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                          Houston, Texas 77030

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

E-MAIL:
mhung@mdanderson.org

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13. ABSTRACT (Maximum 200 Words)

Under support from the US Army / DOD the training program in breast cancer research at MDACC has had a successful first year. The training grant has supported four predoctoral and four postdoctoral fellows (two of these trainees were included in the program due to their outstanding qualifications, but are supported by funds from the BCRP at MDACC). Each trainee has made notable progress as evidenced by publications and presentations at national meetings. Significant strides have been made within the scope of the original specific aims in the following research areas: 1) Therapeutic approaches for breast cancer through regulation of oncogene and tumor suppressor gene expression, and control of signal transduction, apoptosis, and DNA repair; 2) Use of animals to understand the biology of breast cancer and to provide models for preclinical therapeutic and preventive studies; 3) Novel preventive strategies for breast cancer; 4) Population-based studies on breast cancer; 5) Molecular diagnostic/prognostic factors for breast cancer; and 6) The basic biology of breast cancer. In addition to laboratory pursuits each trainee has participated in departmental group meetings, journal clubs, and retreats. The goal of the training program is to further the successful training of fellows who will develop research programs of their own which continue to tackle problems of breast cancer.

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Introduction

The breast cancer training program at the University of Texas MD Anderson Cancer Center is a multidisciplinary research program comprising students and faculty from 20 different departments. The ultimate goal of this training program is to provide support to aid the development of exceptional scientist in the field of breast cancer research. The predoctoral and postdoctoral trainees for the US Army/DOD training grant are chosen from the laboratories of all faculty involved in the entire Breast Cancer Research Program at MDACC which includes over 70 faculty members and 20 departments. The scope of the research conducted by the trainees includes a variety of topics related to breast cancer research. Some trainees are conducting research aimed at developing novel therapeutic approaches for breast cancer through regulation of oncogenes and tumor suppressor genes and control of signal transduction, apoptosis and DNA repair. Others are using animals to understand the biology of breast cancer and to develop models for pre-clinical therapeutic and preventive studies. Still others are studying molecular diagnostic/prognostic factors for breast cancer and developing novel preventive strategies for this disease. The trainees are involved in many departmental and interdepartmental events including journal clubs, group meetings, retreats, and seminars. These activities outside of the laboratory provide opportunities for the trainees to gain a truly multi-disciplinary perspective on their own research projects by communicating and collaborating with researchers from each department at MDACC involved in breast cancer research. The breast cancer training program at MDACC is well on its way to producing top-notch investigators who will continue to probe the problems of breast cancer well into the future.

Body

Support from the US Army/DOD to develop the Breast Cancer Training Program at the University of Texas MD Anderson Cancer Center (UT-MDACC) has led to a successful year in research for several predoctoral and postdoctoral fellows. The Breast Cancer Training Program at UT-MDACC was developed to provide support for exceptional young scientist training in laboratories that are part of the Breast Cancer Research Program (BCRP) at UT-MDACC. The program provides comprehensive interdisciplinary research training to each fellow. The program faculty is comprised of members who cover the full spectrum of breast cancer research, including investigators involved in basic, translation, clinical and population-based studies related to breast cancer. The strength of the training programs at MDACC stem from the strong interactions that take place between basic science researchers and clinicians in the existing environment, which allows for quick transfer of scientific discoveries from the laboratory to the clinic. Predoctoral and postdoctoral trainees at MDACC benefit from this unique environment and are able to gain unparalleled experience in multidisciplinary studies. The ultimate goal of this training program is to train predoctoral and postdoctoral fellows to become highly qualified breast cancer researchers who will develop programs of excellence in breast cancer research.

The training program was instigated after approval of the US Army/DOD training grant awarded to UT-MDACC. This program consists of over 70 faculty members from more than 20 departments at MDACC involved in breast cancer research. The program developed a nomination process that involved the participation of each faculty member in nominating individuals to serve on the US Army Breast Cancer Training Grant Steering Committee. Following the nomination process each BCRP faculty member voted for his or her choices for members to serve on the steering committee. This committee was formed to serve in the selection of US Army Training Grant recipients, the mentoring of the selected fellows, and the monitoring of each fellow's research progress. This process resulted in the election of the following six faculty members to serve on the committee:

Mien-Chie Hung, Ph.D., Professor &Chairman, PI of US Army/DOD Training Grant,
Dept. of Molecular and Cellular Oncology

Melissa Bondy, Ph.D., Associate Professor, Dept. of Epidemiology

Gabriel Hortobagyi. M.D., Professor & Chairman, Dept. of Breast Medical Oncology

Rakesh Kumar, Ph.D., Professor, Dept. of Molecular and Cellular Oncology

Janet Price, Ph.D., Associate Professor, Dept. of Cancer Biology

Di-Hua Yu, M.D., Ph.D., Associate Professor, Dept. of Surgical Oncology – Research

The steering committee commenced its activities by accepting proposals for the US Army/DOD Breast Cancer Training Program Fellowships. The committee received 18 applications from predoctoral and postdoctoral trainees involved in breast cancer research at MDACC (see appendix 2.0). The general notion from each member of the steering committee was that the applicant pool consisted of highly qualified candidates and many excellent proposals. Eventually, after careful review, eight individuals were selected to join the US Army/DOD Breast Cancer Training Program. The awardees include four predoctoral trainees and two postdoctoral trainees who are supported by funds from the US Army/DOD Training Grant. **Due to their exceptional qualifications, the US Army Training Grant Steering Committee awarded an additional two postdoctoral trainees fellowships, and although designated as US Army/DOD**

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Training Grant Fellows, these individuals are funded by MDACC BRCP funds. The top-notch applicant pool truly impressed the steering committee. Their overall consensus is that the US Army/DOD Training Grants in combination with the Breast Cancer Research Program will not only provide each fellow with an excellent orientation to the various aspects of breast cancer research, but will also serve as a strong boaster in their careers.

In addition to the regular monthly journal clubs and seminars organized by BCRP, and the Women's Cancer Research Group, respectively, the trainees also attend weekly lab meetings and seminars in their own individual departments. Furthermore, approximately ten months after receiving funding for training from US Army/DOD, the BCRP held the BCRP Retreat - 2000 at the Four Seasons Hotel in Houston, TX (see Appendix 1.0). This retreat served as a forum for the US Army/DOD Breast Cancer Training Grant fellows to discuss and collaborate with BCRP faculty and other trainees in the various areas of breast cancer research. Each US Army/DOD Training Grant fellow was required to give a ten-minute presentation outlining their research accomplishments and future goals. The talks were followed by open and helpful discussions among the trainees and faculty. In attendance were members of the Breast Cancer Research Program— including each fellow, the steering committee and other BCRP faculty members. See attachment for an agenda outlining the BCRP Retreat – 2000 schedule of events.

The over all feeling among members of the steering committee and other BCRP faculty members was that it was rewarding and exciting to hear all eight US Army/DOD Training Grant fellows give excellent and impressive presentations. Each faculty member agreed that the trainees were making notable progress with their projects as well as in developing critical thinking skills.

In addition to the oral presentations, each trainee was asked to submit a description of their research accomplishments. The achievements of this group of trainees is exceptional and includes publication in several highly reputable journals including *Nature Medicine, Nature Cell Biology, PNAS, Journal of Biological Chemistry, Cancer Research* etc. (see appendix 3.0). A summary of the accomplishments of each trainee is printed below:

Soo-Jeong Lim, Ph.D. – Postdoctoral Fellow

The sensitivity of breast cancer cells to all-trans retinoic acid (ATRA) has been known to be correlated with the estradiol receptor or retinoic acid receptor (RAR)α status. However, the fact that many of EGFR or ErbB2-overexpressing breast cancer cell lines are ATRA-resistant prompted us to investigate the role of EGFR and ErbB2 receptors in ATRA-resistant breast cancer cell lines. We have studied the effect of overexpression of EGFR, ErbB2 or heregulin on the ATRA-sensitivity of parental MCF-7 cells, which were originally sensitive to ATRA. We further investigated the effect of ligands such as EGF and heregulin, which can activate EGFR or ErbB2, on the sensitivity of breast cancer cells to ATRA. We found that ErbB2 receptor, rather than EGFR, is involved in the acquisition of ATRA-resistance in breast cancer cells. Combination treatment with Grb2 antisense oligodeoxynucleotides and Herceptin to block the ErbB2 signaling resensitized ErbB2-activated cells to ATRA. The ATRA-resistance in ErbB2-activated cells was correlated with reduced endogenous expression of RARα protein and further correlated with ATRA-triggered dramatic decrease in RARα protein expression. These results imply that ErbB2 receptors induce the ATRA-resistance mainly by modulating RARα protein expression. We are currently investigating the molecular mechanism how ErbB2 receptors modulate RARα protein expression.

Keishi Makino, Ph.D. – Postdoctoral Fellow

We have identified that Taxol could induce p55Cdc mRNA expression through activation of the p55Cdc promoter, which led to increase p55Cdc protein expression. In addition, Taxol activated p55Cdc-associated kinase. And we have identified that overexpression of the p55Cdc gene resulted in increased cell death in Hela cells in a dose-dependent manner. A dominant-negative mutant of p34cdc2 blocked Taxol-induced p55cdc activation and inhibited p55Cdc-induced and taxol-induced cell death.

Rui-An Wang – Postdoctoral Fellow

Activation of heregulin signaling in the estrogen receptor-positive breast cancer cells leads to the suppression of estrogen receptor element (ERE)-driven transcription, the disruption of estradiol responsiveness, the acquisition of the hormone-independent phenotype and thus, contributes in breast cancer tumor progression to more invasive phenotypes. Steroid receptors have been shown to control gene transcription in conjunction with histone acetylation modifying complexes. Here we report the identification of a metastatic-associated protein 1 (MTA1), a component of histone deacetylase and nucleosome remodeling complexes, as a gene product induced by heregulin-_1. Stimulation of cells with heregulin-_1 was accompanied by a suppression of histone-4 acetylation, and an enhancement of deacetylase activity. In addition, MTA1 was discovered to be a potent corepressor of ERE-transcription as it blocked the ability of estradiol to stimulate the ER-transcription. Histone deacetylase inhibitor trichostatin A blocked the MTA1-mediated repression of ERE transcription. In vivo, MTA1 associates with several components of the NuRD complex, including histone deacetylases 1, 2 and 3, chromodomain proteins 3 and 4, and Sin3. Furthermore, the MTA1 directly interacted with the histone deacetylase-2 in the region of xx – xx amino acids, and also with the activation domain AF2 of the estrogen receptor-_. These results identify ER-mediated transcription as a nuclear target of MTA1 and suggest that histone deacetylase complexes associated with the MTA1 corepressor mediates estrogen receptor transcriptional repression by heregulin-_1.

Binhua Zhou – Postdoctoral Fellow

Amplification/overexpression of the HER-2/neu gene was found in many different types of human cancers. The Her-2/neu protein is a receptor tyrosine kinase, which mediates many signal pathways to regulate cell-growth and cell death. In many of these cellular signal pathways, the PI-3 kinase/Akt pathway is known to play a prominent role in preventing cells from apoptosis, and attributes to the pathogenesis of cancer. Thus, understanding the signal pathways and molecular actions mediated by Her2/neu and Akt, will not only increase our understanding of the biology of cancer, but also shed light on new strategies for developing novel therapeutics for fighting breast cancer.

Kristine Klos – Predoctoral Fellow

ErbB2 over-expression has been found to occur in approximately 30% of human breast cancer cases. This occurrence is correlated with an increase in lymph node metastasis and a poor prognosis for these patients. An early, important step in the process of metastasis is angiogenesis. The most important marker for angiogenesis is VEGF – an endothelial cell mitogen. My hypothesis focuses on the ability of erbB2 overexpresiion to enhance the erbB2 RTK signaling capacity that activated downstream signals to induce angiogenic responses 9such as an increase in VEGF secretion). Which ultimately leads to enhanced metastatic potential.
Using a combination of methods including FACs and Western Blot, I isolated a mutant cell line that overexpressed erbB2 at high levels. I injected 1 x 10^6 selected parental and mutant cell lines into the mammary fat Pad of 180 SCID mice for a spontaneous metastasis assay. The

tumors were grown to 1cm before removal. The mice live for 42 days after primary tumor removal, upon which they are killed and autopsied for metastases. The lungs are perfused with India ink to aid in the counting if metastases.

I have preliminary data on VEGF levels of one set of erbB2 mutant – transfected cell lines. This needs to be repeated with the second set. I have been using western blot, but I am now beginning to use a VEGF assay kit from R&D Systems for a more precise and sensitive method of determining VEGF levels. I have been saving conditioned media from my cell lines for angiogenesis assays. This will be used for the endothelial cell growth rate and migration assays.

Christopher Neal – Predoctoral Fellow

14-3-3 proteins are intracellular, dimeric molecules that can bind to various phosphoserine containing target proteins involved in regulation of cell growth, differentiation, apoptosis, transformation, and checkpoint control pathways. Recently, we inadvertently found that 14-3-3zeta is overexpressed in >70% of human breast cancers by western blot and immunohistochemistry analyses, whereas none of the autologous normal breast tissues overexpressed 14-3-3zeta. We also found that a dominant negative mutant of 14-3-3zeta, 14-3-3ΔN, reduced transforming colony formation and regulated metastasis-related phenotypes. Currently, there are no reports on 14-3-3 overexpression in breast cancers. Thus, our hypothesis that overexpression of 14-3-3zeta may facilitate breast cancer progression/metastasis by regulating specific transformation and metastasis-related properties will bring novel findings to this unexplored area.

Yong Wen – Predoctoral Fellow.

We have successfully generated recombinant adenovirus vector expressing p202, Ad-p202. Ad-p202 infection inhibits cell growth in breast cancer cells *in vitro*, compared with the Ad-luciferase infection. We are going to test the therapeutic effect of p202 gene in an orthotopic breast cancer animal model, using Ad-p202 as well as non-viral delivery system.

Zheming Yu – Predoctoral Fellow

Amplification/overexpression of the Her-2/neu gene is found in approximately 30% of human breast cancers. Overexpression of the Her2/neu oncogene correlates with poor survival for breast cancer patients and enhances metastatic potential and chemoresistance of human cancer cells. Downregulation of Her2/neu suppresses the malignant phenotypes of cancer cells with Her-2/neu overexpression, indicating that Her-2/neu oncogene is an ideal target for development of novel therapeutic agents.

We performed several experiments to test the effect of PEA3 (a member of the Ets family of transcription factors) on Her2/neu promoter activity. Our data indicated that PEA3 could down regulate Her2/neu overexpression at the transcriptional level through competitively binding a positive regulatory motif of the Her2/neu promoter, and could suppress the growth of cancer cells with high but not basal levels of Her2/neu expression. By using an animal model we fond that expression of PEA3 was concomitant with downregulation of Her-2/neu expression.

In conclusion, Pea3 can suppress her2/neu overexpression and inhibit Her-2/neu- mediated tumorigenesis. The results in this study indicate that PEA3 may be important in developing gene therapy for her2/neu overexpressing breast cancers.

Again, after careful review, the US Army/DOD Breast Cancer Training Program steering committee was impressed with the excellent progress made each by training grant recipient in the

areas of laboratory research, development of ideas, and critical thinking. These predoctoral and postdoctoral trainees have taken full advantage of the opportunities provided to them as a result of the US Army/DOD Training Grant awarded to UT-MDACC. All eight of the fellows are making significant strides in their research and agree that the US Army/DOD Breast Cancer Training Program is providing them with a unique opportunity to develop the skills needed to be competitive investigators in the field of breast cancer research. In addition, each US Army/DOD Training Grant fellow's progress will be monitored by the steering committee over the entire two-year period for which the grants were awarded. At the end of the two year award period the BCRP will host a second retreat at which time each fellow will orally present his or her research accomplishments for a second time to the BCRP faculty members. It is expected that some trainees will finish training by the end of their second year on the training grant (Fall 2001). In this case, the steering committee will post a call for additional applications for the US Army/DOD Training Grant and the selection process will be repeated to chose the most qualified candidates. Additionally, the progress of US Army/DOD Training grant recipients continuing studies at MDACC after two years on the grant will be reviewed by the steering committee and decisions will be made regarding whether or not to continuing funding.

In sum, the US Army/DOD Breast Cancer Training Program at the University of Texas MD Anderson Cancer Center has served as an unprecedented opportunity for bright young scientists. Not only have these trainees benefited from the support and multidisciplinary activities offered by the program, but also the program has served as a unique attraction for young scientists from around the world to devote their energy to breast cancer research at MDACC.

Key Research Accomplishments
(see list of publications for details)

- Discovery that Taxol could can induce p55Cdc mRNA.
- Discovery that p55Cdc gene overexpression can increase apoptosis.
- Discovery that Metastasis-Associated Protein 1 represses estrogen receptor transcription via recruiting histone deacetylase complexes.
- Discovery that Her2/neu blocks TNF-alpha induced apoptosis via the Akt/NF-kB pathway.
- Discovery that oncogenic signals from Her2/neu can regulate the stability of cyclin-dependent kinase inhibitor, p27.
- Analysis of VEGF levels in erbB2 overexpressing breast cancer cell lines.
- Generation of a recombinant adenovirus vector that expresses the p202 gene.
- Discovery that p202 contributes to tumor suppression and sensitization to TNF-alpha-induced apoptosis.
- Analysis of Beta-catenin as a novel prognostic marker in breast cancer, and its role in cyclin D1 expression and cancer progression.
- Discovery that Ets protein PEA3 suppresses Her2/neu overexpresiion and inhibits tumorigenesis.
- Discovery that Grb2 downregulation leads to growth inhibition and Akt inactivation in heregulin-stimulated breast cancer cells.
- Analysis of the correlation between decreased RAR alpha expression and induction of resistance to all-trans retenoic acid by ErbB2 in breast cancer.

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Reportable Outcomes

Peer Reviewed Journals:

Lim, S.J., Lopez-Bernstein, G., Hung, M.-C., Lupu, R., and Tari, A.M. Grb2 Downregulation Leads to Akt Inactivation in Heregulin-Stimulated Breast Cancer Cells. *Oncogene. Submitted.*

Lim, S.J., Lopez-Bernstein, G., Zapata, P., and Tari, A.M. Induction of Resistance to All Trans-Retinoic Acid by ErbB2 in Breast Cancer: Correlation with Decreased RAR Alpha Expression. *In preparation*

Lin S.-Y., Xia W., Wang J., Kwong K.-Y., Spohn B., **Wen Y.,** and Hung M.-C.. β-catenin, a Novel Prognositic Marker for Breast Cancer: its Roles in Cyclin D1 Expression and Cancer Progression. *Proc. Natl. Acad. Sci. U.S.A.* 2000; 97: 4262-4266.

Makino, K., Yu, D. and Hung, M.-C., Transcriptional Upregulation and Activation of p55Cdc via p34cdc2 in Taxol Induced Apoptosis. *Submitted.*

Mazmudar, A., **Wang, R.,** Mishra, S., Adam, L., Yarmand, R., Mabdal, A., Vadlamudi, R. and Kumar, R. Transcriptinal Repression of Estrogen Receptor by Metastasis-Associated Protein 1, a Component of Histone Deacetylase and Nucleosome Remodeling Complexes. *Nature Cell Biology. In Press.*

Wen Y., Yan D.-H., Spohn B., Deng J., Lin S.-Y., and Hung M.-C.. Tumor Suppression and Sensitization to Tumor Necrosis Factor α - Induced Apoptosis by an Interferon-Inducible Protein, p202, in Breast Cancer Cells. *Cancer Res.* 2000; 60: 42-46.

Xing, X., Wang, S.C., Xia, W., Zou, Y., Shao, R., Kwong, K.Y., **Yu, Z.,** Zhang, S., Miller, S., Huang, L., & Hung, M.-C. The Ets Protein PEA3 Suppresses Her-2/neu Overexpression and Inhibits Tumorigenesis. *Nature Medicine.* 6:189-194. 2000.

Yin, H., **Zhou, B.P.,** Xia, W., Newman, L., Hung, M.-C., & Lee, M.-H. Oncogenic Signal of Her-2/neu in Regulating the Stability of Cyclin-Dependent Kinase Inhibitor p27. *J. Biol. Chem.* 275, 24735-24739. 2000.

Zhou, B.P., Hu, M., Miller, S., **Yu, Z.,** Xia, W., Lin, S., and Hung, M.-C. Her2/neu blocks TNF-Induced Apoptosis via the Akt/NF-kB Pathway. *J. Biol. Chem.* 275, 8027-8031. 2000.

Zhou, B.P., Liao, Y., Spohn, B., Lee, M.-H., and Hung, M.-C. Cytoplasmic Localization of p21$^{Cip1/WAF1}$ by Akt-Induced Phosphorylation in HER-2/neu- Overexpressing Cells. *Nature Cell Biology. In Revision.*

Meeting Abstracts:

Lim, S.J., Lopez-Bernstein, G., Lupu, R., and Tari, A.M. Grb2 Downregulation Leads to Growth Inhibition and Akt Inactivation in Heregulin-Stimulated Breast Cancer Cells. *Proceedings 91st American Association for Cancer Research.* San Francisco, CA. April 1-5, 2000.

Makino, K. and Hung, M.C. Identification and Characterization of a Taxol Induced Gene. 91st Annual Meeting of AACR. San Francisco, CA. April 1-5, 2000.

Wen Y., Yan D.-H., Spohn B., Deng J., Lin S.-Y., and Hung M.-C.. Tumor suppression and sensitization to tumor necrosis factor α-induced apoptosis by an interferon-inducible protein, p202, in breast cancer cells. AACR-NCI-EORRC International Conference: Molecular Targets and Cancer Therapeutics. November 16-19, 1999, Washington DC.

Yu, Z., Xing, X., Wang, S., Xia, W., Zou, Y., Shao, R., Kwong, K., Zhang, S., Miller, S., Huang, L., and Hung, M.-C. The Ets Protein PEA3 Suppresses Her-2/neu Overexpression and Inhibits Tumorigenesis. DOD Breast Cancer Program Era of Hope Meeting. Atlanta, GA. 2000.

Patents:

U.S. Patent Application No. 09/590,652 entitled "p202 is a Tumor Suppressor" by **Yong Wen et al.** Filing date: June 8, 2000.

Conclusions

In conclusion, the US Army/DOD Breast Cancer Research Training Program at the University of Texas MD Anderson Cancer Center has had a successful first year. Each US Army/DOD Training Grant recipient has benefited from the multi-disciplinary program as evidenced by significant progress in their respective research projects and an outstanding publication record. Additionally, each trainee has gained invaluable knowledge and critical thinking skills as a result of departmental and inter-departmental seminars and group meetings. With continued support for the US Army/DOD the Breast Cancer Research Training Program at UT-MDACC will continue its success in training scientists in the field of breast cancer research.

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Yang, W.M., Yao, Y.L., Sun, J.M., Davie, J.R. & Seto, E. *J. Biol. Chem.* **272**, 28001-28007 (1997).

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Zha, J., Harada, E., Yang, J., Jockel, J., Korsmeyer, S. *Cell* **87**, 619-628 (1996).

Zhang, Y. et al., *Genes Dev.* **13**, 1924-1935 (1999).

Appendices

Appendix 1.0   Agenda - Breast Cancer Retreat 2000

Appendix 2.0   US Army/DOD Breast Cancer Research
                                    Training Grant Applicants 1999;
                                    Biosketches and Abstracts

Appendix 3.0   Cited Publications

Appendix 1.0

Breast Cancer Research Program Retreat – 2000
Agenda

Agenda

Breast Cancer Research Program
Retreat - 2000
August 5, 2000 - 8:00 AM to 5:00 PM

Four Seasons Hotel
1300 Lamar Street
Houston, Texas 77010
Main Phone Number: 713/650-1300

<u>Morning Session</u>

Reception / Continental Breakfast		8:00-8:50 AM
Opening Remarks	Gabriel N. Hortobagyi, M.D.	8:50-9:00 AM
Introduction for Planned PO1: "Novel Signal Pathway in Breast Cancer"	Mien-Chie Hung, Ph.D.	9:00-9:10 AM
Project 1: "Role of STK15 in Chromosomal Instability and Chemotherapy Response of Human Breast Carcinoma Cells"	Subrata Sen, P.I.	9:10-9:40 AM
Project 2: "Molecular Mechanism of HER-2/*neu* Overexpression Mediated Taxol Resistance in Human Breast Cancer"	Dihua Yu, P.I.	9:40-10:10 AM
Project 3: "Mysophospholipids in the Transformation, Proliferation, Prognosis and Diagnosis of Breast Cancer"	Gordon Mills, P.I.	10:10-10:40 AM
COFFEE BREAK		10:40-11:00 AM
Project 4: "The Role of p21 Activating Kinase 1 (PAK-1) in Breast Cancer"	Rakesh Kumar, P.I.	11:00-11:30 AM
Project 5: "Signal Transduction of HER-2/*neu* in Breast Cancer Cells"	Mien-Chie Hung, P.I.	11:30-12:00 PM
Discussion		12:00-12:10 PM
LUNCH		12:10-1:20 PM

<u>Afternoon Session</u>

Project 1: "BCL-2 in Breast Cancer"	Ana Tari, P.I.	1:20-1:50 PM
Project 2: "Novel Anthracyclines Analogues Targeting Apoptosis"	Waldemar Priebe, P.I.	1:50-2:20 PM
Project 3: "Signal Transduction as a Target for Breast Cancer Therapy"	Gabriel Lopez, P.I.	2:20-2:50 PM
Discussion		2:50-3:00 PM
COFFEE BREAK		3:00-3:10 PM
Presentation of DoD/Breast Cancer Research Program Trainees: K. Klos; S-J Lim; K. Makino; C. Neal; R-A Wang; Y. Wen; Z. Yu; P. Zhou (10 minutes per person)		3:10-4:50 PM
Closing Remarks	Gabriel N. Hortobagyi, M.D.	4:50-5:00 PM
Reception		5:00-6:00 PM

Appendix 2.0

US Army/DOD Breast Cancer Research Training Grant Applicants 1999;
Biosketches and Abstracts

Ali Azhdarinia, B.S.

