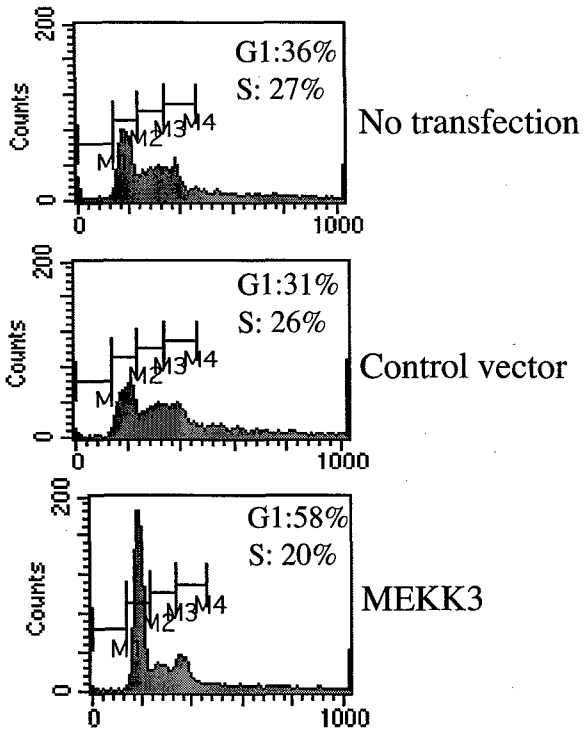

Figure 8 Expression of exogenous MEKK3 in breast cancer cells causes G1 cell cycle arrest. MCF cells were transfected with control vector or MEKK3 expression vector together with a GFP expression plasmid. Transfected cells were stained with PI and analyzed by a FACScaliber for cell cycle progression. 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%).

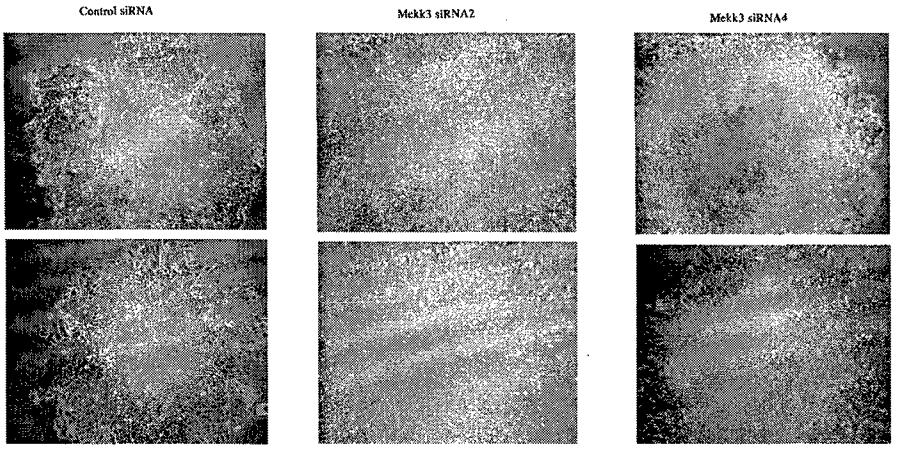

Figure 9 MCF7 cells transduced with control or MEKK3 specific siRNA were selected with hygromycine 48 hr later as indicated. Colonies were visualized under microscope after one-week selection. Cells transduced with MEKK3 specific siRNA have high potential to grow as multiple layers.

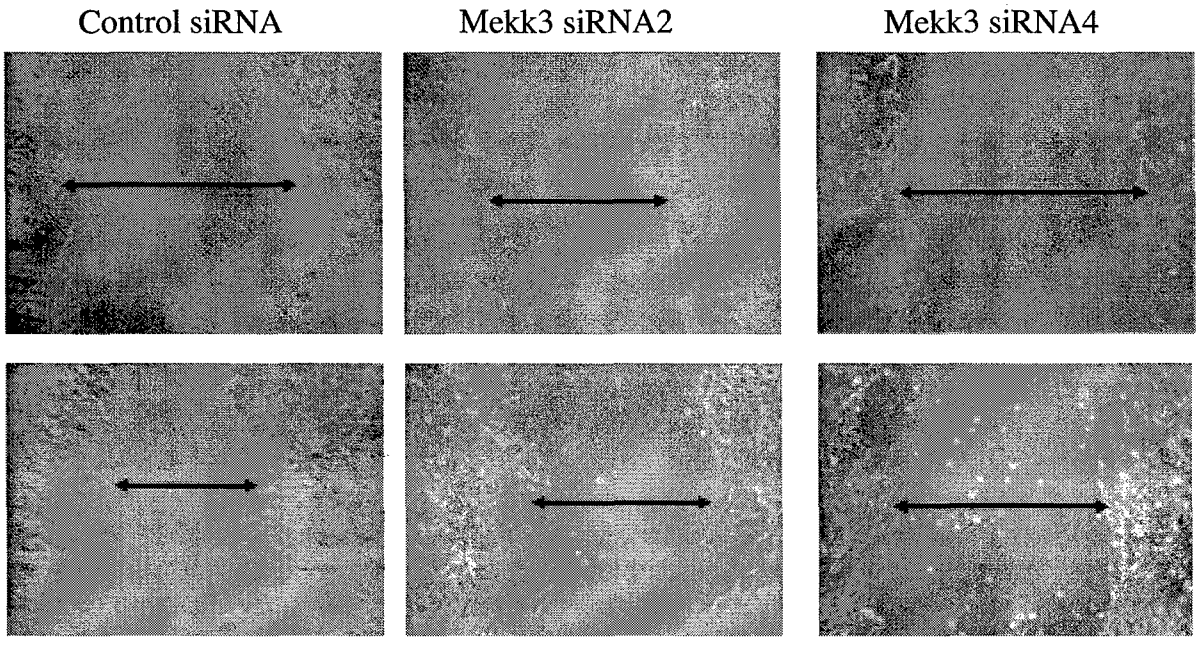

Figure 10 MCF7 cells transduced with control or MEKK3 siRNA were assayed for migration. Specifically, cells were plated as single layer and subjected to an in vitro wound-healing assay. Photographs were taken at 1 hr and 12 hr after injury. Compared with the control siRNA transduced cells that had about 50% decrease in the wounding gap, the two mekk3 siRNA transduced cells had lesser than 10% decrease in the closing of the gap.