Geoffrey A. Bartholomeusz, Ph.D.

Jiong Deng, Ph.D.

Kristine S. Klos, B.S.

Keng-Li Lan, M.D., Ph.D.

Yong Liao, M.D., Ph.D.

Soo-Jeong Lim, Ph.D.

Keishi Makino, M.D., Ph.D.

Stephanie Miller, B.S.

Rhonda J. Moore, Ph.D.

Ulrich W. Mueller, Ph.D.

Christopher L. Neal, Ph.D.

Chunfeng Qu, Ph.D.

Rui-An Wang, Ph.D.

Yong Wen, Ph.D.

Kazumi Yoshida, M.D., Ph.D.

Zhenming Yu, Ph.D.

Binhua P. Zhou, M.D., Ph.D.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Ali Azhdarinia, B.S.	Graduate Student / Research Technician

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

STITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Houston, Houston, TX	B.S.	1999	Biology
Univ. of Texas-Graduate School of Biomedical Sciences, Houston, TX	Predoctoral	Spring 2000	Pharmacology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Professional Experience
1998-1999:   Mentor, University of Houston
1998-1999:   Teaching Assistant, University of Houston, Department of Chemistry
1999:   Research Technician, The University of Texas M.D. Anderson Cancer Center, Houston, TX; graduate student (Ph.D. program in Pharmacology, admitted Spring 2000)

Honors and Awards
Honor Roll: Fall 1996, Fall 1997, Fall 1998, University of Houston
Dean's List: Spring 1998, Spring 1999, University of Houston

Publications
Yang, D.J., Inoue, T., Zakko, S., Kim, C., **Azhdarinia, A.,** Kim, E.E.
     Molecular Targets for Cancer Imaging. Annals of Nuclear Medicine and Sciences (in preparation 1999).
Yang, D.J., **Azhdarinia, A.,** Yu, D-F., Herbst, R., Kim, E.E., Podoloff, D.A.
     [^{131}I]iodoendostatin: Synthesis, biodistribution, and planar imaging in tumor-bearing rodents. Nuclear Medicine Communication (in preparation 1999).
Azhdarinia, A., Yang, D.J., Kim, E.E. Synthesis and Evaluation of [^{131}I]iodopropio-α-methyl tyrosine in tumor-bearing animal models. J. Pharm. Sci. (in preparation 1999).

PHS 398 (REV. 4/98)   (Form Page 6) Page _____   FF

Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 3a, 3b.

Abstract

Breast cancer is the most common type of cancer in women, with approximately 1 in 9 women developing the disease in her lifetime. Currently, the diagnosis of breast cancer is made by pathologic evaluation of tissue obtained by fine-needle aspiration. Mammography is usually performed; however, the detection rate is low among younger women due to denser breast tissue. There is a need to develop agents for functional imaging information.

Angiogenesis, the proliferation of endothelial and smooth muscle cells to form new blood vessels, is an essential component of the metastatic pathway. These vessels provide the principal route by which tumor cells exit the primary tumor site and enter the circulation. For many tumors, the vascular density can provide a prognostic indicator of metastatic potential, with the highly vascular tumors having a higher incidence of metastasis than poorly vascular tumors.

Endostatin, a carboxyl-terminal fragment of collagen XVIII, has been shown to regress tumors in mice. Treatment of cow pulmonary artery endothelial cells by endostatin caused apoptosis (determined by annexin V-fluorescein isothiocyanate staining, caspase 3, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling assay). Moreover, addition of endostatin led to a marked reduction of the Bcl-2 and Bcl-XL anti-apoptotic protein, whereas Bax protein levels were unaffected. These effects were not seen in several non-endothelial cells. These findings provide important mechanistic insight into endostatin action. Measuring angiogenesis (blood vessel density) and/or its main regulators such as VEGF and bFGF, or the levels of apoptosis after treatment in solid tumors provides new and sensitive markers for tumor progression, metastasis and prognosis.

Endostatin tagged with an isotope can increase our understanding of the functions of these molecules in the regulation of physiological and pathological angiogenesis. This provides an important therapeutic strategy for breast cancer treatment. The objective of this proposal is to determine the biodistribution and imaging potential of radiolabeled endostatin. Our hypothesis states that if the tumor uptake of radiolabeled endostatin is related to tumor vascular density, then highly vascular tumors should have more uptake of radiolabeled endostatin than that in poorly vascular tumors. Higher uptake of radiolabeled endostatin in tumors could be a prognostic indicator of a higher incidence of tumor metastasis. The specific aims are: (1). to radiosynthesize ^{131}I- and ^{99m}Tc-endostatin, (2). to evaluate the biodistribution and imaging of radiolabeled endostatin in tumor bearing rodents. The animal models have different vascular density in tumors, and (3). to quantitate tumor uptake changes after antiangiogenic therapy. The long term goal is to apply the radiolabeled endostatin for human use approval.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel listed on the budget page for the initial budget period

NAME Geoffrey A. Bartholomeusz	POSITION TITLE Postdoctoral Fellow

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
University of Zambia, Lusaka, Zambia	M.S.	1988	Microbiology
Rochester Institute of Technology, Rochester, NY	M.S.	1990	Clinical Chemistry
University of Oklahoma, Norman, OK	Ph.D.	1998	Microbiology/Molecular Biology
The University of Texas M. D. Anderson Cancer Center Houston, TX	Pre- & Post-doctoral Fellow	1997-1999	Molecular Genetics
The University of Texas M. D. Anderson Cancer Center Houston, TX	Postdoctoral Fellow	1999	Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 3 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Professional Experience:

1981-1983	Undergraduate Research Assistant, Department of Biological Sciences, University of Zambia, Lusaka, Zambia
1984-1985	Medical Student Assistant, University of Zambia, Lusaka, Zambia
1988-1989	Research Assistant, Department of Surgery, Rochester General Hospital, Rochester, NY
1988-1990	Research Assistant, Clinical Chemistry, Rochester Institute of Technology, Rochester, NY
1989-1990	Laboratory Technician, Department of Biology, University of Rochester, Rochester, NY
1990-1994	Teaching Assistant, Department of Microbiology, University of Oklahoma, Norman, OK
1994-1996	Research Assistant, Department of Microbiology, University of Oklahoma, Norman, OK
1997 (Spring)	Teaching Assistant, Department of Microbiology, University of Oklahoma, Norman, OK
1997-1999	Pre and Postdoctoral Fellow, Department of Molecular Genetics, The University of Texas M. D. Anderson Cancer Center, Houston, TX
1999-present:	Postdoctoral Fellow, Department of Cancer Biology, Section of Molecular Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX

Publications:

1. **Bartholomeusz, G.**, Zhu, Y., and Downard, J. Growth medium-dependent regulation of *Myxococcus xanthus* fatty acid content is controlled by the *esg* locus. J. Barteriol. 180(19):5269-5272, 1998.
2. **Bartholomeusz, G.**, Gilbreth, M., Yang, P., Pimental, R.A., Kansra, S., Gadiraju, T., and Marcus, S. Negative regulation of mitosis in fission yeast by the Shk1 interacting protein Skb1 and its human homolog, Skb1Hs. Proc. Natl. Acad. Sci. USA 95(25);14781-14786, 1998.
3. **Bartholomeusz, G.**, Chang, E., Pimental, R., Chen, J., Lai, H., Wang, L.L, Yang P., and Marcus, S. Direct binding and in vivo regulation of the fission yeast PAK Shk 1 by the SH-3 domain protein Scd2. Molec. Cell Biol. in press, 1999.

Combined BOK Gene Therapy and Hyperthermia
in Breast Cancer

Abstract

It is estimated that one in eight women in this country will develop breast cancer in their life span making breast cancer one of the major causes of death for women in the United States. The resistance of breast cancer to the routinely used chemotherapeutic agents combined with the toxic side effects resulting from this treatment makes the search for novel alternate therapeutic approaches for the treatment breast cancer a necessity. Recent studies on the pro-apoptotic Bcl-2 members have shown that a splice variant of the murine BOK gene as well as amino terminal deletions of BAX, BID and BIM makes them potent induces of apoptosis. Recently the human BOK gene was cloned in our lab. We will generate a series of deletions of this human BOK gene and express these constructs in breast cancer cells using the CMV promoter. Deletions functioning as potent induces of apoptosis will be selected for further studies. We will next identify a promoter whose activation is tumor specific. This is an important step to guarantee that the expression of BOK is tumor specific and minimize its toxic effects on normal cells. Hyperthemia induces apoptosis by regulating the expression of many genes as well as inhibiting the activity of the antiapoptotic molecule NFκB. We will identify a gene in the NFκB signal pathway whose expression is strongly induced by hyperthermia in breast cancer cells and use the promoter of this gene to drive the expression of the mutant BOK gene. We will then identify the optimal in-vitro conditions that can induce rapid apoptosis specifically in BOK overexpressing breast cancer cells. Finally, we will confirm the specific anti-tumor activity in a mouse orthotopic breast cancer model by delivery of the tumor specific promoter / mutant BOK plasmid by the non-viral cationic liposome gene delivery system. We will investigate whether the combination of gene therapy and hyperthermia could achieve an enhanced therapeutic efficacy against breast cancer. In summary, we hope that our approach of combining gene therapy with hyperthermia will result in rapid induction of apoptosis rendering the mice tumor free. The success of the preclinical experiments in this proposal would fulfill the purpose of directing the laboratory research to clinical trials in breast cancer patients.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel listed on the budget page for the initial budget period

NAME Jiong Deng	POSITION TITLE Postdoctoral Fellow

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Nanjing University, Nanjing, P. R. China	B.S.	1982	Biochemistry
Second Military Medical College Shanghai, P. R. China	M.S.	1985	Microbiology
The University of Texas Medical Branch, Galveston, Texas	M.S.	1990	Virology
The University of Texas M. D. Anderson Cancer Center, Houston, TX	Ph.D.	1998	Cancer Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 3 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Professional Experience:

1983 – 1986   Graduate Student, Department of Microbiology, Second Military Medical College, Shanghai, P. R. China

1987 – 1990   Graduate Student, Department of Microbiology, University of Texas Medical Branch, Galveston, Texas

1991 – 1992   Research Assistant, Division of Hematology/Oncology, Department of Internal Medicine, The University of Texas Health Science Center, Houston, Texas

1993 – 1998   Graduate Research Assistant, Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

1998–present Postdoctoral Fellow, Department of Cancer Biology, Molecular Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Honors: James W. McLaughlin Predoctoral Fellowship at The University of Texas Medical Branch

Publications:

1.   **Deng, J.,** and Du, P. Elimination of DNA in Namalva interferon preparation by antibody affinity chromatography. J. Med. Col. PLA 1:151-153, 1985

2.   **Deng, J.,** and Du, P. Purification of human alpha interferon by antibody affinity chromatography. J. Med. Co. PLA 2:1-5, 1987.

3. **Deng, J.,** and Fleischmann, W.R. Determination and partial characterization of an inhibitor of interferon action. M.S. Thesis. The University of Texas Medical Branch, Galveston, TX, 1991

4. Ren, Y., Walker, C., Loose-Mitchell, D.S., **Deng, J.,** Ruan, K.H., and Kulmacz, R.J. Topology of prostaglandin H synthase-1 in the endoplasmic reticulum membrane. Arch. Biochem. Biophy. 323:205-214, 1995.

5. Shao, R.P., Karunagaran, D., Zhou, B.P., Li, K., Lo. S.S., **Deng, J.,** Chiao, P., and Hung, M.C. Inhibition of nuclear factor NF-kB activity is involved in E1A-mediated sensitization of radiation-induced apoptosis. J. Biol. Chem. 272:32739-32742, 1997.

6. Hunt, K.K., **Deng, J.,** Liu, T-J., Wilson-Heiner, M., Swisher, S.G., Clayman, G., and Hung, M.C. Adenovirus-mediated overexpression of the transciption factor E2F-1 induces apoptosis in human breast and ovarian carccinoma cell lines and does not require p53. Cancer Res. 57:4722-4726, 1997.

7. **Deng, J.,** and Hung, M.C. Mechanism of Ad5 E1A-mediated tumor suppression and E1A-mediated apoptosis. Ph.D. Thesis. The University of Texas Health Science Center at Houston, Houston, TX, 1998.

8. **Deng, J.,** Xia, W., and Hung, M.C. Adenovirus 5 E1A-mediated tumor suppression associated with E1A-mediated apoptosis in vivo. Oncogene 17:2167-2175, 1998.

9. Wen, Y., Yan, D-H., Spohn, B., **Deng, J.,** Lin, S.Y., and Hung, M.C. Tumor suppression and sensitization to tumor necrosis factor α-induced apoptosis by an interferon inducible protein, p202, in breast cancer cells. Submitted, 1999.

10. **Deng, J.,** Zhang, H., Kloosterbore, F., Liao, Y., Klostergaard, J., Levitt, M.L., and Hung, M.C. Ultraviolet-induced apoptosis is independent of the ceramide pathway. Submitted, 1999.

The regulatory roles of NF-kB on cyclin D1 overexpression and tumor progression in human breast/ovarian cancers

Postdoctor: Jiong Deng, Ph.D.; Supervisor: Mien-Chie Hung, Ph.D.

ABSTRACT

Amplification and overexpression of cyclin D1 is involved in tumor development of human breast cancers. However, the mechanism of cyclin D1 overexpression is not completely clear. In study of adenovirus E1A-mediated functions, we found that cyclin D1 gene expression was greatly suppressed in all E1A transfectants of different cancer cell lines, which correlates with E1A-mediated tumor suppression *in vivo*. Importantly, this activity is likely to go through E1A-mediated down regulation of NF-kB. We found, by both transient and stable transfection assays, that E1A suppressesed cyclin D1 and NF-kB, and that NF-kB could stimulate cyclin D1 expression. The observation is important since it not only reveals the mechanism of E1A-mediated tumor suppression, but also the mechanism of cyclin D1 overexpression. To further investigate the clinic significance of this observation, we will examine the status of p65 (RelA) and cyclin D1 of a large number human breast tumor tissues by *in situ* immunohistostaining assay that has been shown to be technically feasible. Finally, because constitutive NF-kB activation is involved in oncogenesis of breast/ovarian cancers, targeting of NF-kB may therefore become a novel strategy for cancer therapy. To investigate the potential application value of this strategy, we examined the biological effect of NF-kB inhibitor on cancer cells both *in vitro* and *in vivo*. Treatment of SKOV3 and MCF7 cells with NF-kB inhibitor, sodium salicytate, resulted in suppressed cyclin D1 *in vitro*, which correlates with a suppressed tumorigenicity *in vivo* by an *ex vivo* study. This result suggests that targeting NF-kB may be an effective strategy for cancer therapy. Taken together, we propose to three specific aims in this study: i) to study the mechanism of E1A-mediated suppression of oncogenesis; ii) to study the mechanism of NF-kB-mediated cycln D1 upregulation and to investigate the clinic significance; and iii) to study the potential application of targeting NF-kB as an effective strategy for cancer therapy.

Curriculum Vitae

Kristine Sue Klos

MD Anderson Cancer Center
Department of Surgical Oncology Box 107
1515 Holcombe Blvd.
Houston, TX 77030
713-794-1233
kklos@gsbs3.gs.uth.tmc.edu

Education:

University of Minnesota B.S. Biology   1990-1995

University of Texas-Houston Health Science Center   1998-
     Graduate School of Biomedical Sciences
     Advisor: Dihua Yu, M.D., Ph.D.
     (M.S.)Ph.D. Program

Training:

University of Minnesota

Directed Research   1994-1995
     Advisor: Robert G. McKinnell, Ph.D.
     Thesis: Heart Primordia Transplants in Amphibian Embryos
     Presentations: Poster at the "College of Biological Sciences
            Undergraduate Research Symposium"

Junior Scientist   1996-1998
     Genetics and Cell Biology Department
     R.G. McKinnell Laboratory
       enucleated amphibian oocytes for nuclear transplantation of
       R. pipiens renal carcinoma cells, transplanted frog embryo
       tissues, tissue culture, autopsied frogs with physical deformities,
       fieldwork in MN frog population studies
     Presentations: *Frog Deformities*   1997
            Blessed Trinity Middle School Richfield, MN
            Hot Topics in Science: Cloning and Frog Deformities   1997
            Valley View Middle School Edina, MN

University of Texas - GSBS

Tutorials   1998-1999
    Advisors: Dr. Elizabeth Grimm – Cancer Biology
                    Immunohistochemistry of melanoma cells
               Dr. Dihua Yu – Surgical Oncology
                    ErbB2 expression in breast cancer cells
               Dr. Sharon Roth – Biochemistry and Molecular Biology
                    Histone deletions and the cell cycle in yeast

Other Experience:

University of Minnesota

Teaching Assistant   1997-1998
    Undergraduate Cancer Biology Course

Administrative Assistant   1997-1998
    International Society of Differentiation

Abstracts:

McKinnell, R.G., Klos, K., Christ, C., Rollins-Smith, L.A., and Kowles, R.
"Functional hearts derived from mitotic progeny of a renal carcinoma genome."
Proceedings of the American Association for Cancer Research. Volume 37
March 1996

ABSTRACT

The *erb*B2 (or HER2, *neu*) gene-encoded receptor tyrosine kinase (RTK) belongs to the EGF receptor family. ErbB2 overexpression was previously shown to correlate with poor prognosis and the number of lymph node metastases in breast cancer patients. Previously, our laboratory found that stable transfection of the human *erb*B2 gene into the low *erb*B2-expressing MDA-MB-435 human breast cancer cells (named 435.eB transfectants) enhanced the intrinsic metastatic potential of these cells. Contrary to the well established notion that *erb*B2 enhances metastasis of breast cancer cells, little is known about its underlying molecular mechanisms. Since erbB2 overexpression has been found in ~ 30% of breast tumors, it is very important to examine the molecular mechanisms underlying erbB2-mediated metastasis.

Our laboratory has found that erbB2 overexpression in 435.eB transfectants led to increased cell invasion, matrix metalloprotease (MMP)-2/MMP-9 activities, vascular endothelial growth factor (VEGF) secretion, and apoptosis resistance. We hypothesize that erbB2 overexpression can enhance the RTK signaling capacity that elicits these effects, which all can contribute to enhanced metastatic potential. Recent studies indicate that angiogenic responses are very important in promoting cancer metastasis. This fellowship application focuses on experimental approaches to elucidate the requirement of erbB2 receptor signaling leading to the angiogenic effect of erbB2. Using various mutants of the erbB2 receptor, I will determine the structural requirements of the tyrosine kinase domain and tyrosine autophosphorylation sites in the erbB2 receptor for mediating signals leading to cell invasion, MMP-9 activity, VEGF secretion, angiogenesis (MMP-9 and VEGF are known to facilitate angiogenesis), and ultimately, metastasis in an animal model (mammary fat pad injection of various erbB2 mutant transfected MDA-MB-435 cells) of breast cancer metastasis. I will also investigate the involvement of erbB2 downstream signaling in the angiogenic effects of erbB2 by biochemical and genetic manipulations of the signaling molecules in breast cancer cells.

The completion of the experiments outlined in this application will provide the first detailed analysis of the relationship between erbB2 signal transduction and angiogenic effects in human breast cancer cells, and ultimately will identify critical points in the signaling pathways for future intervention of erbB2-mediated metastasis.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel listed on the budget page for the initial budget period			
NAME Keng-Li Lan	**POSITION TITLE** Postdoctoral Fellow		
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)			
INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
National Yang-Ming University, Taipei City, Taiwan	M.D.	1985-1992	Medicine
University of Michigan, Ann Arbor, MI	Ph.D.	1994-1999	Pharmacology
The University of Texas M. D. Anderson Cancer Center, Houston, Texas	Postdoc	1999-present	Molecular Cell Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 3 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Professional Experience:

1990-1992   Intern Doctor, Taipei and Taichung Veterans General Hospital, Taipei and Taichung, Taiwan

1992-1994   Resident Doctor, Department of Internal Medicine, Chu-Tong Veterans Hospital, Hsin-Chu, Taiwan

1994-1999   Graduate Student Research Assistant, Department of Pharmacology, University of Michigan, Ann Arbor, MI; Mentor: Dr. Richard Neubig

1999-present   Postdoctoral Fellow, Department of Cancer Biology, Section of Molecular Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX

Membership and Honor

1998   American Society of Pharmacology and Experimental Therapeutics Travel Award
1998-present   American Society of Pharmacology and Experimental Therapetuics

Publications:

1.   **Lan, K-L.**, Remmers, A.E., and Neubig, R.R. Roel of Gαo tryptophan in GTP hydrolysis, GDP release and fluorescence signals. Biochem. 37(3):837-843, 1998..

2.   **Lan, K-L.**, Sarvazyan, N.A., Taussig, R., Mackenzie, R.G., Dohlman, H., and Neubig, R.R. A point mutation of Gαo and Gαi blocks interaction with RGS proteins. J. Biol. Chem. 273:12794-12797, 1998.

3. Neubig, R.R., **Lan, K-L.**, Chung, D.A., and Wade, S.M. Inverse agonist activity at the α_{2A}-Adrenergic receptor. Molec. Pharmacol. in press, 1999

4. Wade, S.M., Lim, W.K., Lan, **K-L.**, Nanamori, M., and Neubig, R.R. Gi activator region of α_{2A}-Adrenergic receptor. J. Biol. Chem. submitted, 1999.

5. **Lan, K-L.**, Nanamori, M., and Neubig, R.R. Spectroscopic and biochemical studies of RGS mechanism and specificity. Biochemistry, submitted, 1999

6. Neubig, R.R., and **Lan, K-L**. Magnesium dependent Gα activation. In preparation, 1999.

ABSTRACT

Breast cancer accounts for about 180,000 new cancer cases a year and is one the major causes of death for women in the United States. The multi-steps mechanisms leading to mammary carcinogenesis is not yet fully understood, however, studies support the critical roles of estrogen and concogenes, such as Her-2/neu encoding a tyrosine kinase receptor, in the promotion and development of breast cancer. Tyrosine kinase has been implicated in the estrogen pathway. To study the involvement of tyrosine kinase pathway in estrogen receptor signaling, emodin, a tyrosine kinase inhibitor, was used to treat an estrogen receptor-positive breast cancer cell line, MCF-7. Our data showed that emodin inhibited estrogen-induced mitogenic activity and Rb phosphorylation which were accompanied by depletion of the estrogen receptor. Two other tyrosine kinase inhibitors, RG13022 and genistein, could also deplete the receptor. The decrease in receptor protein was due to enhanced degradation. To examine the mechanism involved, inhibitors of the lysosomal, calpains, and proteasome proteolytic pathways were used. Only proteasome inhibitors blocked emodin-induced depletion of the estrogen receptor indicating the involvement of proteasome pathway.

It has been known that tyrosine residue at 537 of estrogen receptor can be phosphorylated and is important for receptor dimerization and DNA binding. Our studies raised an interesting question that whether tyrosine phosphorylation (such as tyr 537) would be important in regulating the stability of the receptor. Hsp90 is known to play a role in protein degradation. It is also known that hsp90 can form complex with the estrogen receptor, therefore, we will also examine whether emodin could affect this complex formation.

Taken together, our results demonstrated that tyrosine kinase(s) may be involved in the regulation of estrogen receptor and emodin can deplete estrogen receptor through the proteasome pathway, thereby blocking estrogen-induced biological function. Based on our preliminary results, I proposed the following three specific aims to help to understand the cross-talk between receptor tyrosine kinase and estrogen receptor pathways and to develop a potential chemopreventive agent targeting both estrogen and receptor tyrosine kinase pathways. Aim 1: To determine the biochemical and functional properties of tyrosine mutant estrogen receptor. Aim 2: To examine the mechanism of estrogen degradation mediated by tyrosine kinase inhibitor and to define the role of hsp90 in estrogen receptor degradation in the presence of tyrosine kinase inhibitors. Aim 3: To test the potential to use emodin as a chemopreventive agent in transgenic mice model (MMTV-neu).

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel listed on the budget page for the initial budget period	

NAME Yong Liao	POSITION TITLE Postdoctoral Fellow

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
North Sichuan Medical College, Sichuan, P.R. China	M.D.	1981-1989	Medicine
Xinjiang Medical College, Xinjiang, P.R. China	M.Sc.	1989-1992	Medicine
Shanghai Medical University, Shanghai, P.R. China	Ph.D.	1992-1995	Oncology
The University of Texas M. D. Anderson Cancer Center, Houston, Texas	Postdoctoral Fellow	1997-pres	Molecular Cell Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 3 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Professional Experience:

1981-1984   Undergraduate, Department of Medicine, North Sichuan Medical College, Sichuan, P.R. China
1984-1989   Resident, Department of Surgery, Zhongjiang County Hospital, Sichuan, P.R. China
1989-1992   M.Sc. in Medicine, Department of Surgery, 1st Affiliated Teaching Hospital, Xinjiang Medical College, Xinjiang, P.R. China
1992-1995   Ph.D. in Oncology, Liver Cancer Institute, Shanghai Medical University, Shanghai, P.R. China
1995-1996   Researcher, Liver Cancer Institute, , Shanghai Medical University, Shanghai, P.R. China
1997-present   Postdoctoral Fellow, Section of Molecular Cell Biology, Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Honors and Awards:

1993   Winner of "Guang Hua Scholarship" due to excellent credits
1994   Excellent Graduate Student recognized by the Graduate School, Shanghai Medical University
1995   Seed Grant for Gene Therapy, Shanghai Medical University
1995   Excellent Paper certified by the Shanghai Anti-Cancer Society during the Third Meeting of the Shanghai Anti-Cancer Society, Shanghai, 12/1/95
1995   2nd Price in Science & Technology Progress Awards of the Ministry of Health P.R. China, and 1st Prize in Science & Technology Progress of Xinjiang Autonomous Government, Xinjiang, P. R. China
1996   2nd Prize in Achievements in Liver Cancer Research awarded by the Funds of Zhao-You Tang & Reconstruction Bank, Shanghai, P.R. China
1996   1st Prize in Science & Technology Progress Awards of Shanghai, Shanghai Municipality, Shanghai, P.R. China
1996   Grand Prize in "8.5" National Key Project Achievement of Science & Technology awarded by the National Committee of Science & Technology, P.R. China
1992-1997   Seed Grant for Gene Therapy, Shanghai Medical University.

Functional Domains of Adenovirus Type 5 E1A Critical for E1A Tumor Suppression and Sensitization to Taxol Induced Apoptosis in Breast Cancer Cells

Abstract

The adenoviral E1A protein was originally recognized as one of the oncoproteins, which can cooperate with oncogenic ras or c-myc to transform rodent primary cells. However, so far there is little or no evidence to suggest that E1A can transform human cells. In fact, recent publications from our group and several others' suggest that E1A has strong tumor suppressor activity in human breast cancer, ovarian cancer, melanoma and fibrosarcoma cell lines. In addition, it was reported that overexpression of E1A protein in human fibroblast cells induced p53-dependent as well as p53-independent apoptosis. We have also found that introducing E1A protein into human breast or ovarian cancer cells could dramatically sensitize γ-radiation, TNF-α and Taxol cytotoxicity, although the mechanism underlying this effect is unclear. **Recently, a phase I E1A gene therapy protocol for human breast and ovarian cancers has been completed in M.D. Anderson Cancer Center and a phase II study is underway. Therefore, understanding the molecular mechanisms of adenoviral E1A pro-apoptotic function and anti-tumor activity is very important for future clinical trial.** To achieve this goal, a requisite first step is to systematically analysis of how might different functional domains of adenoviral E1A affect cellular functions in human cancer cells, both in cell culture in vitro and in animal models in vivo. We established a serial of E1A functional domain deletion mutation stable cell lines in human breast cancer MDA-MB-231 cells. Our preliminary results from in vitro cell culture study on the different E1A functional domain deletion mutation stable cells indicate that either CR1 domain (p300 binding site and weak pRB binding site) or CR2 domain (pRB binding site) is required for adenoviral E1A tumor suppressive activity, while N-terminal domain (p300 binding site) is dispensable. In addition, we found that CR2 domain is also required for E1A sensitization to Taxol induced apoptosis, while N-terminal or CR1 domain is dispensable. Based on our preliminary results, we hypotheses that adenoviral E1A CR1 and CR2 domains may have differential functions in E1A pro-apoptotic activity and anti-tumor effect. To test our hypotheses, **three specific aims are proposed: a) Specific Aim 1 will extend our study to further confirm whether CR1 domain is also critical for adenoviral E1A tumor suppressive activity in nude mice in vivo. b) If it is true, in Specific Aim 2, we will analysis the molecular genetic basis for E1A tumor suppression, one of the direct approaches is to test whether CR1 domain of E1A through binding with p300 and pRB could directly or indirectly recruit HDAC-1 (histone deacetylase) and repress VEGF transcription. c) In Specific Aim 3, we will investigate whether E1A pro-apoptotic function is through CR2 domain to disrupt pRB-E2F-1 complex and release of free E2F-1.** If it is the case, we will then test whether dominant negative E2F-1 could block E1A pro-apoptotic effect.

The proposed study may not only improve our understanding of the mechanisms underlying E1A pro-apoptotic effect and anti-tumor activity, but also help us to design alternative E1A constructs for cancer gene therapy in the future.

Trainee's Biosketch

NAME	POSITION TITLE
Soo-Jeong Lim, PhD	Postdoctoral fellow

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Seoul National University, Seoul, KOREA	B.S.	1989	Pharmacy
Seoul National University, Seoul, KOREA	M.S.	1991	Pharmaceutics
Seoul National University, Seoul, KOREA	Ph.D.	1996	Pharmaceutics
Seoul National University, Seoul, KOREA	Post-doc	1998	Drug Carriers

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Professional Experience

Sept. 96 – July 98   Postdoctoral fellow, Research Center for New Drug Development, Seoul National Univerisity, Seoul, KOREA

Mar 96 – June 98   Part time lecturer, Graduate School of Pharmacy, Kangwon National Univerisity, Chunchun, KOREA

Aug 98 – present   Postdoctoral fellow, Section of Immunobiology and Drug Carriers, Department of Bioimmunotherapy, U. T. M.D. Anderson Cancer Center, Houston, TX.

Publications

Chong-Kook Kim and Soo-Jeong Lim, Liposome immunoassay (LIA) with antigen-coupled liposome containing alkaline phosphatase. *J. Immunol. Methods*, 159, 101-106, 1993.

Chong-Kook Kim, Yun Jeong Choi, Soo-Jeong Lim, Myung Gull Lee, Sun Hwa Lee and Sung Joo Hwang, Lymph nodes targeting and pharmacokinetics of ^{3}H-methotrexate-encapsulatd neutral large unilamellar vesicles and immunoliposomes, *Int. J. Pharm.*, 98, 9-18, 1993.

Chong-Kook Kim, Eun-Bin Im, Soo-Jeong Lim, Yu-Kyung Oh and Seok-Kyu Han ,Development of the glucose-triggered pH-sensitive liposome for a potential insulin delivery. *Int. J. Pharm.*, 101, 191-197, 1994.

Chong-Kook Kim, Sang-A Ryuu, Kyung-Mi Park, Soo-Jeong Lim and Sung-Joo Hwang, Preparation and physicochemical characterization of phase inverted water/oil micromulsion contaning cyclosporin A. *Int. J. Pharm.*, 147, 131-134, 1997.

Soo-Jeong Lim and Chong-Kook Kim, Homogeneous liposome immunoassay for insulin using phospholipase C from *Clostridium perfringens*. *Anal. Biochem.* , 247, 89-95, 1997.

Soo-Jeong Lim, Eun-Ok Lee, Chong-Kook Kim, Liposome-based assay for phospholipase C. *Bull. Korean Chem. Society*, 18(7) 761-766, 1997.

Zhong-Gao Gao, Hee-Jong Shin, Kyung-Mi Park, Soo-Jeong Lim, Ki-Jun Hwang and Chong-Kook Kim, Physicochemical characterization and evaluation of microemulsion system for oral delivery of cyclosporin A, *Int. J. Pharm.*, 161, 75-86, 1998.

Patents

Invention: Formulation of drug- containing multilamellar microsphere
Inventors: Kim CK, Choi JY, Kong JY, Park KM, Hong MS, Choi HK, Lim SJ and Kim KM
KOREA Patent number: 95-15799
Date of Patent: June 20, 1995

Invention: New formulation for intravenous injection of fluorobiprofen
Inventors: Kim CK, Park KM, Jang JH, Lee MK, Lim SJ and Choi HK.
KOREA Patent number: 97-2834
Date of Patent: January 30, 1997

Invention: Long-circulating thrombi lytic agent prepared by incorporating streptokinase into liposomes
Inventors: Kim CK, Kim IS, Park KM, Lim SJ, Hong MS, Choi HG and Kim BK
KOREA Patent number: 97-2835
Date of Patent: January 30, 1997

Abstract

All-trans retinoic acid (ATRA) can inhibit the proliferation of some breast cancer (BC) cells and breast tumors. BC cells that are resistant to the growth-inhibitory effect of ATRA are generally estrogen receptor (ER)-negative and having lower expression of RARα mRNA and RARα protein. However, some BC cell lines are sensitive to ATRA despite being ER-negative whereas others are resistant to ATRA in spite of having significant levels of RARα proteins, suggesting that there are other mechanisms contributing to the regulation of ATRA sensitivity in BC cells.

Since the erbB receptor tyrosine kinase (RTK) family is known to play a vital role in the growth and progression of human BC, we speculated that they may also regulate the sensitivity of BC cells to ATRA. Indeed using a series of MCF-7 cell lines (parental, transfected with heregulin or erbB2, adriamycin-resistant) and T-47D cells in the presence or absence of erbB RTK-activating ligands, we were able to confirm that the activation of erbB RTKs is correlated with the induction of ATRA resistance in BC cells. In this proposal, the mechanisms by which the activation of erbB RTKs leads to resistance of BC cells to ATRA should be elucidated.

One possibility is that activated erbB RTKs can modulate the expression/function of RARα in BC cells. RARα is known to play a major role in mediating the growth inhibitory effects of ATRA in human BC cells. By northern and western blot, we will investigate whether the decrease in the ATRA-sensitivity is correlated with decreased RARα mRNA or protein expression in transfected and adaptive cell lines, and also in parental cells in which erbB RTKs were ligand-activated. Activated erbB RTKs in BC cells may induce ATRA resistance by impairing RAR function, i.e. its ability to transactivate retinoic acid responsive element (RARE) in target genes. Assessment of the RARE transactivation activity determined by transient transfection assay with RARE-luciferase reporter plasmid among series of cell lines will allow us to investigate this possible mechanism.

Another possibility is that activated erbB RTKs may enhance ATRA degradation, thereby reducing the availability of ATRA to RARα. This will be studied by comparing uptake and retention kinetics of ATRA. Since cytochrome P-450 enzyme is known to play a major role in ATRA degradation, we will also investigate whether ATRA-resistance is correlated with enhanced degradation of ATRA by cytochrome P-450 by comparing the growth inhibition without or with combination treatment of liarozole, one of potent cytochrome P-450 inhibitor.

Besides studying the mechanism, we will also examine whether blocking erbB RTK signaling can resensitize BC cells to ATRA. Activated erbB RTKs can transmit the growth signal by using adapter proteins, such as growth factor receptor-bound protein 2 (Grb2). We have shown that Grb2 protein downregulation by liposomal Grb2 antisense oligodeoxynucleotides (L-Grb2) can block specific erbB RTK signalings including mitogen-activated protein kinase (MAPK) or Akt pathway, leading to the growth inhibition of three different types of BC cells (EGFR-, erbB2-, or HRG-overexpressing). In this study, we will investigate whether the erbB RTK blockade by L-Grb2 can resensitize cells to ATRA. If so, we will then study how Grb2 protein down-regulation can reverse these ATRA resistance mechanisms.

All of these studies will allow us to confirm our hypothesis that the activation of erbB RTKs can stimulate downstream signal transduction pathways, thereby inducing ATRA resistance in BC cells. Understanding the mechanisms by which activated erbB RTK induces ATRA-resistance in BC cells may help us design more successful BC therapeutics by combining ATRA with agents that can block erbB RTKs signaling.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel listed on the budget page for the initial budget period			
NAME Keishi Makino, M.D./Ph.D.		POSITION TITLE Postdoctoral Fellow	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)			
INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Kumamoto University School of Medicine, Japan	M.D.	1990	Medicine
Graduate School, Kumamoto University School of Medicine	Ph.D.	1998	Molecular Biology
The University of Texas M. D. Anderson Cancer Center	Postdoctoral Fellow	1998	Molecular Cell Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 3 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Professional Experience:

1990-1992   Resident, Department of Neurosurgery, Kumamoto University School of Medicine, Japan

1998-present: Postdoctoral Fellow, Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX

Publications:

1.   **Makino, K.**, Murakami, M., Kitano, I., and Ushio, Y. Primary intracranial plasma cell granuloma: a case report and review of the literature. Surg. Neurol. 43:374-378, 1995.
2.   **Makino, K.**, Kuwahara, H., Masuko, N., Nishiyama, Y., Morisaki, T., Sasaki, J., Nakao, M., Kuwano, A., Nakata, M., Ushio, Y., and Saya, H. Cloning and characterization of NE-dlg: a novel human homolog of the Drosophila discs large (dlg) tumor suppressor protein interacts with the APC protein. Oncogene 14:2425-2433, 1997.
3.   Masuko, N., **Makino, K.**, Kuwahara, H., Fukunaga, K., Sudo, T., Araki, N., Yamamoto, H., Yamada, Y., Miyamoto, E., and Saya, H. Interaction of NE-dlg/SAP102, a neuronal and endocrine tissue-specific MAGUK protein, with calmodulin and PSD-95/SAP90: a possible regulatory role in molecular clustering at synaptic sites. J. Biol. Chem. 274:5782-5790. 1999.
4.   Kuwahara, H., Araki, N., **Makino, K.**, Honda, S., Kaibuchi, K., Fukunaga, K., Miyamoto, E., Ogawa, M., and Saya, H. A novel NE-dlg/SAP102-associated protein, p51-nedasin, related to the amidohydrolase superfamily, interferes with the association between NE-dlg/SAP102 and NMDA receptor. J. Biol. Chem. in press, 1999.
5.   Nishiyama, Y., Hirota, T., Morisaki, T., Hara, T., Marumoto, T., Iida, S., **Makino, K.**, Yamamoto, H., Hiraoka, T., Kitamura, N., and Saya, H. A human homolog of Drosophila warts tumor suppressor, h-warts, localized to mitotic apparatus and specifically phoshporylated during mitosis. FEBS Lett. in press, 1999.
6.   **Makino, K.** and Hung, M-C. Identification and Characterization of a Taxol induced gene; Taxol activation of the cell cycle protein p55Cdc promoter. in preparation, 1999.

Abstract

Breast cancer is one of the major causes of morbidity and mortality in women in the United States. Each year, more than 180,000 new cases of breast cancer are diagnosed in this country, and 46,000 women will die due to this malignancy. Paclitaxel (Taxol) is a potent and highly effective antineoplastic agent for the treatment of advanced, drug-refractory, metastatic breast cancers. Taxol induces tubulin polymerization and microtubule formation, blocks the cell cycle in mitosis, and induces apoptosis. Furthermore, an another effect of Taxol is to alter gene expression. In murine macrophages, Taxol can induce the expression of a series of lipopolysaccharide-inducible cytokines, such as IL-1α, IL-1β, TNF-α, and interferon-inducible protein 10. In human monocytes, Taxol also induces cytokine synthesis. However, the molecular pathway of Taxol-mediated gene expression and cytotoxicity remains to be elucidated. Thus, identification and characterization of changes in the gene expression profile in response to Taxol treatment does not only lead to a better understanding of these mechanisms, but also aids in designing novel therapeutic approaches. In this proposal, we plan to study the gene expression profile with Taxol treatment by using the cDNA microarray technique.

Stephanie Miller

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel listed on the budget page for the initial budget period

NAME Stephanie Miller	POSITION TITLE Graduate Research Assistant

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Indiana University, Bloomington, IN	B.S.	1997	Biology
The University of Texas M. D. Anderson Cancer Center, Houston, TX	Predoc.	1998	Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 3 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Professional Experience:

1996 – 1997   Undergraduate Research, Department of Molecular Biology, Indiana University, Indiana

1997-present   Graduate Research Assistant, Department of Cancer Biology, Section of Molecular Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Honors: Spring 1995, Dean's List; Fall 1996, Dean's List; Spring 1997, Dean's List

Publications:

1. Zhou, B.P., Hu, MC-T., **Miller, S.**, Yu, Z., Xia, W., Lin, S-Y., and Hung, M-C. Her-2/neu blocks TNF-induced apoptosis via the Akt/NF-κB pathway. Submitted to Science, 1999.

Abstract:

HER-2/neu oncogene is overexpressed in many cancers, including breast cancer. It correlates with poor prognosis and chemoresistance. Therefore it is an important target for therapy. It is known to activate many different signaling pathways, which lead to growth and survival. However the mechanism of Immortalization in these cells is unknown. One molecule known to be involved in Immortalization is telomerase. This enzyme is involved in maintenance of telomere, which naturally shorten through replication. Shortening of telomeres leads to senescence, and telomerase is a way for the cell to escape senescence, thus extending the life of the cell, and the number of replications the cell goes through. I propose that telomerase is the mechanism in which HER-2/neu overexpressing cells are immortalized. I plan to determine that HER-2/neu can activate the telomerase catalytic subunit(hTERT) promoter, increasing the amount of mRNA transcribed to increase telomerase activity in breast cancer. I will determine the mechanism in which this occurs by looking at the different pathways in which HER-2/neu is known to be involved it. I will also determine the promoter region that is responsible for this action. This will help elucidate the mechanism in which HER-2/neu can lead to Immortalization of cells, which will help in treatment of HER-2/neu overexpressing breast cancer.

Biographical Sketch

Rhonda J. Moore

Present Address:
7326 Staffordshire # 4
Houston, TX 77030
(713) 797-1404

Work Address:
Department of Epidemiology, Box 189
MD Anderson Cancer Center
1515 Holcombe Blvd.
Houston, TX 77030
(713) 792-4650
Email: rjmoore@leland.stanford.edu;
rhmoore@notes.mdacc.tmc.edu

EDUCATION:

1998-

THE UNIVERSITY OF TEXAS M. D. ANDERSON CANCER CENTER, Houston, TX
- Post-Doctoral Fellow in Epidemiology
- Research on the cultural experience of pain in early and late stage survivors of upper areodigestive tract cancers and ovarian cancer
- Issues include racial, ethnic and gender differences in health, social support, suffering, pain and culture.

1996-1998

STANFORD UNIVERSITY, Stanford, CA.

- Fellow in Psychiatry
- Research on cultural differences in breast cancer
- Issues include spirituality, culture, race, social support, gender and health.

1990-1997

STANFORD UNIVERSITY, Stanford, CA.

- Ph.D. in Cultural Anthropology (Conferral date: 1/97)
- MA degree in Cultural Anthropology (6/91)
- Courses taken include pain and emotion, race, social support, health, organizational cultures, gender and sexuality.

1984-1988

NORTHWESTERN UNIVERSITY, Evanston, Ill.

- BS Program in Performance Studies (6/88)
- Courses include performance studies, interpersonal communications, drama, and comparative literature.
  Minors in Art (lithography and silk-screen) Comparative Literatures (African and Japanese Literatures) and Existential Philosophy.

EXPERIENCE:

RESEARCH EXPERIENCE:

1998-

UNIVERSITY OF TEXAS M. D. ANDERSON CANCER CENTER

POST-DOCTORAL RESEARCH, EPIDEMIOLOGY DEPT.

- Conceived pilot study describing the experiences of pain and suffering in African-American and White early and late stage head and neck and lung cancer survivors

- Developed pilot study on Risk and Quality of Life in African-American and WhiteOvarian Cancer Survivors

- Implemented qualitative research design

- Collected in-depth interviews

- Negotiated use of clinic visits.

	• Field research on cultural differences on health outcomes in African-American and White American lung, head and neck, and ovarian survivors with primary and metastatic cancers.
1996-1998	STANFORD UNIVERSITY

POST-DOCTORAL RESEARCH, PSYCHIATRY DEPT.
- Conceived independent research project on the relationships between spirituality, social support, gender and cancer progression in African - American Women
- Implemented qualitative research design
- Collected in-depth interviews
- Negotiated use of field sites and field visits
- Field research on cultural differences amongst African-American and White women with primary and metastatic breast cancer.

1990-1997   **STANFORD UNIVERSITY**
DOCTORAL RESEARCH, ANTHROPOLOGY DEPT.
- Dissertation: Bonding With Pain: An Ethnographic Study of Identity, and the Cultural Construction of Belonging in the United States Marine Corps
- Through negotiations designed independent research project
- Reviewed literature on emotion, identity, social support and ritual
- Implemented research design
- Collected interviews from enlisted personnel and officers, completed focus groups and analyzed data
- Negotiated use of field sites and field visits
- Field Research on the USMC.

MA PROGRAM, ANTHROPOLOGY DEPT. (STANFORD)
- Master's thesis: Nationalism and the Public Sphere in the Proslavery Writings of George Fitzhugh (1850-1860) [completed 6/91]
- Successfully analyzed historical data
- Gained significant writing and research experience.

DANFORTH FELLOWSHIP FOR SUMMER RESEARCH (STANFORD) (summer 1991 and 1992)

- Predoctoral research on the US Army (summer 1991)
- Prepared research paper on National Service (summer 1992)
- Developed efficient research methods and research agendas
- Sharpened interviewing skills.

TEACHING EXPERIENCE: **LECTURER**
1988 - present   **Stanford University**, Anthropology Department (6/97-8/97)
- The Body, Health and Illness: Perspectives in Medical Anthropology
San Francisco State University, Anthropology Department (1/97-5/97)
- Contemporary Perspectives on American Racism

TEACHING ASSISTANT, Stanford University
        (1/91-4/92)
- Introduction to Afro-American Studies
- Medical Anthropology.

LECTURER (GUEST)
- Introduction to Afro-American Studies at Stanford (5/95)
- Introduction to Cultural Anthropology at SF State (11/96).

CONSULTING EXPERIENCE:

1992-   **RAND CORPORATION, SUMMER INTERN PROGRAM**
        SANTA MONICA, CA (summer 1994)

- Summer Consultant to project for RAND/ Office of the Secretary of Defense. Paper and Presentation entitled: Keep on Keepin' On: the Cultural Effects of Exclusion from Social Network: Implications for Women and Minority Officers in the US Military
- Conducted research and informal interviews
- Analyzed and synthesized relevant materials
- Presented findings to social scientists and peers.

STANFORD ATHLETIC DEPARTMENT, STANFORD UNIVERSITY (5/94-6/94)

- Helped facilitate focus groups and interviews
- Observed interview process
- Served as a notetaker.

STANFORD COMMUNITY PARTNERSHIP FOR ALCOHOL AND OTHER DRUG ABUSE PREVENTION, COWELL STUDENT HEALTH CENTER, STANFORD UNIVERSITY (4/94-5/94)

- Taught interview methodology
- Developed and suggested qualitative methodologies.

COUNCIL FOR UNDERGRADUATE EDUCATION, STANFORD UNIVERSITY (4/94-5/94)

- Served as a notetaker
- Observed interview process.

PROJECT ANALYST, CONFERENCE FOR COMMUNITIES ON NATIONAL SERVICE, WASHINGTON, DC. (4/92-6/92)

- Analyzed, proofread and assessed proposals for government funding
- Participated in debates
- Recommended proposals for government funding.

FELLOWSHIPS:

1988-present

POST-DOCTORAL FELLOWSHIPS

- NCI Cancer Prevention and Education Post-Doctoral Fellowship, Department of Epidemiology, MD Anderson Cancer Center (1/98-)

- NIH Minority Supplemental Grant, Psychiatry Department, Stanford University Medical Center (9/96-1/98).

DISSERTATION FELLOWSHIPS
- Mellon Dissertation write-up Grant (9/94-6/95)
- Stanford University Fellowship (9/90-6/94)
- Danforth Fellowship for Summer Research and Study (Summer 1991 and Summer 1992).

PRESENTATIONS:

1996-

- Bonding with Pain: the culture of pain in the US Marine Corps. Department of Psychiatry and Behavioral Sciences, Stanford Medical Center. Stanford, CA. September 1996.

- African-American Women, the Media and Disordered Eating: A critical review. American Association for Anthropologists. San Francisco, CA, November 1996.

- Black Women and Breast Cancer: what we know and what we don't. Northern California Cancer Center. Union City, CA, August 1997
- Black Women and Breast Cancer: What's Social Support got to do with it? American Association for Anthropologists. Washington, DC. November 1997.
- Differential mortality in African-American Women and White women with Breast Cancer in the US. International Union against Cancer (UICC) Rio De Janeiro, Brazil. August 1998.
- Paradoxes in the Cultural Narration of Pain in the Late Stages of Head and Neck Cancer. Division of Surgery and Head and Neck Oncology, UT MD Anderson Cancer Center, Houston, TX, September 1998.
- Historicizing Cancer risk factors—Minorities and Cancer. PHNMA. Houston, TX November 1998.
- Narrating Breast Cancer, Narrating Race. American Association for Cancer Education. Portland, Oregon, November 1998.
- Cultural Encounters with Pain in White and African-American early stage Head and Neck Cancer Patients in the US: Discourse of Survival. American Anthropological Association, Philadelphia, PA, December 1998.
- Narrating Pain and Culture in the Early Stages of head and neck cancer. 9th world Congress on Pain, Vienna, Austria, August 1999.
- Narratives of time and the experience of social suffering in African-American and White American advanced lung cancer survivors American Anthropological Association. November 1999.

Abstracts accepted: 1999

- Culture and the Pain Experience in early stage Lung Cancer Survivors. Pain in the Mediterranean Corner. HIT (Hellenic, Israeli, Turkish) Congress on Pain. Heraklion, Greece. March 1999.

- Narrating Culture and Pain in the Early Stages of Head and Neck Cancer. International Association for the Study of Pain. Vienna, Austria. August 1999.

- Narrating the cultural experience of time and social suffering in African-American and White American advanced lung cancer survivors. American Association of Anthropologists. Chicago, Ill. November 1999. (Chair of panel: Narratives of Sickness).

Meetings Attended: 1999

- 9th World Congress on Pain. International Association for the Study of Pain. Vienna, Austria. Aug 1999
- Ovarian Cancer National Alliance meeting: silence no more. Washington, DC, Sept 1999.
- American Association for Anthropologists. Chicago, Ill. November 1999.
- The World Foundation for Pain relief and Research, Inc. Current Concept in Acute, Chronic, and Cancer Pain Management. New York, December1999,

SUBMISSIONS/ PUBLICATIONS:

- Imagery and Hypnosis in the Treatment of Cancer Patients (With Dr. David Spiegel, MD.) In Oncology, 11:8:1179-1190.

- Black Women and Breast Cancer in the US. In Preventing and Controlling Cancer (ed. D. Weiner et al). Denver, Co: Greenwood Press, 1999 (in press).
- The Uses of Guided Imagery for Pain Control by African-American and White Women with Metastatic Breast Cancer (With David Spiegel, MD) Submitted to Integrative Medicine, 10/99.

- Suffering, the care of the self and a place in the text--- the ethical implications of excluding African-American breast cancer survivors. Submitted 09/99 to The Journal of the Medical Humanities.
- Narrating Pain and Culture in the Early Stages of head and Neck Cancer. (With Fadlo R. Khuri, MD). Submitted 10/99 to Qualitative Health Research.
- Conducting Ethnography in Applied Settings: from Cancer Prevention to Hi-Tech Environments (with Jared Braiterman, Ph.D.) (forthcoming).
- Transformation through Pain: an ethnographic study of gender and the cultural construction of social suffering in the United States Marine Corps (submitted to Stanford University Press for review).

References available upon request
C:\WINDOWS\DESKTOP\CV--1999.doc

Familial Breast Cancer:
A Study of Risk and Quality of Life in African-American and Jewish American Breast and Ovarian Cancer Survivors."

Abstract:

Risk Factors for ovarian cancer after breast cancer, and the effects of these diseases on the subsequent quality of life of cancer survivors vary by race, ethnicity, gender, age, and class, including access to adequate medical care. Nevertheless, there have been no studies to date that have combined qualitative and quantitative methods to examine the effects of being high-risk for breast and ovarian cancer on the quality of life of African-American and Jewish American survivors of ovarian cancer after breast cancer. The purpose of this two-part study is to first assess risk factors for ovarian cancer in a population of premenopausal African-American and Jewish American women who have already had breast cancer. The second part of this study is qualitative and descriptive and will evaluate the impact of familial breast and ovarian cancer on the experience of pain, suffering and quality of life of the women in this unique patient population.

Biographical Information: Fellowship Candidate

Ulrich W. Mueller, Ph.D.

BIRTHDATE   February 28, 1967

BIRTHPLACE   Germany

ADDRESS   Department of Biochemistry and Molecular Biology - 117
University of Texas M.D. Anderson Cancer Center
1515 Holcombe Boulevard
Houston, Texas 77030-4095
Telephone Number: (713) 794-1165
FAX Number: (713) 791-9478
E-mail: umueller@odin.mdacc.tmc.edu

CITIZENSHIP   Permanent Resident

EDUCATION   University of Texas M.D. Anderson Cancer Center, 1998-present
Postdoctoral Fellow
Department: Biochemistry and Molecular Biology

Baylor College of Medicine, 1991-1998
Ph.D. Degree, 1998
Department: Cell and Molecular Biology

Texas A&M University, 1989-1991
Master of Science Degree, 1991
Department: Biology

New Mexico State University
Bachelor of Science Degree, 1988
Department: Microbiology

AWARDS AND   Claude W. Smith Fellowship Award for Outstanding
FELLOWSHIPS   Research Performance
Baylor College of Medicine, 1994, 1995

Molecular and Cellular Biology Graduate Fellowship
Texas A&M University, 1989

Crimson Scholar

New Mexico State University, 1984-1988

RESEARCH EXPERIENCE

Department of Biochemistry
Baylor College of Medicine, 1991-1998

My dissertation project focused on evaluating the regulation of the mitosis to interphase transition using fission yeast as a model organism. Using a combination of genetic, biochemical, and cell biological techniques, I characterized a novel essential gene involved in coordinating cytokinesis with the proper progression of the cell cycle. Furthermore, I also analyzed the role of a highly conserved nuclear small GTP-binding protein in the mitosis to interphase transition. By mutational analysis I was able to identify sites on the GTPase essential for binding of effector molecules. These findings provided new means by which the molecular targets of this GTPase switch could be identified.

Department of Biology
Texas A&M University, 1989-1991

Both plants and photosynthetic bacteria contain genes that are either activated or inactivated by changes in light intensity. I studied the light-mediated transcriptional regulation of a gene family in cyanobacteria. Several regulatory factors were partially purified from cells grown under high light conditions that specifically bound the promoter region of a high-light inducible gene. By mobility shift and DNA footprinting methods, I was able to identify the sequences bound by these factors.

Department of Microbiology
New Mexico State University, 1987-1988

Several species of fish contain light organs which are filled with a single species of bacteria that produce light by bioluminescence. The nature of this symbiotic relationship is of particular interest. I studied how the *lux* operon which contains the genes that encode the enzymes for producing light is regulated in *Vibrio fisheri*. To do this, I performed a genetic screen for mutants that did not produce light and found that light production is regulated by the level of cAMP in the cells. Thus it appears that the fish can regulate the intensity of light produced in the light organs by altering the carbon source provided to the bacteria.

PUBLICATIONS

Mueller, U. W. and S. Sazer (1999) A *Schizosaccharomyces pombe* EH-domain protein involved in septation and cell fission. Mol. Biol. Cell (submitted).

Mueller, U. W., H. Ji, and P. D. McCrea (1998) Identification of novel transcriptional targets of β-catenin mediated signaling in MDCK cells. Mol. Biol. Cell 9(S): (poster abstract).

Matynia, A., U.W. Mueller, N. Ong, J. Demeter, A. L. Granger, K. Hinata, and S. Sazer (1998) Isolation and characterization of fission yeast *sns* mutants defective at the mitosis to interphase transition. Genetics **148**:1799-1811.

Mueller, U. W., S. S. Salus, D. Ensenat, and S. Sazer (1996) Isolation and characterization of three novel fission yeast cell-cycle mutants that are rescued by the spi1 nuclear GTP-binding protein. Mol. Biol. Cell 7(S): 97a (poster abstract) .

Matynia, A., K. Dimitrov, U. Mueller, X. He, and S. Sazer (1996) Perturbations in the spi1 GTPase cycle of *Schizosaccharomyces pombe* through its GAP and GEF components result in similar phenotypic consequences. Mol. Cell. Biol. **16**:6352-6362..

Noguchi, E., N. Hayashi, Y. Azuma, T. Seki, M. Nakamura, N. Nakashima, M. Yanagida, X. He, U. Mueller, S. Sazer, and T. Nishimoto (1996) Dis3, implicated in mitotic control, directly binds to Ran and enhances the GEF activity of RCC1. EMBO J. **15**:5595-5605.

Turi, T. G., U. W. Mueller, S. Sazer, and J. K. Rose (1996) Characterization of a nuclear protein conferring Brefeldin A resistance in *Schizosaccharomyces pombe*. J. Biol. Chem. **271**:9166-9170.

Ouspenski, I.I., U. W. Mueller, A. Matynia, S. Sazer, S. J. Elledge, and B. R. Brinkley (1995) Ran-binding protein-1 is an essential functional component of the Ran/RCC1 molecular switch system in budding yeast. J. Biol. Chem. **270**:1975-1978.

Li, R., N. S. Dickerson, U. W. Mueller, and S. S. Golden (1995) Specific binding of *Synechococcus* sp. PCC 7942 proteins to the enhancer element of *psbAII* required for high-light-induced expression. J. Bacteriol. **177**:508-516.

Mueller, U. W. and S. Sazer (1993) Identification of effector molecules that interact with a ras-like GTPase involved in regulating the exit of mitosis in fission yeast. Poster Abstract: FASEB Summer Research Conference: "Ras Superfamily of proteins".

Golden, S. S., R. D. Kulkarni, R. Li, M. R. Schaefer, S. A. Bustos, N. Tsinoremas, N. S. Dickerson, and U. W. Mueller (1993) Light-responsive regulation of cyanobacterial photosynthesis genes. *In* Industrial Microorganisms: Basic and Applied Molecular

Genetics. R. H. Baltz, G. D. Hegeman, and P. L. Skatrud, editors. American Society for Microbiology, Washington, DC.

Kulkarni, R. D., U. W. Mueller, and S. S. Golden (1993) Nucleotide sequence of *psbB* from *Synechococcus* sp. strain PCC 7942. Biochim. Biophys. Acta **1173**:329-332.

Dunlap, P. V., U. Mueller, T. A. Lisa, and K. S. Lundberg (1992) Growth of the marine luminous bacterium *Vibrio fischeri* on 3':5'-cyclic AMP: correlation with a periplasmic 3':5'-cyclic AMP phosphodiesterase. J. Gen. Micro. **138**:115-123.

Ulrich W. Mueller, Ph.D.

Abstract

Components of the Wnt/Wingless signal transduction pathway were initially identified as factors that play central roles in the development and differentiation of animal tissues. More recently the role of these factors in the development of cancer has become a major focus of research. Mutation of two central regulatory factors, APC and/or β-catenin, has been associated with the onset of cancer in several tissues types including those in the colon, liver, skin, breast, and kidney. In all cases, the molecular events preceding tumorigenesis are associated with the activation of specific transcriptional targets of this oncogenic pathway.

The primary goal of this research project is to identify novel transcriptional targets of this signal transduction pathway in a mammalian cell type. Mammalian kidney cell lines that stably express a dominant active form of β-catenin were isolated and characterized. Using these lines, a subtractive hybridization screen was performed to identify genes activated upon overexpression of β-catenin. A number of positive clones were isolated, identified, and are currently under further investigation. Preliminary analysis of these clones by Northern blotting and RT-PCR revealed which genes show increased expression upon β-catenin stimulation. Further work to test whether these clones are direct targets of the signaling pathway is currently in progress. One of the clones has been identified to encode cytokeratin 19 (K19). Identification of K19 as a possible β-catenin target is of particular relevance in breast cancer research. Historically, K19 overexpression has been found in breast cancer patients in whom the tumors had spread. Current efforts are underway to determine whether β-catenin can regulate K19 expression in a human breast cancer cell line.

Following characterization of target clones, the project will focus on isolating mouse and human homologs. Human clones will be used to analyze the expression patterns of these clones in primary cancer tissues. By identifying which clones are expressed at high levels in specific types of cancer, we will be able to define new markers to be used by physicians in the early diagnosis of possible cancers. Because activation of components of the Wnt signaling pathway is one of the earliest events in tumor progression, these markers will provide the greatest opportunity for physicians to prevent further progression of possible tumors. The mouse clones will become useful in development of transgenic experiments to further analyze the molecular events resulting from improper activation of the Wnt signaling pathway. The availability of tissue specific transgenic systems will allow us to focus on specific organs. For example, further analysis concerning the role of K19 in breast cancer can be explored using a mouse mammary model system.

Curriculum Vitae

Christopher L. Neal

Work Address: M D Anderson Cancer Center
Department of Surgical Oncology, Box 107
1515 Holcombe Blvd.
Houston, TX 77030

Work Phone: (713) 794-1233

E-mail Address: cneal@gsbs3.gs.uth.tmc.edu

Education:
1997-present   Ph.D. (M.S.), University of Texas-Houston Health Science
Center, Graduate School Of Biomedical Sciences,
Houston, TX 77054
Program: Cancer Biology
Advisor: Dihua Yu, M.D., Ph.D.

1991-1996   B.S., East Tennessee State University,
Johnson City, TN 37614
Major: Biology   Minor: Philosophy
GPA in Major: 3.37   Cumulative GPA: 3.17

Tutorials at UTH-GSBS:
Aug. 1997-Dec. 1997   Role of p16 promoter methylation in senescence
Department of Carcinogenesis,
Science Park Research Division
Advisor: Marcelo Aldaz, Ph.D.

Jan. 1998-Mar. 1998   Development of a novel gene therapy delivery system
Department of Surgical Oncology
Advisor: Dihua Yu, M.D., Ph.D.

Mar. 1998-May 1998   Role of 14-3-3 sigma (HMe1) in cell cycle regulation
Department of Surgical Oncology
Advisor: Mong-Hong Lee, Ph.D.

Research Honors at ETSU: Ronald McNair Research Program
Intern 1993-1994

James H. Quillen School of Medicine
Research Forum
2nd place Undergraduate Poster Division, 1994

<u>Honor Societies at ETSU</u>:   Tri-Beta Biological Society

<u>Research and Work Experience at ETSU</u>:

Sept. 1995-Aug.1997	Research Technician, NIH funded project Department of Internal Medicine, ETSU College of Medicine Advisor: Elaine Walker, Ph.D.
May 1995-April 1997	Research Technician, NIH funded project Department of Biological Science, ETSU Advisor: Foster Levy, Ph.D.
Aug. 1992-April 1997	Undergraduate research, projects funded by Ronald McNair Research Program and student worker program Department of Biological Sciences, ETSU Advisor: Foster Levy, Ph.D.

<u>Scientific Publications at UTH-GSBS</u>:

Abstracts:	Larango C, Neal CL, Lee MH. HMe1, a p53 inducible protein, plays a key role in cell cycle regulation. Society of Surgical Oncology, March 1999.
	Larango C, Neal CL, Lee MH. HMe1, a 14-3-3 family member, is a regulator of the cell cycle. Proc. Amer. Assoc. Cancer Res., April 1999.
Papers:	Larango C, Yang HY, Neal CL, Lee MH. Association of the cyclin-dependent kinases and 14-3-3 Sigma negatively regulates cell cycle progression (submitted May 1999)

<u>Scientific Publications at ETSU</u>:

Abstracts:	Neal C, Berk S, Holtzclaw-Berk S, Laffan E, Kalbfleisch JH and Walker ES. Temporal trends in antimicrobial resistance in a local population of Moraxella catarrhailis. Amer. Soc. Micro. 97:321, 1997.
Papers:	Levy F and Neal CL. Spatial and temporal genetic structure in chloroplast and allozyme markers in Phacelia dubia implicate genetic drift. Heredity 1999 Apr;82 Pt 4:422-31.
	Walker ES, Neal CL, Berk S, Holtzclaw-Berk S, Laffan E, Kalbfleisch JH. Trends in Susceptibility of Moraxella catarrhailis. J. Antimicrobial Chemotherapy. (manuscript in preparation)

Abstract

The 14-3-3 proteins are a family of ubiquitously expressed acidic proteins highly conserved through evolution and there are nine 14-3-3 isoforms in humans. 14-3-3 can bind to various phospho-serine containing target proteins involved in many important cellular processes. For example, 14-3-3 proteins are known to bind to c-Raf-1, MEKK, KSR, Cdc25, Bad, FKHRL1, p53, Bcr, and the middle T antigen of the polyoma virus. These target proteins are involved in regulation of cell growth, differentiation, apoptosis and transformation, respectively. Thus, 14-3-3 can participate in these important cellular processes. The most well studied 14-3-3 isoform, 14-3-3 sigma, has been indicated as a negative regulator of the cell cycle. Paradoxically, the less studied 14-3-3 zeta seems to function differently from 14-3-3 sigma. Recently, we inadvertently found that 14-3-3 zeta is overexpressed in >60% of human breast cancers versus autologous normal breast tissues. We also found that a dominant negative mutant of 14-3-3 zeta, 14-3-3C, impaired metastasis-related properties in HT-1080 cells. Thus, we hypothesize that overexpression of 14-3-3 zeta may facilitate breast cancer metastasis by regulating specific metastasis-related properties. This proposal will first determine whether 14-3-3 (14-3-3 will be used to denote 14-3-3 zeta, unless specified for other isoforms) promotes breast cancer tumorigenicity/metastasis. If so, we can develop reagents that block 14-3-3 functions. The specific aims of the proposal are:

1.	**To investigate the biological role of 14-3-3 in breast cancer cells in vitro.** First, we will transfect into MDA-MB-435 breast cancer cells HA-tagged full length 14-3-3 (FL), 14-3-3C mutant, 14-3-3 antisense (AS) constructs, along with vector controls to establish stable transfectants. Then, we will examine these transfectants for metastasis-related properties, and their growth in hard agar, which has previously been shown to correlate well with *in vivo* metastatic potential.
2.	**To determine the tumorigenic and metastatic potential of 14-3-3 transfectants in vivo.** We will compare the ability of the 14-3-3FL, 14-3-3C, 14-3-3AS transfectants to induce tumors and metastases in ICR-SCID mice. Metastasis assays will be accomplished by injecting cells into the tail vein of the mice. Tumorgenicity and spontaneous metastasis assays will be done by injecting cells to the mammary fat pad (m.f.p.) of the mice.

To the best of our knowledge, there is no previous report on 14-3-3 overexpression in breast cancers. Moreover, the *consequence of 14-3-3 overexpression in human breast cancers, especially in breast cancer metastasis, has never been investigated.* 14-3-3 is overexpressed in >60% of breast tumors, whereas none of the currently intensively studied genes in breast cancers, such as p53, erbB2, BRCA1 and BRCA2 etc., affects breast cancers at this high rate. Thus, it is imperative to investigate the unexplored area of the role of 14-3-3 in breast cancer tumorigenicity/metastasis. The pursuance of this proposal will bring original and important contributions to this unexplored area. It will bring timely findings regarding the role of 14-3-3 in breast cancers and ways to block the function of overexpressed 14-3-3 in breast cancers

CURRICULUM VITAE

NAME: **Chunfeng Qu**

DATE OF BIRTHE: March 11,1963

NATIONALITY: Chinese

SEX: Female,

Health: Excellent

ADDRESS: Molecular Immunology Branch,

                  National Laboratory of Molecular Oncology

                  Cancer Institute

                  Chinese Academy of Medical Sciences

                  &Peking Union Medical College

                  P.O. Box 2258.   Beijing 100021,China

Telephone & Fax: 86-10-67713917

E-mail: **vannaqu@public.bta.net.cn**

EDUCATION:

9/1996   Ph.D. Medical Science (expected in 6/1999)

to present   Peking Union Medical College

              Beijing, China

              Major: Immunology

9/1987   M.S. Medical Science

to 6/1990   Hubei Medical University

              Wuhan, Hubei, China

              Major: Medical Microbiology and Immunology

9/1980   B.S. Medicine

to 7/1985   Weifang Medical College,

              Weifang, Shandong, China

RESEARCH EXPERINCE:

9/1996   Ph. D student, **Supervisor: Zong-Tang(Tsung Tang) Sun**

to present   Cloning, Characterization of Human IL-12 and Functional Studies on IL-12
              Engineered Human Dendritic Cells

- Cloned and sequenced human IL-12 p40 cDNA
- Constructed recombinant human IL-12 bi-cistronic retroviral expression vector
- Identified the expressed human IL-12 biological activity and specific immunity
- Propagated and characterized human dendritic cell from cord blood cells

- Transfected hematopoietic progenitor with IL-12 bi-cistronic expression vector by using DOTAP. Identified the cytokine expression by intracellular cytokine staining and FACS analysis. Quantify the production of IL-12 by ELISA.
- Studied the antigen presenting function of IL-12 transfected dendritic cells by using cellular immunity methods including mixed leukocytes reaction, cell proliferation, Th1 induction and its cytokine assay, CTL induction and its cytotoxity assay.
- Identified the HLA-A201 individual by using specific sequence primers PCR

12/1994 to 8/1996	Research Associate, Administrative Assistant Immunology Department & Provincial Laboratory of Molecular Immunology of Shandong Province, Weifang Medical College, Weifang , Shandong , China

- **Principal Investigator** of the project " Immunology study of HBeAg-minus hepatitis B virus infected patients" (Subsidized by the Education Department of Shandong Province)
  - ▸ Detected the HBV precore region mutation by using PCR combination with digoxin-tagged oligonucleotide probe dot blotting
  - ▸ Detected the $HBcAg_{50-69}$ specific and PHA stimulated no-specific T lymphocyte proliferation in HBeAg-minus HBV infected patients
  - ▸ Tittered anti-HBe in patients sera
  - ▸ Quantified IL-4, IL-2 and IFN-γ production by PBMC after PHA stimulation
- **Co-principal Investigator** of the project "the role of immune response in the hepatic cell damage after HDV super or co-infection with HBV"
  - ▸ HBV-DNA, HDV-RNA *in situ* hybridization
  - ▸ HBcAg, HDAg immunohistochemistry staining

9/1994 to 12/1994	Research Assistant Molecular Virology Department Shanghai Medical University, Shanghai, China

- Took advanced course of molecular virology
- Engaged in the advanced study of virology (mainly in Hepatitis B Virus)

8/1992 to 11/1994	Lecture, Research Assistant Medical Microbiology and Immunology Department Weifang Medical College, Weifang , Shandong, China

- Participated in the project "study of Pseudomonous Cocovenenans classification and its phylogene" with the partial 16s rRNA sequence

7/1990 To 7/1992	Teaching Assistant Medical Microbiology and Immunology Department Weifang Medical College, Weifang Shandong , China

| 9/1987 | Graduate student, **Supervisor: Jinmin Xiang** |
| to 6/1990 | Virus Research Institute, Hubei Medical University |

- Studies on antiviral effect of extract from *Alternanthera Philoxeroides* Griseb on epidemic haemorrhagic fever *in vivo*

8/1985	Teaching Assistant
To 8/1987	Medical Microbiology and Immunology Department
	Weifang Medical College, Weifang Shandong , China

TECHNICAL SKILL:
MOLECULAR VIROLOGY AND BIOLOGY:

- DNA/RNA isolation and purification
- Southern Blotting and Northern Blotting
- cDNA Cloning
- *In situ* Hybridization
- Western Blotting
- Probe Preparation and Dot Hybridization

- PCR/RT-PCR
- Expression Vector Construction
- Sequencing
- Mammalian Cells Transfection
- Cell Culture and Virus Infection
- Virus Isolation and titration

IMMUNOLOGY

- Dendritic Cells Culture
- Mixed Lymphocyte Culture
- in vitro Peptides Specific CTL Induction
- Immunohistochemistry
- HLA typing
- Cloning with limiting dilution

- CTL Cytotoxicity Assay
- Cytokine Assay
- EIA or ELISA and IFA
- FACS
- T cell epitope peptide binding assay
- Protein Characterization and Purification

AWARD

- Young Investigator Award, the Educational Department of Shandong Province, 1995
- Outstanding Young Investigator Award, Weifang City ,1996
- Excellent Teacher Award of Weifang Medical College,1994,1996

MAIN PUBLICATION

1. **Chunfeng Qu** and Zongtang Sun. Cloning, sequencing analysis and biological characterization of human recombinant IL-12. **Chin. J. Immunol.** 1999,15(1); 13-15
2. **Chunfeng Qu**, Zongtang Sun and Peidi Gu Studies on the human dendritic cells transduced by rhIL-12 expression vector. **Chin. J. Immunol.** in Press.
3. **Chunfeng Qu**, Zongtang Sun, Guoting Liu et al. Functional studies on IL-12 engineered human dendritic cells. Submit.
4. Yafei Hou, Zongtang Sun, Ettore Appella, **Chunfeng Qu**, et al. HLA-A2 subtype distribution among Chinese and its effect on CTL response in vitro to p53 based

synthetic peptides loaded on dendritic cells. **Chin. J. Microbiol. Immunol.** 1999;19(1):47-50

5. Xiaoyan Wang, **Chunfeng Qu,** Yanjun Wang et al. Study on cellular immunity of HBeAg-minus HBV infected patients. Reported on 3rd Chinese Immunology Congress July 1998, Lanzhou, China. **Chin. J. Hepatol.** 1999, in Press

6. **Chunfeng Qu,** Naifa Miao, Enyu Xuan et al. The role of immune response in the hepatic cell damage after HDV super or co-infection with HBV. **Chin. J. Hepatol.** 1999; 15(2): 61-64

7. Hong Tu, **Chunfeng Qu,** Pingyang Li et al. Study on the effect of anti-HBe titers on HBV HBeAg-minus variant. **Chin. J. Microbiol. Immunol.** 1996;16(5):377-380

8. Naixin Zhao and **Chunfeng Qu.** Phylogenetic evidence for the transfer of Pseudomonas Cocovennenans(van Damme et al. 1960) to the Genus Burkholderia as Burkholderia Cocovennenans (van Damme et al. 1960) com. nov. **Inter. J. System. Bacter.** 1995;45(3)600-633

9. **Chunfeng Qu,** Naixin Zhao, En'Tao Wang et al. Pseudomonas Cocovennenans Partial 16s rRNA sequencing and its phylogene **Chin. J. Microbiol. Immunol.** 1994; (6)387-390

10. **Chunfeng Qu** , Zhanqiu Yang, Yanwen Liu et al Studies on antiviral effect of extract from *Alternanthera Philoxeroides* Griseb on epidemic hemorrhagic fever *in vivo*. **Chin. J. Exp. Clin. Virol.** 1993□7□1□□55-59

11. Zhanqiu Yang, **Chunfeng Qu,** Zhiming Zheng et al. Characteristic of propagation of epidemic hemorrhagic fever virus (Hantan virus) in the Vero-E6 cells. **J. Hubei Medical College.**1991;12(4):308-311.

NAMES OF REFERENCES:

Zong-Tang Sun, M.D, Chief and Professor
Molecular Immunology Branch
National Laboratory of Molecular Oncology
Cancer Institute
Chinese Academy of Medical Sciences
P.O. Box 2258, Beijing 100021, China
Tel. & Fax: 86-10-6771-3917
E-Mail: ztsun@public3.bta.net.cn

Ningzhi Xu, M.D, Chief and Professor
Molecular and Cellular Biology Department
Cancer Institute
Chinese Academy of Medical Sciences
P.O. Box 2258,Beijing 100021, China
E-Mail: xningzhi@public.bta.net.cn

Lihua Ming Ph.D. Assistant Professor
Molecular Immunology Branch
National Laboratory of Molecular Oncology
Cancer Institute
Chinese Academy of Medical Sciences
P.O. Box 2258,Beijing 100021, China

Abstract

A Gene Vaccine for Metastatic Breast Cancer

HER2/*neu* expression in a breast cancer biopsy indicates poor prognosis. How HER2/*neu*, a tyrosine kinase receptor accelerates the growth and metastasis of breast cancer cells is not yet understood; this lack of knowledge reduces treatment options. A vaccine targeting HER2/*neu* could have significant therapeutic and preventative application by controlling the growth and spread of the highly aggressive HER2/*neu*⁺ cells. Anti-tumor immunity has been developed using gene vaccines, a new form of gene therapy, by immunizing with bacterial expression plasmids encoding the DNA sequence for antigens such as HER2/*neu*. We have shown that vaccination of mice with a novel plasmid expressing the DNA sequence for HER2/*neu* protected mice challenged with a HER2/*neu*-expressing murine breast tumor cell line injected directly into mammary tissue or intravenous injection, a model of tumor metastasis. The plasmid we used for vaccination, called ELVIS, was created by Chiron Technologies (San Diego, CA) and is the first step of a two-stage vaccination protocol that uses Virus Like Particles (VLP) as the second step. Non-pathogenic VLP are similar to attenuated viruses that induce long-lasting immunity to childhood diseases. We propose to test the strategy of primary vaccination with ELVIS-HER2/*neu* followed by boosting vaccination with VLP containing the sequence for HER2/*neu*. We expect this strategy to much more effective than our existing strategy of both priming and boosting with only ELVIS-HER2/*neu*. The goals of this study are: **1)** To establish the optimum prime and boost strategy using ELVIS-HER2/*neu* and VLP-HER2/*neu* to generate humoral and T cell immunity against mouse mammary tumor cells expressing HER2/*neu* and **2)** To demonstrate that the optimum vaccination protocol can protect mice from tumor expansion and metastasis when delivered after the tumor has already been established, a condition more representative of the clinical situation for women with metastatic breast cancer.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Rui-an Wang	Postdoctoral Fellow

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
First Military Medical University, China	BS	1984	Medicine
Fourth Military Medical University, Xi'an Shaanti, China	MS	1990	Medicine-Gut Endocrinology
Third Military Medical University, Chongqing, PR China	PhD	1994	Medicine-Gut Endocrinology
Nagasaki University School of Medicine, Japan	Postdoctoral Fellow	1996-97	Programmed Apoptosis
University of Missouri-Columbia, Missouri	Postdoctoral Fellow	1998-99	Germ Cell Development
UT-MD Anderson Cancer Center, Houston, TX	Postdoctoral Fellow	1999--	Molecular mechanism of Cancer cell metastasis

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Research and Profession Experience:
1984 – 87   Instructor, Taught Histology and Human Embryology, The Fourth Military Medical University, Xi'an, China
1994 – 96   Lecturer: Histology and Human Embryology, The Fourth Military Medical University, Xi'an, China
1996 – 97   Postdoctoral Fellow, Department of Histology and Cell Biology, Nagasaki University School of Medicine, Japan
1997 – 98   Assistant Professor, Assistant Professor, Department of Histology and Embryology, The Fourth Military Medical University, Xi'an, China
1998 – 99   Visiting Research, Department of Pathobiology, University of Missouri-Columbia, Columbia, MS
1999 -   Postdoctoral Fellow, Cell Growth Regulation Section, Clinical Investigation, Division of Medicine, The University of Texas M. D. Anderson Cancer Center, Houston, TX

Awards and Honors:
1995   Advanced Teacher of the 4th Military Medical University, Xi'an, China
1996   Second Prize of the Chinese Army Medical System – Study on the somatostatin gene expression in gut-pancreatic endocrine system.

Memberships:
Member, Chinese Anatomy Society
Member, Chinese Society of Cell Biology

Publications:
1.   Wang RA, Su HC, Zhang, YQ, and Wang WC. Immunohistochemical localization of pancreastatin in human and guinea pig gastroentero-pancreatic system (in English). Proc 1st China-Japan Histochem Cytochem Seminar, 209-210, 1989.
2.   Wang RA and Su HC. Immunohistochemical localization and distribution of pancreastatin in guinea pig, porcine and human gastrointestinal tract. Acta Anatomica Sinica, 21(Suppl): 39-44, 1990.
3.   Zhang YQ, Wang RA, and HC. A comparative study of pancreatic polypeptide (PP) immunoreactive cells in the body of the pancreas of mammalia. Acta Zoologica Sinica, 37(2): 198-202, 1991.
4.   Fu CG, SU HC, Wang RA, Zhang LF and Zhang CJ. Changes of 5-HT immunoreactive cells in the gastric antrum and duodenum during simulated weightlessness in rats (in English). Acta Anatomica Sinica, 22(Suppl): 225-226, 1991.
5.   Wang RA and Su HC. Pancreastatin (Review). Progress in Physiological Science (Beijing), 23(1): 68-70, 1992.
6.   Su HC and Wang RA. Immunocytochemical localization of pancreastatin in guinea pig, pig and human pancreas. Acta Anatomica Sinica, 23(1): 58-62, 1992.

7. Wang RA, Cai WQ and Yao J. Application of in situ hybridization with digoxigenin-labeled cRNA probe for detection of somatostatin mRNA in gastroentero-pancreatic system (in English). Chin J Histochem Cytochem, 2(Suppl): 158-159, 1993.

8. Yao J and Wang RA. Application of Dig-labeled cRNA probe in situ hybridization for detecting the somatostatin gene expression in brain. Chin J Histochem Cytochem, 3(1): 22-28, 1994.

9. Wang RA, Cai WQ and Su HC. Immunohistochemical localization and distribution of cholecystokinin octapeptide in porcine, guinea pig and rat intestinal tract. Acta Zoologica Sinica, 41(2): 431-437, 1995.

10. Wang RA, Cai WQ and Zhang CG. Mutual relationships between the contents of the cholecystokinin octapeptide, serotonin and chromogranin A in individual EC cells of the porcine duodenum (in Chinese). Chin Sci Bull, 39(23): 2184-2187, 1994.

11. Wang RA, Cai WQ and Zhang CG. Mutual relationships between the contents of the cholecystokinin octapeptide, serotonin and chromogranin A in individual EC cells of the porcine duodenum (in English). Chin Sci Bull, 41(5): 417-421, 1995.

12. Wang RA, Cai WQ and Su HC. A comparative study on the morphology and distribution of somatostatin-immunoreactive cells in the human, guinea pig, rat, pig, dog and ox pancreas. Acta Anatomica Sinica, 26(2): 176-179, 1995.

13. Wang RA, Cai WQ and Su HC. Changes of gastrin, serotonin, somatostatin and chromogranin A immunoreactive cells in the gastric antrum of simulated weightless rat. Space Medicine and Medical Engineering (Beijing), 8(1): 49-52, 1995.

14. Wang RA, Cai WQ and Su HC. The somatostatin gene expression in the stomach, duodenum and pancreas during the postnatal development in the rat. Chin J Histochem Cytochem, 4(1): 4-8, 1995.

15. Wang RA and Zhang YQ. Genetic controls on the cell death (Review). Progress in Anatomical Sciences, 1(2): 142-144, 1995.

16. Huang WQ, Sun L and Wang RA. Immunocytochemical identification of serotonin anti-idiotype antibodies and the distribution of serotonin receptors. J Fourth Milit Med Univ, 16(3): 1995-198, 1995.

17. Zheng PJ, Huang WQ, Wang RA and Sun L. Immunocytochemical localization of serotonin and its receptors in the colon cancer. Chin Sci Bull, 40(21): 2001-2003, 1995.

18. Wang RA, Nakane PK and Koji T. Autonomous cell death of mouse male germ cells during fetal and postnatal period. Biology of Reproduction, 58(5): 1250-1256, 1998.

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Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 3a, 3b.

ABSTRACT

Antagonists of steroid hormones are clinically important in the management of breast cancer. However, the duration of response is limited due to the development of hormone-independent tumors. Growth of human breast cells is closely regulated by steroid hormone as well as peptide hormone receptors. Deregulation of HER2/HRG pathway into the estrogen receptor-positive breast cancer cells leads to alteration/disruption of estrogen receptor (ER) signaling, endocrine resistance and thus, contribute in breast cancer tumor progression.

A group of transcription cofactors for nuclear hormone receptors, referred to as corepressors and coactivators, has been shown to induce transcriptional silencing and hormone-induced activation, respectively, of genes that contain positive hormone response elements. Ligand-dependent transcriptional activation involves the recruitment of coactivators (CoAs), which possess or recruit histone acetyltransferases (HATs). However, transcriptional silencing by corepressors involves the recruitment of histone deacetylases (HDACs). Since HDACs-mediated deacetylation of nucleosomal histones is known to be associated with transcriptional repression of some genes, it is being proposed that the deregulation of HDAC recruitment to promoters could serve as a potential mechanism by which these enzymes contribute to tumorigenesis. A role of HDACs in tumor progression was also suggested by findings, demonstrating the identity of NURD70 (a component of nucleosome remodeling and histone deacetylase complex) to a candidate metastatic factor 1 (MTA1), a gene originally identified by differential expression in rat mammary adenocarcinoma metastatic cells.

Since HRG promotes invasiveness of non-invasive MCF-7 breast cancer cells and MTA1 was identified as differentially expressed gene in metastasized breast cancer, we initially investigated the possibility whether HRG could regulate MTA1 expression. On-going studies from the mentor's laboratory have demonstrated- 1) HRG regulation of MTA1 in a non-invasive breast cancer cells; 2) cloning and expression of MTA1 cDNAs; 3) differential localization of a MTA1 variant; 4) lack of histone acetylation by HDAC inhibitor in HRG-treated cells; and 5) repression of ERE-driven transcription by MTA1.

Since MTA1 is known to be closely related with metastasis, we hypothesized that "HRG/HER2 pathway may regulate the expression and function of MTA1, and MTA1 may be involved in the repression of ER transcription and thus hormone-independence of breast cancer". Studies are proposed to examine the modulation of ER transcription by MTA1 (AIM 1), and regulation of endocrine resistance by MTA1 (AIM 2) in breast cancer cells.

This postdoctoral fellowship will establish, for the first time, the role of histone acetylation modifying components in the regulation of ER pathways and sensitivity of breast cancer cells to anti-estrogen therapy. It is expected that our studies will provide a long-awaited opportunity to reverse the HER2- or HRG- associated endocrine resistance by HDAC inhibitors, and open a new translation avenue of a therapeutic value. This application is based on the original findings in the mentor's laboratory (preliminary studies).

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel listed on the budget page for the initial budget period

NAME Yong Wen	POSITION TITLE Graduate Student

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Beijing Medical University, P.R.China	M.D.	1988-1993	Medicine
Beijing Medical University, P.R.China	Ph. D. candidate	1993-1997	Histology
The University of Texas, Houston, TX	Graduate Student	1997-present	Cancer Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 3 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Research Training (tutorials):

Fall 1997	Mien-Chie Hung, Ph.D.	Dept. Cancer Biology
Spring 1998	Li-Kuo Su, Ph.D.	Dept. Cancer Biology
Spring 1998	Sue-Hwa Lin, Ph.D.	Dept. Molecular Pathology

Courses and Scores (*required courses)

Fall 1997	Experimental Genetics	(A)
Fall 1997	Current topics in Gene and Development	(P)
Fall 1997	Ethical Dimensions on Biomedicine	(P)
Spring 1998	*Advanced Biochemistry	(B)
Spring 1998	Current Topics in Oncogene Research	(P)
Summer 1998	*Biomedical Statistics	(A)
Fall 1998	*Cell Biology	(A)
Spring 1999	Human Gene Therapy	(A)
Spring 1999	Current Topics in Oncogene Research	(P)
Spring 1999	Seminar in Tumor Suppressor Genes	(P)
Summer 1999	Computer on Research and Medicine	(A)
Fall 1999	*Cancer Biology (on going)	

Honors and Awards:

1996   Outstanding Graduate Student Award, Bejing Medical University, P.R.China.
1993   National Outstanding Student Award, P.R.China
1992 Outstanding Student Award, Beijing, P.R.China.

Provisional Patent:

Hung, M.-C., Yan, D.-H., **Wen, Y.,** Spohn, B. Provisinal SN 60/139,039 "p202 is a tumor suppressor" (UTMDACC:611PZ1). Filing date: June 10, 1999.

Publications:

Wen, Y., Yan, D.-H., Spohn, B.,Deng, J., Lin, S-Y., and Hung, M.-C. (1999) Tumor suppression and sensitization to tumor necrosis factor α-induced apoptosis by an interferon inducible protein, p202, in breast cancer cells. *Cancer Res.* (in press)

Yan, D.-H., **Wen, Y.,** Spohn, B., Choubey, D., Gutterman, J. U., and Hung, M.-C. (1999) Reduced growth rate and transformation phenotype of the prostate cancer cells by an interferon-inducible protein, p202. *Oncogene* 18:807-811.

Yan, D.-H., **Wen, Y.*,** Lin, G., Su, L.-K., Xia, W., Wang, S.C., Zhang, S., Hortobagyi., G., and Hung, M.-C.. (1999) A delayed chemical-induced tumorigenesis in BRCA$_2$ mutant mice. (submitted) (* co-first author)

Lin, S.-Y., Xia, W, Wang, J, Kwong, K-Y., Sphon, B., **Wen, Y.,** Pestell, R., and Hung, M.C. (1999) β-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc Natl Acad Sci U S A* (submitted).

Lin, S.-Y., Matin, A., Xia, W., **Wen, Y.,** and Hung, M.-C.. (1999) Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nature* (submitted).

Wen, Y., and Liu, B. (1997) The expression of transforming growth factor α in the mouse embryos and preimplantation uterus. *J. Beijing Medical. University.* 29:12-14.

Wen, Y., and Liu, B. (1997) Transforming growth factor α and its receptor in reproduction process. (in Chinese) *Foreign Medical Sciences.* 16:27-30.

Wen, Y., and Liu, B. (1996) The role of platelet activating factor in reproduction. (in Chinese) *Progress of Anatomical Sciences.* 2: 204-211.

ABSTRACT

Title: p202 as a Potential Therapeutic Gene for Breast Cancer

p202, an interferon (IFN)-inducible protein, interacts with several important cell cycle, differentiation, and signal transduction regulatory proteins. These protein-protein interactions result in mainly transcription repression of the target genes leading to growth arrest or differentiation. Our previous studies have shown that p202 suppresses both *in vitro* and *in vivo* cell growth and tumorigenicity of human breast cancer and prostate cancer cells. In addition, we found that p202 can physically interact with NF-κB and inhibit TNF-α induced NF-κB activation. The inactivation of NF-κB by p202 sensitizes breast cancer cells to TNF-α induced apoptosis. Based on the previous observations, we propose to: (1) further investigate the anti-tumor activity of p202 on breast cancer and develop a p202 gene therapy approach for breast cancer; (2) determine the effects of p202 on the sensitivity of breast cancer cells to TNF-α, radiotherapy, and chemotherapy to develop appropriate combination therapies. Three studies will be carried out to accomplish this objective.

1. To determine the effects of p202 on the sensitivity of breast cancer cells to anticancer agents. The p202 stable transfectants and the parental cells will be exposed to therapeutic agents, such as ionizing irradiation and chemotherapy drugs, especially those have been known to be able to induce nuclear NF-κB activity and result in resistance. The cell growth and apoptosis will be examined to determine if p202 could induce certain sensitization to radiotherapy or chemotherapy in breast cancer cells.

2. To test the anti-tumor activity of p202 in breast cancer cells using preclinical gene therapy strategies in an orthotopic breast cancer animal model. Orthotopic breast cancer model will be generated by mammary fat pad inoculation of human breast cancer cells in female nude mice. The non-viral delivery systems, such as LPD1 (a cationic lipid) will be used for p202 gene transfer by intratumoral injection or systemic treatment through intravenous injection. The tumor volume, metastasis, and angiogenesis will be examined to determine the therapeutic efficacy.

3. To evaluate the therapeutic efficacy of p202 gene therapy in combination with other anti-cancer agents in an orthotopic breast cancer animal model. The tumor-bearing mice (orthotopic breast cancer model) will be treated with the combination of p202 gene therapy and TNF-α. Therapeutic efficacy will be compared among the combination therapy and each single treatment. Once we identify any other anticancer agents with enhanced efficacy in combination with p202 *in vitro* as described in Specific Aim 1., we will also test the feasibility of the combination therapies in animals.

Success of this study will constitute a scientific basis for p202 mediated gene therapy for breast cancer cells and will enable us to develop novel combination therapeutic approaches with enhanced efficacy against breast cancer.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Photocopy this page or follow this format for each person.

NAME Kazumi Yoshida, M.D., Ph.D.	POSITION TITLE Postdoctoral Research Fellow

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
The Jikei University School of Medicine, Tokyo, Japan	M.D.	1987	Medical Science
Graduate School of The Jikei University School of Medicine, Tokyo, Japan	Ph.D.	1996	Cell Biology (Morphology)

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

PROFESSIONAL EXPERIENCE

May 1987-March 1992	Surgical Residency, Mitsui Memorial Hospital, Tokyo, Japan
April 1996-December 1996	Assistant Surgeon, Department of Surgery, The Jikei University School of Medicine, Tokyo Japan
January 1997-March 1997	Ships' Doctor, Institute for Sea Training, Ministry of Transport, Tokyo, Japan
April 1997-June 1998	Assistant Surgeon, Department of Surgery, The Jikei University School of Medicine, Tokyo Japan
July 1998-present	Postdoctoral Research Fellow, The University of Texas, M.D. Anderson Cancer Center Houston, Texas, U.S.A.

HONORS AND AWARDS:

Poster Presentation Award, University of Texas M.D. Anderson Cancer Center, 1999

PUBLICATIONS:

1. Yoshida K, Wanibuchi Y, Kanda T, Shimoyama Y, Furuta S. Valve replacement in infective endocarditis with mycotic aneurysm. Japanese Journal of Thoracic Surgery, 38:146(2162)-149(2165), 1990.

2. Yoshida K, Nishi T, Miyamoto H, Obara T, Maruyama Y. Non-recurrent inferior laryngeal nerve in two patients with thyroid tumor. Endocrine Surgery, 8:319-322, 1991.

3. Sugimto K, Fujii S, Sago H, Yoshida K, Takemasa T, Yamashita K. Role of marginal stress fibers formed in the rat vascular endothelial cells. Tissue and Cell, 25:825-831, 1993

4. Yoshida K, Hataba Y, Suzuki T, Tanaka H, Sakurai K. Adaptation of vascular endothelial cell under different hemodynamic conditions. Recent Development of Electron Microscopy, 141-143, 1993.

5. Sugimoto K, Yoshida K, Fujii S, Takemasa T, Sago H, Yamashita K. Heterogenous responsiveness of the in situ rat vascular endothelial cells to mechanical stretching in vitro. European Journal of Biology, 68:70-77, 1995.

6. Yoshida K, Sigimoto K. Morphological and cytoskeletal changes in endothelial cells of vein grafts under arterial hemodynamic conditions in vivo. Journal of Electron Microscopy, 45:428-435, 1996.

7. Yoshida K, Uchida K, Inoue Y, Nakano S, Yamashita A, Takeyama H, Yoshida K, Yamazaki Y, Nomura K. Four cases of breast cancer associated with neurofibromatosis type 1. Japanese Journal of Breast Cancer, 13:615-620, 1998.

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Number pages consecutively at the bottom throughout the application. Do <u>not</u> use suffixes such as 3a, 3b.

Abstract

The Retinoblastoma (*RB*) gene is known to be a tumor suppressor gene that plays an important role in regulating the activities of cells. These activities include inhibition of inappropriate cell growth and inhibition of genes that inappropriately activate cell death. One of the best-described functions of the *RB* protein product (pRb) is its regulation of the transcription factor E2F-1. E2F-1 is responsible for turning on numerous genes within the cell that cause the cell to replicate. In addition, when E2F-1 is produced in the cell at superphysiologic levels, it can lead to inappropriate signals that cause death of the cell (cell suicide). Previous work from our laboratory has demonstrated that giving breast cancer cells superphysiologic doses of E2F-1 can cause the cancer cells to commit suicide. We investigated the effect of giving superphysiologic doses of pRb (the normal regulator of E2F-1 within the cells) on breast cancer cell lines to determine if we could inhibit growth of the cells by supplying this tumor suppressor gene to the cells in excess amounts. We utilized recombinant adenovirus vectors in order to deliver the tumor suppressor genes to the cancer cells. Adenovirus vectors are responsible for causing the common cold virus. The vectors utilized in our study have been modified so that they can not replicate in the cells but they can deliver the tumor suppressor genes at superphysiologic levels. We constructed two vectors, one with the full-length (normal) *RB* gene (Rb-1) and one with a *RB* gene that has been shortened and modified so that it remains active within the cell for a longer period of time (Rb56m). After infection with the adenovirus vector containing the shortened and modified *RB* gene (Rb56m), we observed a marked reduction in growth in almost all of the breast cancer cell lines that we tested. We show here that this growth inhibition is a result of apoptotic cell death (cell suicide). This cell suicide appears to be caused by an increase in levels of E2F-1 within the cell, triggered by the Rb56m. We also found activation of key cellular proteins that are responsible for initiated the cell suicide pathway. These novel observations provide insight into the mechanisms of Rb56m-mediated cell death in breast carcinoma cells. Further exploration of these important cellular regulatory mechanisms will contribute to the development of alternative treatment strategies for breast cancer patients who fail standard therapies. The potential outcome of the proposed research is preclinical experimentation that can serve as the basis for a novel gene therapy strategy using Rb56m as a therapeutic gene in the treatment of breast cancer patients.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel listed on the budget page for the initial budget period			

NAME Zhenming Yu		POSITION TITLE Graduate Research Assistant	

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Nankai University, Tianjin, P. R. China	B.S..	1990-1994	Microbiology
Institute of Microbiology, Chinese Academy of Sciences, Beijing, P. R. China	M.S.	1994-1997	Microbiology
The University of Texas M. D. Anderson Cancer Center, Houston, Texas	Ph.D.	1997-present	Cancer Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 3 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Professional Experience:

1990-1994   B.S., Department of Biology, Nakai University, Tianjin, P. R. China.

1994-1997   M.S., Institute of Microbiology, Chinese Academy of Sciences, Beijing, P. R. China

1997-present: Graduate Research Assistant, The University of Texas M. D. Anderson Cancer Center, Houston, TX
Ph.D. Candidate, The University of Texas Graduate School of Biomedical Science at Houston, Houston, TX

Honors and Awards:

1998-1999   Principal Investigator of the Training Award in support of research entitled "Development of PEA3 as a Potential Gene Therapy Agent for Breast Cancer", US Army Medical Research DAMD17-96-1-6223

1992-1993   Scholarship for Academic Excellence in Nankai University

Publication:

1.   Xing, S., Wang, S.C., Miller, S., Huang, L., Xia, W.Y., **Yu, Z.**, Shao, R.P., Kwong, S. Zou, Y., Zhang, S., and Hung, M.C. The *Ets* protein *PEA3* suppresses *HER-2/Neu* overexpression and inhibits tumorigenesis. Nature, submitted, 1999.

2.   Zhou, B.P., Hu, M., Miller, S., **Yu, Z.**, Xia, W., Lin, S-Y., and Hung, M.C. *HER-2/neu* blocks TNF-induced apoptosis via the *Akt/NF*-κB pathway. Science, submitted, 1999.

ABSTRACT

Breast cancer is a major cause of death for women in the United States. Overexpression of the HER-2/neu oncogene was reported to correlate with poor survival for breast cancer patients, enhance metastatic potential of human breast cancer cells and induce resistance to chemotherapeutic agents. Therefore, HER-2/neu oncogene is an excellent target for development of novel anti-cancer agent for the HER-2/neu-overexpressing breast cancers.

We have previously shown that PEA3, a transcriptional factor from ETS family, can suppress HER-2/neu transforming phenotype in vitro and inhibit tumor growth in vivo. Based on our data, we also proposed PEA3 represses the HER-2/neu promoter activity through competitively binding to a positive regulatory motif of the promoter. Consistent with this competition model, we found that DNA binding domain of PEA3 (PEA3DBD) along is sufficient to down-regulate HER-2/neu promoter activity and suppress HER-2/neu mediated focus formation of mouse fibroblast NIH3T3.

Since a significant fraction of PEA3 target genes encode proteases which are associated with metastatic propensity of tumor cells, one side-effect of using PEA3 as a gene therapy agent is that the metastatic potential of cancer cells may possibly be enhanced. Therefore, here we hope to explore the feasibility of development of PEA3DBD as a novel gene therapy agent. **Our hypothesis is that PEA3DBD may function as a tumor suppressor for HER-2/neu-overexpressing breast cancer cells and sensitize response of breast cancer cells to chemotherapeutic drugs through PEA3DBD-mediated down-regulation of HER2/neu.**

Our specific aims are:

1. Examination of transformation suppression activity of PEA3DBD in breast cancer cell lines through generation of PEA3DBD stable transfectants.

2. Examination of preclinical therapeutic effects of PEA3DBD in an orthotopic breast cancer model, using PEA3DBD DNA-liposome complex and adenovirus vector carrying PEA3DBD.

3. Testing the potential synergistic effects of gene therapy using PEA3DBD and chemotherapy for breast cancer with overexpression of HER-2/neu.

This proposal is designed to develop PEA3DBD as a potential gene therapy agent for HER2/neu-overexpressing breast cancers and investigate the therapeutic effect of combination of PEA3DBD and chemotherapy agent.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel listed on the budget page for the initial budget period

NAME	POSITION TITLE
Binhua P. Zhou	POSTDOCTORAL FELLOW

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Guangzhou Medical College, Guangzhou, China	M.D.	June, 1986	Medicine
The University of Texas at Austin, Austin, TX	Ph. D.	July, 1996	Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 3 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Research and Professional Experience:

Sep. 1996-Present	Postdoctoral Fellow, Section of Molecular Cell Biology, Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center
Jan. 1991-July, 1996	Graduate Research Assistant, Division of Medicinal Chemistry, College of Pharmacy, The University of Texas at Austin
1987-Oct. 1990	Research Medical fellow, The Hepatoma research Center, and Department of Biochemistry, Guangzhou Medical College, Guangzhou, China

Honor:

1997	Young Investigator Award for Breast Cancer Research, The University of Texas M. D. Anderson Cancer Center
1993-1996	Tuition-Waived fellowship, College of Pharmacy, The University of Texas at Austin
1986	Graduate with Honor, Guangzhou Medical College, China

Membership:

Associate Member	American Association for Cancer Research (AACR)
Member	American Association for Advancement of Science (AAAS)
Member	American Chemical Society (ACS)

Publications:

1. **Binhua P. Zhou**, Duane A. Lewis, Sau-Wah Kwan, and Creed W. Abell (1995) Flavinylation of Monoamine Oxidase B, *J. Biol. Chem.* **270**, 23653-23660

2. **Binhua P. Zhou**, Duane A. Lewis, Sau-Wah Kwan, Timothy J. Kirksey, and Creed W. Abell (1995) Mutagenesis at a Highly Conserved Tyrosine in Monoamine Oxidase B Affects FAD Incorporation and Catalytic Activity, *Biochemistry*, **34**. 9526-9531

3. Sau-Wah Kwan, Duane A. Lewis, **Binhua P. Zhou**, and Creed W. Abell (1995) Characterization of a Dinucleotide-Binding Site in Monoamine Oxidase B by Site-Directed Mutagenesis, *Arch. Biochem. Biophys.* **316**, 385-391

4. Ruping Shao, Devarajan Karunagaran, **Binhua P. Zhou**, Kaiyi Li, Su-Shun Lo, Jiong Deng, Paul Chiao, and Mien-Chie Hung (1997) Inhibition of NF-κB Activity is Involved in E1A-mediated Sensitization of Radiation-induced Apoptosis. *J. Biol. Chem.* **272**, 32739-32742

5. **Binhua P. Zhou**, Bo Wu, Sau-Wah Kwan and Creed W. Abell (1998) Characterization of a New FAD Binding Domain in Monoamine Oxidase B. *J. Biol.Chem.* **273**, 14862-14868

6. **Binhua P. Zhou**, Bo Wu, Sau-Wah Kwan and Creed W. Abell (1999) Role of Two Tyrosine Residues Flanking in the Covalent Binding Site of Monoamine Oxidase B. *J. Biol. Chem.*, Accepted with minor revesion

7. Mien-Chie Hung and **Binhua P. Zhou** (1999) Comment on Breast Cancer, *Breast Diseases: A Year Book Quarterly*, **10**, 45

8. Ruoping Shao, Micky C-T. Hu, **Binhua P. Zhou**, Shiaw-Yih Lin, Paul J. Chiao, Ryan H. von Lindern, Bill Spohn, And Mien-Chie Hung (1999) E1A Sensitizes Cells to Tumor Necrosis Factor-Induced Apoptosis through Inhibition of IkB Kinases and Nuclear Factor kB Activities, *J. Biol. Chem.* **274**, 21495-21498.

9. **Binhua P. Zhou**, Mickey C.-T. Hu, Stephanie Miller, Zhenming Yu, Weiya Xia, Shiaw-Yih Lin, and Mien-Chie Hung, *HER-2/neu* Blocks TNF-induced Apoptosis via the Akt/NF-κB Pathway, Submitted

Patents:

1.Creed W. Abell, Sau-Wah Kwan, Binhua P. Zhou, Blain M. Mamiya, and Duane A. Lewis (1996) Novel Flavin Adenine Dinucleotide Analog Inhibitors of Monoamine Oxidase. International Publication Number: W0 96/20946, Docket Number: D5717

2. Creed W. Abell, Binhua P. Zhou, Sau-Wah Kwan, Blain M. Mamiya, and Duane A. Lewis (1996) Novel Method of Preparing Apoenzyme. Application Serial No: 60/003,921, Docket NO: D5803CPA, USSN: 08/714,922, approved

Principal Investigator: Binhua P. Zhou, M.D., Ph.D.
Proposal Abstract

Amplification/overexpression of the HER-2/neu gene was found in many different types of human cancers. The HER-2/neu protein is a receptor tyrosine kinase which mediates many signal pathways to regulate cell-growth and cell-death. In many of these cellular signal pathways, the PI 3-kinase/Akt pathway is known to play a prominent role in preventing cells from apoptosis, and attributes to the pathogenesis of cancer. Akt was initially described as an oncogene and its overexpression was also found to correlate with different cancers such as breast cancer, ovarian cancer and some pancreatic cancer. In our recent studies, we found that overexpression of HER-2/neu can activate the Akt activity, and the HER-2/neu overexpressed breast cancer cell lines have a overall enhancement of the Akt activity. In addition, we found that cell growth was inhibited when the Akt pathway was blocked by the dominant-negative Akt (DN-Akt) in either the HER-2/neu transformed cells or the HER-2/neu overexpressed breast cancer cells. Furthermore we found that these cells lost their abilities to grow in an anchorage-independent manner, underwent anoikis, and were sensitized to apoptosis induced by chemotherapeutic drugs. Thus, understanding the signal pathways and molecular actions mediated by HER-2/neu and Akt, will not only increase our understanding of the biology of cancer, but also shed light on new strategy for developing novel therapeutics for fighting breast cancer. Therefore, in this proposal, we plan to study the molecular mechanism of the cell growth inhibition mediated by DN-Akt, and we also propose to identify the other survival pathways from the extracellular matrix that cooperate with Akt to regulate apoptosis. Finally, we propose to develop a novel therapeutic approach to specifically target the HER-2/neu overexpressed breast cancer cells by combining the potential synergistic effects between DN-Akt and chemotherapeutic drugs. We will test this novel approach at the molecular and cellular levels to determine the synergistic effects both *in vitro* and *in vivo*. If effective, this approach should be able to benefit breast cancer patients with HER-2/neu overexpressed tumors clinically in the future.

Appendix 3.0

Cited Publications

Grb2 downregulation leads to Akt inactivation in heregulin-stimulated and ErbB2-overexpressing breast cancer cells

Soo-Jeong Lim[1], Gabriel Lopez-Berestein[1], Mien-Chie Hung[2], Ruth Lupu[3], and Ana M. Tari[1]

[1]Department of Bioimmunotherapy, Section of Immunobiology and Drug Carriers, [2]Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX, and [3]Lawrence Berkeley National Laboratories, University of California Berkeley, Berkeley, CA.

Correspondence: Ana Maria Tari, Dept. of Bioimmunotherapy, Section of Immunobiology and Drug Carriers, Box 60, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030. Phone: (713) 794-4856. FAX: (713) 796-1731. Email: atari@notes.mdacc.tmc.edu

Running Title: Grb2 downregulation leads to Akt inactivation

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Abstract

ErbB2 can be activated by its own overexpression or be transactivated by the heregulin polypeptide growth factor. Activation of ErbB2 leads to breast cancer cell proliferation, presumably by inducing the activation of extracellular signal-regulated kinases 1,2 (Erk1,2) and Akt. We have previously reported that the growth factor receptor bound protein-2 (Grb2) is required for the proliferation of ErbB2-overexpressing breast cancer cells. We investigated here whether Grb2 protein plays a role in heregulin-stimulated proliferation. Grb2 protein inhibition led to growth inhibition of heregulin-stimulated breast cancer cells, but not Erk1,2 inactivation. These findings are similar to our earlier observations in ErbB2-overexpressing cells. Since Akt can also be activated by heregulin, the effects of Grb2 inhibition on Akt were examined. Akt was inactivated following Grb2 downregulation in heregulin-stimulated breast cancer cells. We then examined the effects of Grb2 downregulation on Akt in ErbB2-overexpressing cells in the absence of heregulin. Similar to heregulin-stimulated cells, Grb2 inhibition also led to Akt inactivation in ErbB2-overexpressing breast cancer cells. Our results indicate that the activation of ErbB2 by heregulin or by its overexpression requires Grb2 to stimulate the Akt pathway to propagate mitogenic signals.

Introduction

Amplification of the ErbB2 (also known as Her2/neu) receptor tyrosine kinase gene is found in 20-30% of breast cancer patients and is associated with poor patient prognosis (Slamon *et al.*, 1987; Slamon *et al.*, 1989; McCann *et al.*, 1991; Paterson *et al.*, 1991). Unlike its other family members (EGFR, ErbB3, and ErbB4), no ligand has yet been identified for ErbB2. However, ErbB2 can be activated by its own overexpression (Pierce *et al.*, 1991) or be transactivated by heregulin (Holmes *et al.*, 1992; Lupu *et al.*, 1992; Peles *et al.*, 1992). Heregulin is a family of proteins identified as ligands for the ErbB3 and ErbB4 receptor tyrosine kinases. Heregulin induces the formation of heterodimers between ErbB3 and ErbB2 or between ErbB4 and ErbB2, thereby transactivating ErbB2 (Plowman *et al.*, 1993a; Plowman *et al.*, 1993b; Sliwkowski *et al.*, 1994; Carraway *et al.*, 1995). In fact, the effects of heregulin are predominantly mediated by ErbB2 since inhibiting the function of ErbB2 can block the cellular transformation and proliferation processes induced by heregulin (Alimandi *et al.*, 1995; Lewis *et al.*, 1996). Heregulin stimulates these cellular processes, probably by activating multiple pathways, including those involving extracellular signal-regulated kinases 1,2 (Erk1,2) and phosphoinositol-3 (PI3) kinase (Fiddes *et al.*, 1995; Marte *et al.*, 1995; Sepp-Lorenzino *et al.*, 1996).

Upon activation by heregulin or its overexpression, ErbB2 becomes phosphorylated (Hazan *et al.*, 1990), and bound to the Src homology 2 (SH2) domain of the growth factor receptor bound protein-2 (Grb2) (Lowenstein *et al.*, 1992). Grb2 then uses its Src homology 3 (SH3) domains to bind to the guanine nucleotide exchange factor, Son of Sevenless (Sos) (Bonfini

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et al., 1992; Chardin *et al.*, 1993; Li *et al.*, 1993; Simon *et al.*, 1993). Sos stimulates Ras by increasing the amount of guanine nucleotide triphosphate (GTP) on Ras (Downward *et al.*, 1990). GTP-bound Ras then interacts with Raf (Dickson *et al.*, 1992; Vojtek *et al.*, 1993), leading to the stimulation of MEK and Erk1,2 (de Vries-Smits *et al.*, 1992; Howe *et al.*, 1992). Besides binding to Raf, GTP-bound Ras may bind to PI3 kinase (Rodriguez-Viciana *et al.*, 1994), leading to the stimulation of Akt (Burgering & Coffer, 1995; Franke *et al.*, 1995). Thus, Grb2 can potentially link activated ErbB2 to the activation of Erk1,2 and Akt in a Ras-dependent manner. Stimulation of the kinase activities of Erk1,2 and Akt have been shown to be important for mitogenesis (Marais & Marshall, 1996; Treisman, 1996; Alessi & Cohen, 1998). Grb2 may therefore play a vital role in transducing the mitogenic signals of heregulin-activated ErbB2 to Erk1,2 and Akt.

We have previously used liposome-incorporated nuclease-resistant antisense oligodeoxynucleotides specific for the GRB2 mRNA (Tari *et al.*, 1997; Tari *et al.*, 1999) to inhibit Grb2 protein expression. We found that Grb2 downregulation led to growth inhibition of breast cancer cells that have overexpression of ErbB2 (Tari *et al.*, 1999), thus indicating that Grb2 is important for the proliferation of this type of breast cancer cells. Since the effects of heregulin are predominantly mediated by ErbB2 (Alimandi *et al.*, 1995; Lewis *et al.*, 1996), we propose that Grb2 will also be important for the proliferation of heregulin-stimulated breast cancer cells. Here, we provide further evidence that Akt is involved in the mitogenic signaling mediated by Grb2.

Results

Liposomal Grb2 antisense oligodeoxynucleotides inhibited the growth of breast cancer cells stimulated by heregulin

Before examining the effects of Grb2 downregulation upon heregulin treatment, the growth-stimulatory effects of heregulin were investigated in two different breast cancer cell lines, T-47D and BT-474. These cells do not express endogenous heregulin but express ErbB3 and/or ErbB4 receptors and thus are able to bind to heregulin (Lewis *et al.*, 1996). These cells also differ in their ErbB2 expression levels (BT-474>>T-47D) (Lewis *et al.*, 1996). Increasing doses of heregulin stimulated the proliferation of both cell lines even in cell culture medium supplemented with fetal bovine serum (FBS) (Figure 1). At 80 and 100 ng/ml of heregulin, the proliferation of T-47D cells and BT-474 cells were stimulated up to 180 and 160% of untreated cells in mediums supplemented with 5 and 10% FBS, respectively.

We then investigated the effects of liposomal Grb2 antisense oligodeoxynucleotides (L-Grb2) on the growth of T-47D and BT-474 cells in the absence and presence of heregulin. Previously we had reported that Grb2 inhibition induced growth inhibition in breast cancer cells that have high, but not low, expression of ErbB2 (Tari *et al.*, 1999). Therefore, the low ErbB2-expressing T-47D cells were not expected to be growth inhibited by L-Grb2 but the high ErbB2-expressing BT-474 cells were expected to be inhibited by L-Grb2. Indeed in the absence of heregulin, the growth of T-47D cells was not decreased by L-Grb2 (Figure 2A), but the growth of BT-474 cells was selectively decreased by L-Grb2. BT-474 cell growth was decreased by 50%

at 12 μM of L-Grb2 (Figure 2B). Under identical conditions, liposomal control oligodeoxynucleotides (L-control) did not decrease the growth of T-47D and BT-474 cells (Figures 2A and B).

In the presence of heregulin, L-Grb2 selectively decreased the proliferation T-47D cells. When 12 μM of L-Grb2 was used, the growth of T-47D cells decreased by 75% while the same concentration of L-control decreased growth only by 20% (Figure 2A). A dose-dependent decrease in the proliferation of BT-474 cells was also observed when cells were coincubated with L-Grb2 and heregulin (Figure 2B). At 12 μM of L-Grb2, growth inhibition of BT-474 cells was similar in the absence or presence of heregulin (50% versus 58%). Our data indicate that L-Grb2 can inhibit the heregulin-stimulated proliferation of breast cancer cells regardless of the expression levels of ErbB2.

To further investigate the involvement of Grb2 protein in the heregulin-stimulated proliferation of breast cancer cells, MCF-7 cells transfected with heregulin (MCF-7/T7) were used. MCF-7/T7 cells produce high levels of heregulin and thus have constitutively activated ErbB2 receptors in spite of their low ErbB receptors expression (Tang *et al.*, 1996). A dose-dependent decrease in cell proliferation was observed when MCF-7/T7 cells were incubated with various concentrations of L-Grb2, but not with L-control (Figure 3A). At 12 μM of L-Grb2, the growth of MCF-7/T7 cells was decreased by 40%, while the same concentration of L-control only decreased growth by 4%. MCF-7 cells transfected with the control neomycin vector, MCF-7/V, were also obtained from Dr. Ruth Lupu and were used as a negative control cell line. These cells have low levels of ErbB2 but do not express any heregulin; therefore ErbB2

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is not activated in these cells (Tang *et al.*, 1996). As expected, growth inhibitory effects were not observed in MCF-7/V cells incubated with either L-Grb2 or L-control (Figure 3B). These data demonstrate that L-Grb2 can selectively inhibit the proliferation of MCF-7 cells which have activated ErbB2 due to the transfected heregulin gene.

Downregulation of Grb2 protein expression predominantly inhibited Akt activation in heregulin-transfected breast cancer cells

Western blot analysis was carried out to confirm that L-Grb2 could selectively downregulate the expression of Grb2 protein as we had previously reported (Tari *et al.*, 1997; Tari *et al.*, 1999). Compared with untreated cells, MCF-7/T7 cells treated with 10 μM of L-Grb2 for 3 days had a 39% decrease in Grb2 protein expression, while cells treated with the same concentrations of L-control had a 9% decrease in Grb2 protein expression (Figure 4). These data indicate that growth inhibition by L-Grb2 (Figure 3) was mediated by specific downregulation of Grb2 protein expression.

We were interested in whether disruption of heregulin-induced growth signaling by Grb2 protein downregulation was due to inactivation of Erk1,2 and Akt, two of the downstream kinases associated with heregulin signaling (Fiddes *et al.*, 1995; Marte *et al.*, 1995; Sepp-Lorenzino *et al.*, 1996; Liu *et al.*, 1999). So, we determined the phosphorylation, i.e. activation, levels of Erk1,2 and Akt in the same protein lysates in which Grb2 protein expression was downregulated. The protein levels of Erk1,2 and Akt did not change in MCF-7/T7 cells upon incubation with L-Grb2 or L-control. The phosphorylation levels of Erk1,2 were also not

significantly changed after L-Grb2 treatment (Figure 4). Compared with untreated cells, 10 μM of L-Grb2 decreased Erk1,2 activities by 15% while 10 μM of L-control decreased Erk1,2 activities by 1%. On the other hand, L-Grb2 decreased the phosphorylation levels of Akt (Figure 4). Akt activity was decreased by 62% in cells treated with 10 μM of L-Grb2 but only 12% by L-control. Our data indicate that Grb2 protein downregulation predominantly induces Akt inactivation in heregulin-transfected breast cancer cells.

Grb2 protein downregulation also led to Akt inactivation in ErbB2-overexpressing breast cancer cells

We have demonstrated that Grb2 downregulation could induce growth inhibition but not Erk1,2 inactivation in ErbB2-overexpressing breast cancer cells (Tari *et al.*, 1999). These findings are similar to what we had just described above for the heregulin-stimulated cells. Therefore, we speculate that Grb2 downregulation may also lead to Akt inactivation in ErbB2-overexpressing breast cancer cells. We examined the effects of Grb2 downregulation on Akt activities in MDA-MB-453 and BT-474 breast cancer cells, which express high levels of ErbB2 but do not express any heregulin. Similar to what was observed in breast cancer cells stimulated with heregulin, Grb2 inhibition also induced Akt inactivation in ErbB2-overexpressing breast cancer cells (Figure 5). When MDA-MB-453 cells (Figure 5A) and BT-474 cells (Figure 5B) were incubated with 8-12 μM of L-Grb2, Akt activity was inhibited by 34-47%. But Akt activity was inhibited by < 4% when same concentration of L-control were used. In all cases, L-Grb2 induced a decrease in Akt activity without decreasing Akt protein expression.

Discussion

Our results indicate that downregulation of Grb2 protein expression induced growth inhibition and Akt inactivation in ErbB2-overexpressing breast cancer cells as well as in heregulin-stimulated breast cancer cells. It is not surprising that Grb2 downregulation would lead to growth inhibition of heregulin-stimulated cells since there is considerable overlap in signaling between heregulin-stimulated and ErbB2-overexpressing breast cancer cells. Both cell types require the presence and the activation of ErbB2 to induce cellular transformation and mitogenesis (Alimandi et al., 1995; Lewis et al., 1996). The signaling induced by heregulin is initiated mainly from ErbB2 heterodimers while the signaling of ErbB2 overexpression is mainly from ErbB2 homodimers (Muthuswamy et al., 1999).

We postulate that Grb2 regulates Akt activity in a Ras-dependent manner. Grb2 can bind directly to ErbB2 upon ErbB2 activation (Meyer et al., 1994). Alternatively, Grb2 can bind indirectly to ErbB2 via Shc (Meyer et al., 1994; Ravichandran et al., 1995). This is because activation of ErbB2 leads to increased tyrosine phosphorylation on Shc (Segatto et al., 1993), which can bind to Grb2 and ErbB2 simultaneously (Meyer et al., 1994; Segatto et al., 1993). So either directly or indirectly, Grb2 can link activated ErbB2 to Sos, thereby increasing Ras activity (Bonfini et al., 1992; Chardin et al., 1993; Li et al., 1993; Simon et al., 1993). Activated Ras may bind and activate PI3 kinase (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996), which can in turn stimulate Akt activity (Burgering & Coffer, 1995; Franke et al., 1995). Inhibition of Grb2 expression may therefore disrupt Ras/PI3 kinase/Akt signaling, and induce

Akt inactivation. Stimulation of Ras/PI3 kinase activities, for example by expressing consitutively activated forms of Ras/PI3 kinase, should then block the inactivation of Akt by Grb2 downregulation.

On the other hand, Grb2 may also regulate Akt activity in a Ras-independent manner. This is because in addition to binding to Sos, Grb2 can use its SH3 domain to bind to other proline-rich containing proteins, such as PI3 kinase (Wang *et al.*, 1995), and Grb2-associated binder protein-1 (Gab-1) (Holgado-Madruga *et al.*, 1996). Both PI3 kinase and Gab-1 can stimulate Akt activity (Holgado-Madruga *et al.*, 1997; Wang *et al.*, 1995). The activation of Akt by PI3 kinase or Gab-1 appears to be Ras-independent because the binding of Grb2 to PI3 kinase or Gab-1 can exclude Sos from binding to Grb2, and could potentially stimulate Akt activity without stimulating Erk1,2 activity (Holgado-Madruga *et al.*, 1997; Wang *et al.*, 1995). In that case, inhibition of Grb2 expression may induce Akt inactivation without inducing Erk1,2 inactivation. This postulation is similar to what we have described here for our ErbB2-activated breast cancer systems. However, since Grb2 inhibition was found to decrease Ras activity in fibroblasts transfected with ErbB2 (Xie *et al.*, 1995), we believe that Grb2 inhibition induces Akt inactivation via Ras inactivation.

Even though Akt is known to be activated in response to PI3 kinase activation, some studies have suggested that Akt could be activated by PI3 kinase-independent mechanisms. For example, Filippa et al.'s studies suggested that Akt could be activated by protein kinase A (Filippa *et al.*, 1999). When Akt is activated by PI3 kinase, Ser[473] is always phosphorylated (Franke *et al.*, 1997; Alessi & Cohen, 1998); however in Filippa et al.'s studies, protein kinase A

activation of Akt did not induce Ser[473] phosphorylation (Filippa *et al.*, 1999). Since our studies showed that the Ser[473] phosphorylation of Akt was modulated by Grb2, we believe that Grb2 inhibition is followed by PI3 kinase inactivation, and thus Akt inactivation. We are currently investigating whether Ras and/or PI3 kinase is involved in the regulation of Akt activity by Grb2 protein.

Heregulin can stimulate the activation of Erk1,2 and Akt (Fiddes *et al.*, 1995; Marte *et al.*, 1995; Sepp-Lorenzino *et al.*, 1996; Liu *et al.*, 1999); both Erk1,2 and Akt are expected to play major roles in heregulin-induced cellular processes. However, here we report that activation of ErbB2 by heregulin (as well as by ErbB2 overexpression) uses Grb2 to preferentially stimulate Akt, not Erk1,2, activity to effect breast cancer mitogenesis. There have been reports demonstrating that Akt is preferred to Erk1,2 in other heregulin-induced cellular processes, such as cell cycle progression (Daly *et al.*, 1999) and cellular aggregation (Tang *et al.*, 1999). Wortmannin and LY294002, which can inhibit PI3 kinase and therefore Akt activities, can inhibit cell cycle progression and aggregation of breast cancer cells induced by heregulin (Daly *et al.*, 1999; Tang *et al.*, 1999). On the other hand, PD98059, which can inhibit MEK and therefore Erk1,2 activities, cannot inhibit these same breast cancer cellular processes induced by heregulin (Daly *et al.*, 1999; Tang *et al.*, 1999). So, it appears that heregulin may prefer PI3 kinase/Akt pathway to MEK/Erk1,2 pathway in regulating mitogenesis, cell cycle progression, and aggregation of breast cancer cells. It will be very interesting to determine what kinds of cellular processes heregulin will use Erk1,2 for, and the identity of those adaptor proteins that are involved in mediating the activation of Erk1,2 by heregulin.

Materials and Methods

Cell lines

T-47D, BT-474, and MDA-MB-453 cells were obtained from ATCC (Manassas, VA). MCF-7 cells transfected with the control neomycin vector (MCF7/V) or the heregulin β-2 gene (MCF7/T7) were obtained from Dr. Ruth Lupu (Tang *et al.*, 1996). Cells were cultured in DMEM/F12 medium supplemented with 5 or 10% heat-inactivated FBS at 37°C in a 5% CO_2 humidified incubator.

Antibodies

Monoclonal Grb2 and Actin antibodies were purchased from Transduction Laboratories (Lexington, KY) and Sigma Chemical Co. (St. Louis, MO), respectively. Polyclonal antibodies specific for Erk1,2, phosphorylated Erk1,2 (Thr^{202}/Tyr^{204}), Akt, and phosphorylated Akt (Ser^{473}) were purchased from New England Biolabs (Beverly, MA). These antibodies had been reliably used by us (Tari *et al.*, 1999; Tari & Lopez-Berestein, 2000) and others (Li *et al.*, 1998; Majeti *et al.*, 1998) to measure the phosphorylation, i.e. activation, levels of Erk1,2 and Akt. Antimouse or antirabbit secondary antibodies were obtained from Amersham Life Sciences (Cleveland, OH).

Liposomal oligodeoxynucleotides (oligos)

Nuclease resistant P-ethoxy oligos (18 bases) were purchased from Oligos Etc., Inc. (Willsonville,

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OR). We have previously reported the sequences of the Grb2 antisense and the control oligos (Tari *et al.*, 1997; Tari *et al.*, 1999). Grb2 antisense: 5'-ATATTTGGCGATGGCTTC-3'; Random control oligo: 5'-GGGCTTTTGAACTCTGCT-3'. Grb2 antisense and control oligos were mixed with dioleoyl-phosphocholine (Avanti Polar Lipids, Alabaster, AL) in the presence of tertiary butanol and prepared as described (Tari *et al.*, 1997; Tari *et al.*, 1999).

Cell growth and viability assay

Breast cancer cells were seeded between 2 to 8 X 10^3 cells/well in 96-well plates in 0.1 mL of DMEM/F12 medium supplemented with 5 or 10% FBS. The next day, heregulin β1 (Neomarkers, Union city, CA) and/or liposomal oligos were added to cells for 5-6 days before cell growth and viability was measured by the CellTiter 96® AQueous nonradioactive assay (Promega, Madison, WI). This assay measures the conversion of a tetrazolium compound, MTS, into water-soluble MTS-formazan. Each experiment was done in triplicates and repeated at least 3 times.

Western Blots

Breast cancer cells were incubated with liposomal oligos for 3 days. Untreated and treated cells were lysed, and protein lysates were prepared. Proteins were electrophoresed and electrotransferred as described (Tari *et al.*, 1999; Tari & Lopez-Berestein, 2000). The membranes were incubated with anti-Grb2 antibodies, anti-phosphorylated Erk1,2 antibodies, or anti-phosphorylated Akt antibodies, and protein bands were visualized by enhanced

chemiluminescence (Amersham Life Sciences, Cleveland, OH). The membranes were then stripped and reincubated with antibodies specific for Actin, Erk1,2, or Akt. Protein bands were visualized by enhanced chemiluminescence. Images were scanned and quantitated by an Alpha Innotech densitometer using the Alpha Imager application program (Alpha Innotech, San Leandro, CA).

Acknowledgement

Figure Legend

Figure 1 Heregulin induced the proliferation of breast cancer cells. T-47D and BT-474 cells were incubated with different concentrations of heregulin β1 in DMEM/F12 medium supplemented with 5 and 10% FBS, respectively. Cell growth was determined by the MTS proliferation assay. Growth of treated cells was compared with that of untreated cells cultured under the same FBS conditions, and was expressed as % of untreated cells.

Figure 2 L-Grb2 selectively inhibited the heregulin-stimulated proliferation of breast cancer cells. (A) T-47D and (B) BT-474 cells were plated in medium supplemented with 5 and 10% FBS, respectively. They were incubated with 0-12 μM of L-Grb2 (O, ●) or L-control (□, ■) for 5-6 days in the absence (O, □) or presence (●, ■) of heregulin. Cell growth was determined by the MTS proliferation assay. Growth of treated cells was compared with that of untreated cells, and was expressed as % of untreated cells cultured under the same heregulin conditions.

Figure 3 L-Grb2 selectively inhibited the proliferation of breast cancer cells transfected with heregulin. (A) MCF-7/T7 and (B) MCF-7/V cells were incubated with 0-12 μM final concentration of L-Grb2 (O) or L-control (□) for 5 days. Cell growth was determined by the MTS proliferation assay. Growth of treated cells was compared with that of untreated cells, and was expressed as % of untreated cells.

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Figure 4 Grb2 downregulation predominantly induced Akt inactivation in heregulin-transfected breast cancer cells. MCF7/T7 cells were incubated with liposomal oligos for 3 days. Protein lysates were obtained and western blots were performed as described. [a]Densitometric scans were performed on western blots to measure the ratios of Grb2 to Actin, phosphorylated Erk1,2 to Erk1,2, and phosphorylated Akt to Akt. [b]Grb2 inhibition was obtained by (1-ratio of Grb2:Actin in treated cells/ratio of Grb2:Actin in untreated cells) X 100%. [c]Erk1,2 inactivation was obtained by (1-ratio of phosphorylated Erk1,2 : Erk1,2 in treated cells/ratio of phosphorylated Erk1,2 : Erk1,2 in untreated cells) X 100%. [d]Akt inactivation was obtained by (1-ratio of phosphorylated Akt : Akt in treated cells/ratio of phosphorylated Akt : Akt in untreated cells) X 100%.

Figure 5 Grb2 downregulation led to Akt inactivation in ErbB2-overexpressing breast cancer cells. (A) MDA-MB-453 and (B) BT-474 cells were incubated with liposomal oligos for 3 days. Protein lysates were obtained and western blots were performed as described. [a]Densitometric scans were performed on western blots to measure the ratios of phosphorylated Akt to Akt. [b]Akt inactivation was obtained by (1- ratio of phosphorylated Akt : Akt in treated cells / ratio of phosphorylated Akt : Akt in untreated cells) X 100%.

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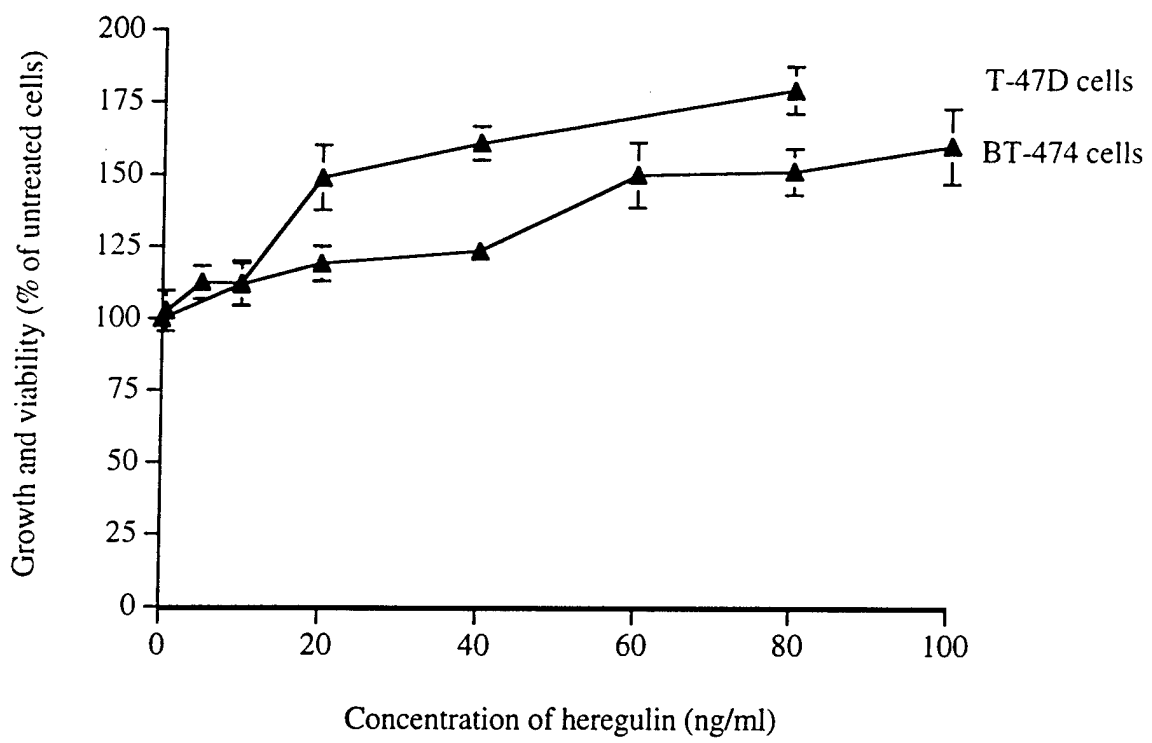
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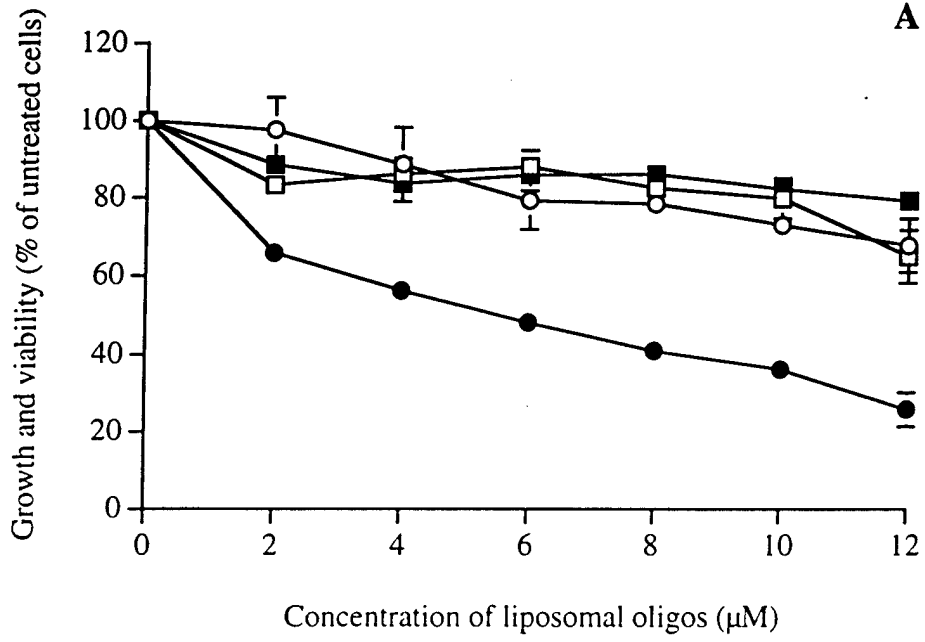
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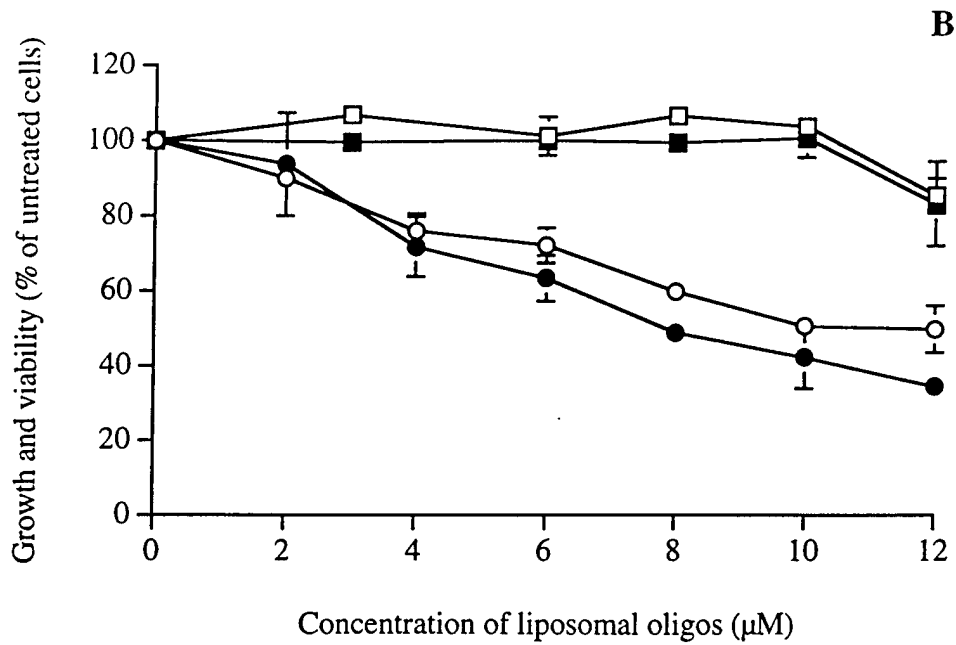
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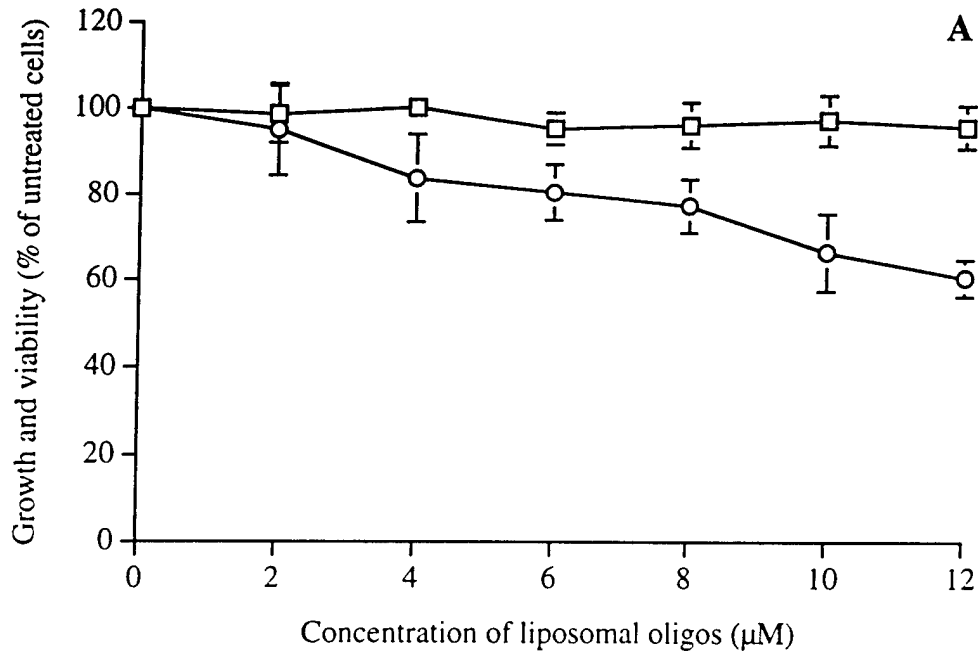
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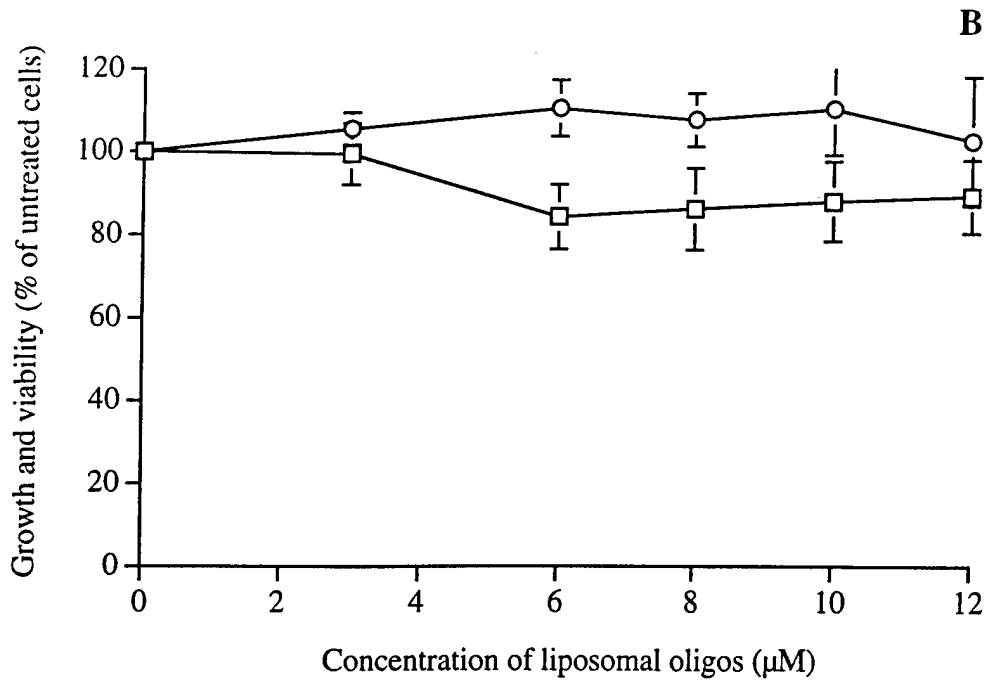
Treatment	None	L-control	L-Grb2
Concentration (µM)	0	10	10
Grb2 protein			
β-actin protein			
Grb2 : β-actin ratio[a]	51	47	31
% of Grb2 inhibition[b]	---	8	39
Phosphorylated Erk1,2			
Erk1,2 proteins			
Phosphorylated Erk1,2 : Erk1,2 ratio[a]	178	176	152
% of Phosphorylated Erk1,2 inhibition[c]	---	1	15
Phosphorylated Akt			
Akt protein			
Phosphorylated Akt : Akt ratio[a]	78	69	30
% of Phosphorylated Akt inhibition[d]	---	12	62

Treatment	None	L-Grb2	L-control
Concentration (μM)	0	8	8
Grb2 protein			
Phosphorylated Akt			
Akt protein			
Phosphorylated Akt : Akt ratio[a]	126	67	121
% of Phosphorylated Akt inhibition[b]	---	47	4
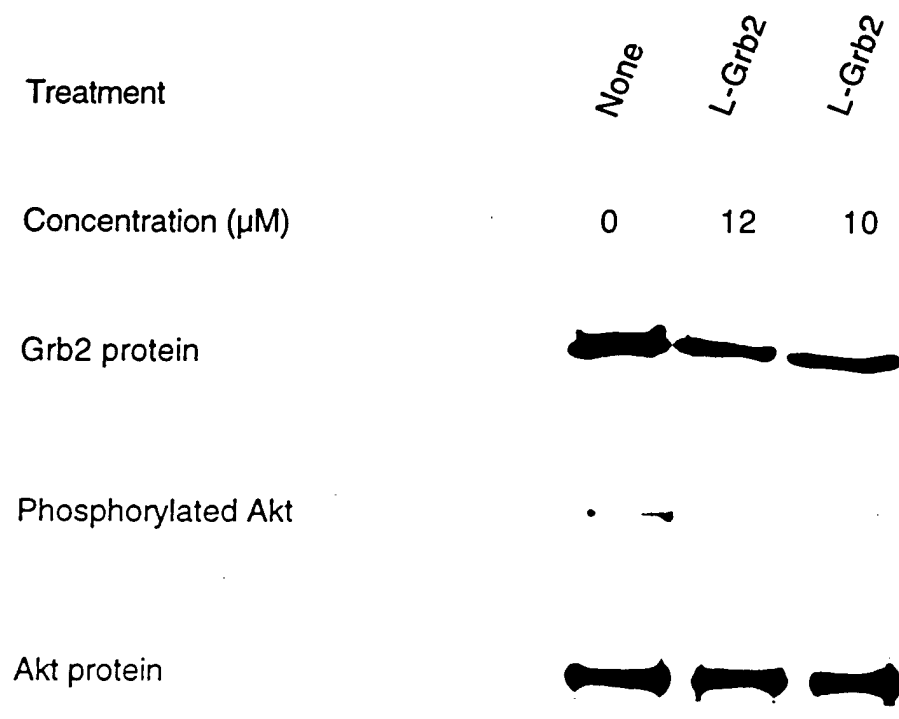
Treatment	None	L-Grb2	L-Grb2
Concentration (µM)	0	12	10
Grb2 protein			
Phosphorylated Akt			
Akt protein			
Phosphorylated Akt : Akt ratio[a]	32	19	21
% of Phosphorylated Akt inhibition[b]	---	40	34

Induction of resistance to all-trans retinoic acid by ErbB2 in breast cancer: Correlation with decreased RAR alpha expression.

Soo-Jeong Lim, Gabriel Lopez-Berestein, Pablo Zapata and Ana M. Tari

Department of Bioimmunotherapy, Section of Immunobiology and Drug carriers, The University of Texas M.D. Anderson Cancer Center, Houston, TX

Correspondence: Ana Maria Tari, Dept. of Bioimmunotherapy, Section of Immunobiology and Drug Carriers, Box 60, The University of Texas MD Anderson Cancer Center, Houston, TX 77030. Phone: (713) 794-4856. FAX: (713) 796-1731. Email:atari@mdanderson.org

Running title: ATRA resistance by ErbB2 receptor in breast cancer

Keywords: all-trans retinoic acid, breast cancer, ErbB2, EGFR, heregulin, RARα

Abstract

The sensitivity of breast cancer cells to all-trans retinoic acid (ATRA) has been known to be correlated with the estradiol receptor or retinoic acid receptor (RAR)α status. However, the fact that many of EGFR or ErbB2-overexpressing breast cancer cell lines are ATRA-resistant prompted us to investigate the role of EGFR and ErbB2 receptors in ATRA-resistance in breast cancer. We have studied the effect of overexpression of EGFR, ErbB2 or heregulin on the ATRA-sensitivity of parental MCF-7 cells, which was originally sensitive to ATRA. We further investigated the effect of ligands such as EGF and heregulin, which can activate EGFR or ErbB2, on the sensitivity of breast cancer cells to ATRA. We found that ErbB2 receptor, rather than EGFR, is involved in the acquisition of ATRA-resistance in breast cancer cells. The ATRA-resistance in ErbB2-activated cells was correlated with reduced endogenous expression of RARα protein and further correlated with ATRA-triggered dramatic decrease in RARα protein expression. These results imply that ErbB2 receptors induce the ATRA-resistance mainly by modulating RARα protein expression. The ATRA-resistance in ErbB2-overexpressing cells could be partially overcome by combination treatment with Herceptin®, which can modulate ErbB2 signaling, providing strong implications for novel treatment strategies.

Introduction

All-trans retinoic acid (ATRA), a compound structurally related to vitamin A, has been shown to induce growth inhibition, differentiation, and development in various types of cancers. ATRA functions mainly by binding to retinoic acid receptors (RAR α, β, γ), which are the members of the steroid nuclear receptor superfamily. Upon ATRA binding, these receptors bind to the retinoic acid responsive elements (RAREs) in the promoters of target genes to induce transcription. In addition to RARE transactivation, ATRA effect may also be mediated by transrepression of AP-1 activity (Toma et al., 1998; Yang et al., 1999).

ATRA can also induce growth inhibition and/or apoptosis in human breast cancer (Toma, 1997) but not in every cell line and tumor. ATRA-resistant breast cancer cell lines are generally estrogen receptor (ER)-negative and/or express low levels of RARα mRNA (Roman et al., 1992; van der burg et al., 1993; Fitzgerald et al., 1997) and RARα protein (Han). When ATRA-resistant breast cancer cell lines were transfected with ER or with RARα, they became sensitive to ATRA (Sheikh et al., 1993; Sheikh et al., 1994).

While we were comparing the characteristics of ATRA-sensitive and resistant breast cancer cell lines, we found that many ATRA-resistant cell lines have high expression of epidermal growth factor receptor (EGFR) such as MDA-MB-468, BT-20, Hs578T and MDA-MB-231, or ErbB2 receptors such as MDA-MB-453, MDA-MB-361 and BT-474 (De Luca et al., 1997 and unpublished observation) or heregulin (MDA-MB-231) (Holmes et al., 1992). Overexpression of receptor tyroine kinases such as EGFR and ErbB2 (also known as HER2/neu) has been correlated with poor prognosis and poor survival outcome in breast cancer patients (Slamon et al., 1987). Overexpression of heregulin, a ligand that can transactivate ErbB2, has

also been shown to be involved in the progression of breast cancer to a more malignant phenotype (Lupu; Adam, 1998, JBC). The EGFR and ErbB2 receptors are also involved in the acquisition of resistance to some chemotherapeutic drugs in breast cancer (Nicholson et al., 1994; Pusztai et al, 1999). Therefore, we have proposed that EGFR and ErbB2 may modulate the ATRA-sensitivity of breast cancer cells. Here we found that ErbB2 was mainly involved in the induction of resistance to the growth inhibitory effects of ATRA in breast cancer cells. Modulation of expression of RARα protein by ErbB2 was strongly suggested as the main mechanism mediating ATRA-resistance.

Materials and Methods

Cell lines

MCF-7 cells were obtained from American Type Cell Culture (Manassas, VA). Doxorubicin-resistant MCF-7 cells (MCF-7/ADR) was from Dr. Kapil Metha (Houston, TX). MCF-7 cells transfected with the heregulin β-2 gene (MCF-7/HRG), the erbB2 gene (MCF-7/HER2), and the EGFR gene (MCF-7/EGFR) were kindly obtained from Dr. Ruth Lupu (Berkeley, CA) , Dr. Mien-Chie Hung (Houston, TX) and Dr. Francis G. Kern (Washington, DC), respectively. These cells were originally named as MCF-7/T7 (Tange et al., 1996), MCF-7/HER2-18 (Benz et al., 1992) and MCE5 (Miller et al., 1994). Cells were cultured in DMEM/F12 medium supplemented with 5% heat-inactivated fetal bovine serum. MCF-7/EGFR cells were cultured in phenol red-free DMEM/F12 medium supplemented with 10% charcoal-stripped fetal bovine serum (CSS).

Antibodies and ligands

Polyclonal RARα, β, γ antibodies were purchased from Santa Cruz Co. (Santa Cruz, California). β-actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Antimouse or anti-rabbit secondary antibodies were obtained from Amersham Life Sciences (Cleveland, OH).
All-trans retinoic acid (ATRA), epidermal growth factor (EGF) and tumor growth factor α (TGF-α) were obtained from Sigma Chemical Co. (St. Louis, MO). Heregulin β1 was purchased from Neomarkers (Union city, CA). Herceptin® was kindly provided by Genetec.

Cell growth and viability assay

Breast cancer cells were seeded between 1 to 3×10^3 cells/well in 96-well plates in 0.1 ml of DMEM/F12 medium supplemented with 5% FBS. The next day, ATRA was added to cells. When the sensitivity to ATRA was studied in EGFR-transfected cells, the culture medium was changed to phenol red-free DMEM/F12 supplemented with 10% CSS before adding ATRA to maintain the high EGFR protein expression in transfected cells (Miller et al., 1994). After 5 days of incubation, cell growth and viability was measured by the Celltiter 96® AQueous nonradioactive assay (Promega, Madison, WI). Each experiment was done in tripilcates and repeated at least three times.

Western blots

Cell lysates were obtained from untreated, exponentially growing cells or from cells treated with vehicle alone or with ATRA. Proteins were electrophoresed and electrotransferred as described (Tari et al., 1999; Tari and Lopez-Berestein, 2000). Membranes were incubated with antibodies specific for RARα, RARβ or RARγ antibodies. To ensure even loading of proteins, membranes were also incubated with antibodies specific for β-actin. Protein bands were visualized by enhanced chemiluminescence (Amersham Life Sciences, Cleveland, OH). Images were scanned and quantitated by an Alpha Innotech densitometer using the Alpha Imager application program (Alpha Innotech, San Leandro, CA).

RT-PCR

Results

Overexpression of ErbB2 or heregulin reduced the sensitivity of breast cancer cells to ATRA

To investigate the involvement of ErbB receptors in the induction of ATRA resistance in breast cancer, the sensitivity of parental or vector-transfected MCF-7 cells to ATRA was compared with those transfected with EGFR, ErbB2 or heregulin. We chose MCF-7 cells because these cells are known to be sensitive to ATRA and have low but detectable levels of EGFR and ErbB2 and nondetectable level of heregulin (Tang et al., 1996). After 5 days of treatment with ATRA, the proliferation of parental MCF-7 cells (MCF-7/WT) was inhibited in a dose-dependent manner as reported by various groups (Fig. 1A). However, the proliferation of ErbB2-, heregulin-transfected cells was hardly inhibited (Fig. 1A). The sensitivity of EGFR-transfected cells was partially decreased (Fig. 1B). At 1 μM of ATRA, the growth of parental MCF-7 cells was reduced by 63.3%, while that of ErbB2-, heregulin-, or EGFR-transfected cells was reduced by 0.3, 6.1 and 39.8%, respectively. The sensitivity of vector-transfected cells (MCF-7/Ctl) to ATRA was similar to that of parental cells (data not shown). Under our experimental conditions, MCF-7/HER2 cells were the most resistant to ATRA, followed by MCF-7/HRG and MCF-7/EGFR.

Heregulin, but not EGF, reduced the sensitivity of breast cancer cells to ATRA

In addition to the receptor overexpression, EGFR can be activated by direct binding to ligands such as EGF and TGF-α while ErbB2 can be transactivated by heregulin bound to ErbB3/4 receptors. To provide further evidence that ATRA-resistance could be induced by activation of ErbB2 receptors, heregulin was added to MCF-7/WT cells simultaneously with

ATRA. Again, MCF-7/WT cells became more resistant to ATRA in the presence of heregulin (Fig. 2A). The sensitivity of T-47D cells, an another breast cancer cell line which is also sensitive to ATRA, was compared in the absence or presence of estradiol, epidermal growth factor (EGF), tumor growth factor α (TGF-α), and heregulin. These ligands were used at a concentration that can induce maximum stimulation of growth. The sensitivity of T-47D cell to ATRA was not affected by the presence of EGF, TGF-α and estradiol. However, in the presence of heregulin, T47D cells became more resistant to ATRA (Fig. 2B, data not shown for TGF-α). At 100 nM of ATRA, the growth of T-47D cells was reduced by 54.7%, 50.2, 51.4, 15.1% without any ligands, with estradiol, EGF, and heregulin, respectively. Taken together, these data further demonstrate that ErbB2, rather than EGFR receptor, induces the ATRA-resistance in breast cancer cells.

Overexpression of ErbB2 or heregulin led to the lower RARα protein expression.

Since there have been numerous reports demonstrating that the sensitivity of breast cancer cells to ATRA is correlated with the expression RARα protein (Van der burg et al., 1993), we then examined the expression of RAR expression in the MCF-7 transfectants. Western blot was used to compare the expression levels of RAR α, β, γ proteins among the various MCF-7 cell derivatives. Figure 3 shows that RARα protein expression of MCF-7/Ctl, MCF-7/EGFR, MCF-7/HRG and MCF-7/HER2 cells was 90.3, 48.0, 41.1 and 41.1% of that of MCF-7/WT cells. RARα protein was even undetectable in MCF-7/ADR cells that were completely resistant to ATRA in our study (data not shown for ATRA-sensitivity). Again, RARα protein expression was lower in BT-474 cells and barely detectable in MDA-MB-453 cells which have overexpression of ErbB2, compared with MCF-7/WT cells. RARβ protein was not detectable in any of the cell lines except very low level of RARβ was detected in MCF-7/ADR cells. The expression of RARγ

protein was similar among all the MCF-7 cell lines except MCF-7/ADR cells (Fig. 3). Taken together, ATRA-resistance was correlated with decreased expression of RARα protein, suggesting that ATRA-resistance by ErbB2 receptors was mediated, at least partly, by downregulating RARα expression.

ATRA induced the decrease in RARα protein mainly in ErB2-overexpressing cells

Recently, it has been shown that in some cell types, RARα protein, but not mRNA, expression is rapidly decreased in response to ATRA (van der Leede, MCE, 1995; Zhu, PNAS, 1999; Toma, 1998; Shang, 1999; yang, 1999). Thus, we have also examined the levels of RARα protein upon ATRA treatment. Within 1 hr of ATRA treatment, a rapid decrease in RARα protein expression could be observed in a dose-dependent manner in both MCF-7/WT and MCF-7/HER2 cell lines (Fig. 4A). However, the decrease in RARα protein expression was much faster in MCF-7/HER2 cells than in MCF-7/WT cells. After treating MCF-7/WT and MCF-7/HER2 cells with 1, 10, 100, 1000 nM of ATRA for 1 hr, 11.9, 30.8, 42.2, 56.0% and 28.3, 53.5, 67.4, 78.3% decrease in RARα protein expression were observed, respectively. No change was observed in cells treated with vehicle alone (data not shown). We then examined time-dependent change of RARα protein expression in response to ATRA. With 100 nM of ATRA, in MCF-7/WT cells, the reduced RARα protein expression rapidly recovered more than basal level within 24 hr of treatment. On the other hands, in MCF-7/HER2 cells, reduced RARα protein expression still maintained low up to 24 hrs and then began to be recovered (Fig. 4B); the RARα protein expression was 68.0, 230.0, 232.3% of untreated cells in MCF-7/WT cells and 59.1, 26.0, 165.9% of untreated cells in MCF-7/HER2 cells, after 2, 24 and 48 hr of treatment. Upon treatment with higher concentration (1 μM) of ATRA, the difference was more evident; the

RARα protein expression was 41.4, 127.9, 114.8% of untreated cells in MCF-7/WT cells and 40.8, 31.0, 31.2% of untreated cells in MCF-7/HER2 cells, after 2, 24 and 48 hr (Fig. 4B). As reported in other studies, no significant change in RARα mRNA level was observed even when cells were treated even with 1 μM of ATRA for 48 hrs (data not shown). Even with 100 μg of protein lysates, we could not detect any RARβ protein in both cell lines after treatment with 1 μM of ATRA, in spite of the previous reports showing the induction of RARβ mRNA transcripts by same concentration of ATRA. ATRA also decreased the RARγ protein expression but it was much slower in both cell lines and thus no difference was observed in both cell lines until 48 hrs (data not shown). Taken together, these data demonstrate that in MCF-7/HER2 cells, ATRA triggered further decrease in RARα protein expression, which was already lower, compared with MCF-7/WT cells.

Herceptin® increased the ATRA-sensitivity of ErbB2-overexpressing cells

Since Herceptin® has been shown to increase the sensitivity of some chamotherapeutic drugs by modulating ErbB2 siganling, we have tried to determine whether Herceptin® can increase the ATRA-sensitivity of ErbB2-overexpressing cells. MDA-MB-453 cells were treated with ATRA together with Herceptin®. Figure 5 shows that MDA-MB-453 cells was completely resistant to ATRA within all the concentration used, but the sensitivity was significantly increased in the presence of Herceptin®: no inhibition of proliferation was observed by 100, 500 and 2500 nM of ATRA alone while it was inhibited by 37.7, 54.1, 57.8% together with 1000 nM of Herceptin®, which could inhibit 16.0% of proliferation by itself.

Discussion

We found that ErbB2 receptors induce the ATRA-resistance in breast cancer as a novel mechanism. Despite the occurrence of ATRA-resistance in both EGFR- or ErbB2-overexpressing natural breast cancer cell lines, we could not find the involvement of EGFR in the ATRA-resistance. Since EGFR-overexpressing natural breast cancer cell lines are generally ER negative/ATRA-resistant but our transfected cells were still positive and quite sensitive to ATRA, in those cells, ATRA-resistance may result from ER negativity rather than directly from EGFR overexpression.

ATRA resistance can be caused by the defect in RARE transactivation or AP-1 transrepression. Reduced expression of RAR protein may limit the ability to activate RARE in the promoter of target genes which are involved in the regulation of cell growth. Either it may induce weaker repression on the activity of AP-1 transcription factors, which are involved in the stimulation of cellular proliferation. Indeed, we found lower RARα protein expression in ErbB2-activated breast cancer cells. It is not believed that RARE transcriptional activation induced by ATRA is specific for RARα. Rather, RARα or β have been shown to play a major role in the repression of AP-1 activity (Agadir, 1997; Fanjul, 1996). RARβ protein expression is barely detectable, but inducible by RARα, in breast cancer cells (Shang et al., Oncogene, 1999). Thus, lower RARα expression may lead to ATRA-resistance due to less ability to repress AP-1. Recent paper suggesting AP-1 rather than RARE as the major mechanism of ATRA effect in breast cancer provides evidence for our postulation. We are currently investigating whether the lower RARα expression, via less repression of AP-1 or less activation of RARE activities, led to the ATRA-resistance in our cells.

Our study further demonstrated that, in ErbB2-overexpressing breast cancer cells, RARα protein expression was decreased much faster in response to ATRA and the decrease prolonged for longer period, compared with MCF-7/WT cells, thereby further limiting the amount of RARα available for ATRA. It has recently been shown that ATRA can induce the degradation of RARα protein in a caspase- or proteasome- dependent manner although it activates RARα at the same time. Thus, we postulate that some pathway involved in the RARα-degradation is activated as a downstream from ErbB2 receptors.

Another mechanism may also contribute to the induction of ATRA-resistance by ErbB2 receptors, together with repressed RARα expression. Recently, it has been shown that MCF-7 cells transfected with cJun, a member of AP-1 complex, showing increased basal level of AP-1 activity, became resistant to ATRA (Yang, 1997). Basal AP-1 activity has been shown to be increased by constitutive activation of ErB2 (Galang et al., 1996) and in ATRA-resistant breast cancer cells. Thus, ErbB2 activation may increase the basal activity of AP-1 transcriptional factors, causing cells bypass the ATRA-induced AP-1 transrepression.

ATRA has been successfully used for treating APL patients. Our study suggests that ErbB2 receptor may be a major obstacle in using ATRA for breast cancer treatment. Thus, blockade of ErbB2 signaling may add therapeutic value of ATRA in breast cancer. Indeed, modulation of ErbB2 signaling by Herceptin increased the ATRA-sensitivity in our study, providing strong implications for novel treatment strategies.

Figure legends

Figure 1. Overexpression of ErbB2 or heregulin inhibited the growth-inhibitory effect of ATRA on MCF-7 cells. (A) Parental (MCF-7/WT), ErbB2-transfected (MCF-7/HER2), heregulin-transfected (MCF-7/HRG) and (B) EGFR-transfected (MCF-7/EGFR) cells were treated with different dose of ATRA for 5 days and the growth and viability of cells were determined by Celltiter 96® AQueous nonradioactive assay. Growth of treated cells was compared with that of untreated cells and expressed as % of untreated cells.

Figure 2. Heregulin predominantly decreased the growth-inhibitory effect of ATRA on (A) MCF-7 (B) T-47D cells. Cells were treated with different dose of ATRA in the presence or absence of estradiol (25 nM), EGF (100 ng/ml) or heregulin (100 ng/ml) in phenol red-free DMEM/F12 medium supplemented with 10% CSS. After 5 days, cell growth assay was done and the growth of treated cells was compared with that of untreated cells cultured under the same condition, and expressed as % of untreated cells.

Figure 3. Decrease in ATRA-sensitivity was correlated with reduced RARα expression. Protein lysates were obtained from exponentially growing untreated cells. Western blots were performed for determining RARα, β, γ protein expression as described in materials and methods. [A]Densitometric scans were performed on western blots to measure the ratio of RARα to Actin. % of RAR expression was obtained by (ratio of RAR:Actin in transfected cells/ratio of RAR:Actin in parental cells)×100%

Figure 4. ATRA-induced decrease in RARα protein expression was faster and prolonged in ErbB2-overexpressing breast cancer cells. 800,000 cells of MCF-7/WT cells and MCF-7/HER2 cells were plated at cell culture flask. Two days later, cells were treated with vehicle alone or 1, 10, 100, 1000 nM of ATRA for 1 hr (for A) or with 100 or 1000 nM of ATRA for 0, 2, 24, 48 hrs (for B). Twenty five and forty μg of protein lysates were obtained from MCF-7/WT and MCF-7/HER cells, respectively. Western blot was done as described. Following electrophoresis and eletrotransfer, the membranes were incubated with RARα and then β-actin antibody. After stripping, the membrane was then incubated with antibodies specific for RARγ. [A]Densitometric scans were performed on western blots to measure the ratio of RARα to Actin. % of RAR expression was obtained by (ratio of RAR:Actin in treated cells/ratio of RAR:Actin in untreated cells)×100%

Figure 1

A)

Growth and viability (% of untreated cells)

MCF-7/WT
MCF-7/HER2
MCF-7/T7

Concentration of ATRA (nM)

B)

Growth and Viability (% of untreated cells)

MCF-7/WT
MCF-7/EGFR

Concentration of ATRA (nM)

Figure 2

A)MCF-7/WT

Growth and Viability (% of untreated cells)

HRG +

HRG -

Concentration of ATRA (nM)

B) T-47D

Growth and viability (% of untreated ells)

10% CSS

EGF (100 ng/ml)

HRG (100 ng/ml)

Estradiol(25 nM)

Concentration of ATRA (nM)

Figure 3

Figure 4A

A) MCF-7/WT cells

ATRA (nM)   0   1   10   100   1000

RARα

β-actin

B) MCF-7/HER2 cells

ATRA (nM)   0   1   10   100   1000

RARα

β-actin

Figure 4B

1) 1000 nM of ATRA

Cell line	← MCF-7/WT →	← MCF-7/HER2 →
Time (hr)	0 2 24 48	0 2 24 48
RARα		
β-actin		

2) 100 nM of ATRA

Cell line	← MCF-7/WT →	← MCF-7/HER2 →
Time (hr)	0 2 24 48	0 2 24 48
RARα		
β-actin		

Figure 4B

1) 1000 nM of ATRA

Cell line	← MCF-7/WT ►	← MCF-7/HER2 ►
Time (hr)	0 2 24 48	0 2 24 48
RARα		
β-actin		

2) 100 nM of ATRA

Cell line	← MCF-7/WT ►	← MCF-7/HER2 ►
Time (hr)	0 2 24 48	0 2 24 48
RARα		
β-actin		

β-Catenin, a novel prognostic marker for breast cancer: Its roles in cyclin D1 expression and cancer progression

Shiaw-Yih Lin*†, Weiya Xia*†, Jo C. Wang*, Ka Yin Kwong*, Bill Spohn*, Yong Wen*, Richard G. Pestell‡, and Mien-Chie Hung*§

*Department of Molecular and Cellular Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030; and ‡Department of Medicine and Developmental and Molecular Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461

β-Catenin can function as an oncogene when it is translocated to the nucleus, binds to T cell factor or lymphoid enhancer factor family members, and transactivates its target genes. In this study, we demonstrate that cyclin D1 is one of the targets of β-catenin in breast cancer cells. Transactivation of β-catenin correlated significantly with cyclin D1 expression both in eight breast cell lines *in vitro* and in 123 patient samples. More importantly, we found that high β-catenin activity significantly correlated with poor prognosis of the patients and was a strong and independent prognostic factor in breast cancer. Our studies, therefore, indicated that β-catenin can be involved in breast cancer formation and/or progression and may serve as a target for breast cancer therapy.

Cyclin D1 overexpression has been found in ≈50% of patients with breast cancer (1, 2), whereas gene amplification accounted for only 15–20% of these cases (3). Therefore, other mechanisms such as up-regulation of gene transcription must have played a substantial role in the overexpression of cyclin D1. By analyzing the promoter region of cyclin D1, we identified a perfect T cell factor 4 (Tcf4)-binding site (CTTTGATC) located between nucleotides −80 and −73, suggesting the potential involvement of the β-catenin/Tcf4 pathway in the regulation of cyclin D1 expression. β-Catenin was first found to be a cell–cell adhesion molecule. However, recent studies have indicated that β-catenin also could be translocated to the nucleus, where it binds to Tcf/lymphoid enhancer factor (Lef) architecture factor family members and activates genes whose promoters contain the binding sites for Tcf/Lef (4–6).

Several mechanisms have been reported to cause this deregulation, including deletion of the adenomatous polyposis coli (*APC*) gene, mutation of β-catenin, and activation of the Wnt pathway (7). Although deletion of *APC* and mutation of β-catenin have been found in many types of cancers (7), so far no such defects have been reported in breast cancer. However, many studies have indicated a possible role for the Wnt pathway in breast cancer. For example, mouse Wnt1, Wnt3, and Wnt10b have been found to be among the oncogenes activated by the insertion of mouse mammary tumor virus (MMTV) (8, 9). Mammary hyperplasias also have occurred in Wnt1 transgenic mice (10). In addition, several members of the Wnt family have been shown to induce cell proliferation (11, 12). Moreover, the expression of different Wnt members has been reported to correlate with abnormal cell proliferation in human breast tissue, suggesting the possible involvement of Wnt and the β-catenin pathway in breast cancer (13–15).

Materials and Methods

Cell Lines and Transfections. All cell lines were obtained from the American Type Culture Collection and maintained in DMEM/F-12 (HyClone) with 10% (vol/vol) fetal bovine serum. Transient transfections were performed by using DC-Chol liposome provided by Leaf Huang, University of Pittsburgh. In brief,

exponentially growing 293 cells and MCF7 cells were cultured in six-well plates and transfected with 0.4 μg of reporter, 0.2 μg of pCMVβGal control, and 1 μg of effector constructs or different amounts of β-catenin expression vectors in the dose-dependent experiment or transfected with the control vector pcDNA3 (Invitrogen). The β-catenin, GSK-3β (16), and dnTcf4 effector plasmids have been described (4). Luciferase assays were performed 40 h after transfection and normalized through β-galactosidase activity. Each assay was performed triplicate. The β-catenin stable cell lines were generated by transfecting the 293 cells with the β-catenin phosphorylation mutant (S45Yβ-catenin). Individual clones were selected for resistance to 500 μg/ml G418 (Geneticin, GIBCO/BRL).

Western Blot Analysis. Cell lysates were separated by SDS/PAGE and transferred onto the nitrocellulose membrane. Protein levels were determined by using antibodies that recognized myc-tagged β-catenin, cyclin D1 (purchased from NeoMarkers, Union City, CA), and α-actin (purchased from Oncogene Science).

Gel Mobility Shift Assays. The gel-shift assays for β-catenin/Tcf4 were performed as described (4). Extracts were prepared from intact nuclei of different breast cancer cell lines. The probe was a double-stranded 15-nt oligomer, CCCTTTGATCTTACC; the control oligomer was CCCTTTGGCCTTACC. The binding reaction contained 5 μg of nuclear protein, 10 ng of radiolabeled probe, and 1 μg of poly(dIdC) in 25 μl of binding buffer (60 mM KCl/1 mM EDTA/1 mM DTT/10% glycerol). Samples were incubated on ice for 30 min, and the probes were added and incubated further at room temperature for 30 min. The β-catenin/Tcf4 bands were confirmed by the competition assays with the excess of cold wild-type or control oligomers and by comparing the complexes derived from the nuclear extract of 293 cells and its β-catenin transfectants.

Immunohistochemical Staining. Immunohistochemical staining was done by using a modification of the avidin–biotin complex technique described previously (17). The results were analyzed and confirmed by two individuals.

Results

Up-Regulation of Cyclin D1-Promoter Activity and the Protein Expression by β-Catenin. We first sought to determine whether cyclin D1 could be transcriptionally regulated by β-catenin. We found that
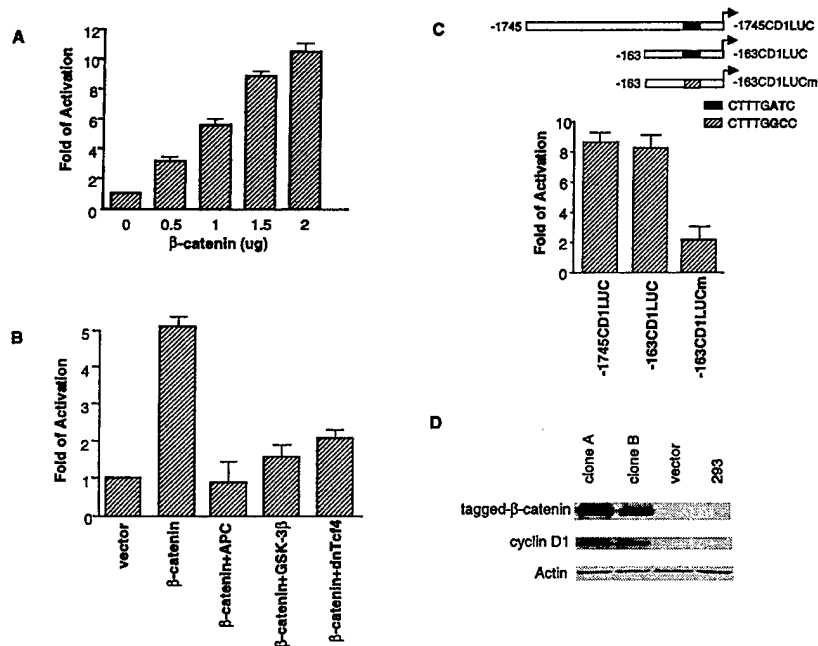

Fig. 1. Up-regulation of cyclin D1 promoter activity and the protein expression by β-catenin. (*A*) The 293 cells were transfected with 0.4 μg of cyclin D1 reporter (−1745CD1LUC) along with increasing amounts of the wild-type human β-catenin expression plasmid. (*B*) Cells were transfected with 0.4 μg of cyclin D1 reporter (−1745CD1LUC) with 1.0 μg of β-catenin expression plasmid and 1.0 μg of different negative β-catenin/Tcf regulators or with control pcDNA3 plasmid. (*C*) Cyclin D1 reporters (0.4 μg) (−1745CD1LUC, −163CD1LUC, and its mutant, 163CD1LUCm, which had an AT to GC change at nucleotides −75 and −74) and 1.5 μg of β-catenin expression plasmid were transfected into 293 cells. (*D*) The whole cell lysate of 293 cells, 293 cells transfected with empty vector, and two constitutively-activated β-catenin stable transfectants were separated by SDS/PAGE and analyzed by Western blotting with antibodies that recognized myc-tagged β-catenin, cyclin D1 (purchased from NeoMarkers), and α-actin (purchased from Oncogene).

transient transfection of exogenous human β-catenin in human embryonic kidney 293 cells could activate a cyclin D1 reporter, containing 1,745 bp of the cyclin D1 promoter, up to 11-fold in a dose-dependent manner (Fig. 1*A*). This activation was blocked when various inhibitors of the β-catenin/Tcf4 pathway were coexpressed such as APC, GSK-3β, and a dominant negative mutant of human Tcf4 (dnTcf4) (Fig. 1*B*) (18). We chose the 293 cell line to perform our studies because of its low background of β-catenin activity and its previous use for studying the response to β-catenin/Tcf4-mediated transcription (19).

To confirm that the Tcf4 site on cyclin D1 promoter was responsible for the activation by β-catenin, we used a deletion construct containing 163 bp of the cyclin D1 promoter as the reporter. Many known transcription factor binding sites had been eliminated from this construct, but it still contained the putative Tcf4 site (−163CD1LUC). As shown in Fig. 1*C*, expression of β-catenin activated this reporter to a similar extent, suggesting that the responsive element remained within this deletion construct. When the Tcf4 site was mutated so that the AT was changed to GC at nucleotides −75 and −73 (163CD1LUCm), β-catenin no longer sufficiently activated the cyclin D1 gene promoter. These data indicated that the putative Tcf4 site located at −80 to −73 was responsible for the β-catenin-mediated transactivation of the cyclin D1 promoter. In addition to transient transfection, we also generated a stable cell line by transfecting the 293 cells with the β-catenin phosphorylation mutant (S45Yβ-catenin). This mutant has been shown to resist degradation and to increase its activity to transactivate β-catenin/Tcf4-dependent transcription (19). As shown in Fig. 1*D*, cyclin D1 protein expression in both individual stable transfectants was substantially increased (lanes 1 and 2) com-

pared with the vector control cells (lane 3) and the parental cells (lane 4).

Correlation Between Cyclin D1 Expression and β-Catenin/Tcf4 Activity in Breast Cancer Cell Lines. After identifying cyclin D1 as the target gene for β-catenin, we next asked whether β-catenin played an important role in up-regulating the expression of cyclin D1 in breast cancer. We first tested this possibility in breast cancer cell lines *in vitro*. Eight breast cancer cell lines were chosen to compare their cyclin D1 expression level and their β-catenin/Tcf4 activity. We used reporter constructs that contained three repeats of wild-type (TOP) or mutant (FOP) Tcf4-binding sites (4) to determine the transactivational activity of endogenous β-catenin/Tcf4. Higher ratios of these two reporter activities (TOP/FOP) indicated a higher β-catenin/Tcf4 activity. As shown in Fig. 2*A* (*Top* and *Middle*), cyclin D1 expression in breast cancer cells highly correlated with the β-catenin/Tcf4 activity. The eight cell lines tested could be roughly divided into three groups. BT549 and HBL100 cell lines, which expressed almost no detectable cyclin D1, had the background transactivating activity of β-catenin/Tcf4 (TOP/FOP = 1). In contrast, MCF-7, which expressed the highest level of cyclin D1 protein, had the most significant β-catenin/Tcf4 activity (TOP/FOP = 10). In the other five cell lines, cyclin D1 expression was consistently moderate, as were β-catenin/Tcf4 activities. By linear regression, we demonstrated that cyclin D1 expression indeed was proportionally correlated with β-catenin/Tcf4 activity (*r* = 0.97). In addition to the reporter assay, we also confirmed the β-catenin/Tcf4 activity by gel-shift assay. Consistent with reporter activity and cyclin D1 expression levels, β-catenin/Tcf4-binding activity was not detectable for either BT549 or HBL100
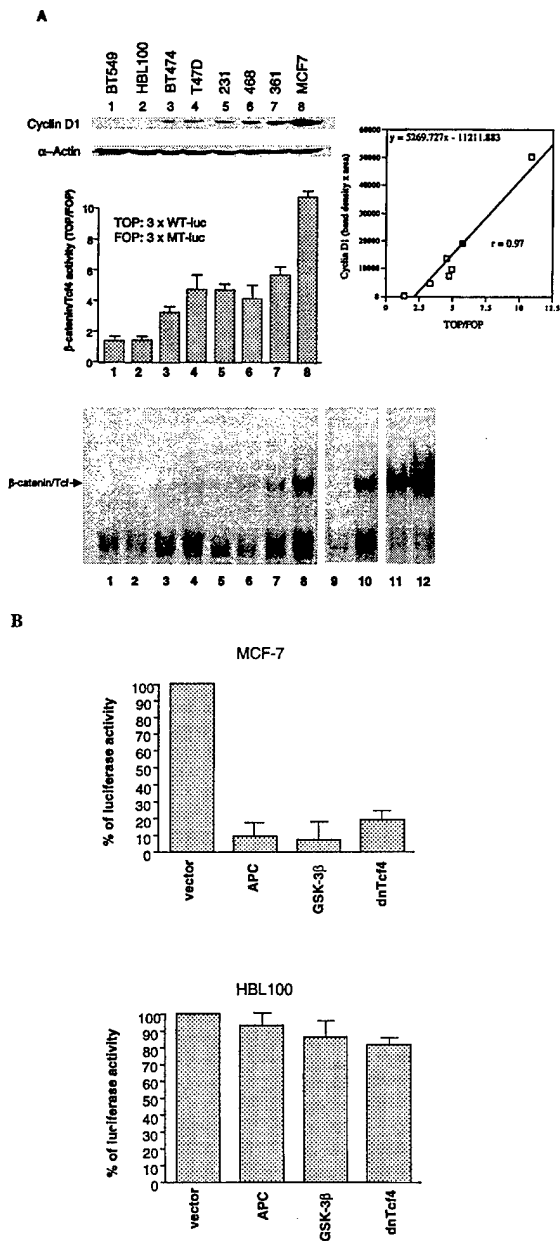
Fig. 2. Correlation between cyclin D1 expression and β-catenin/Tcf4 activity in breast cancer cell lines. (A) Cell lysates from different breast cell lines were separated by SDS/PAGE and analyzed by Western blotting with antibodies, which recognized cyclin D1 and α-actin (Top). The relative β-catenin/Tcf4 activity in different cell lines were determined by the TOP/FOP luciferase activities in each cells (Middle). The density of cyclin D1 bands were quantitated (by NIH IMAGE, an analyzing software) and plotted with β-catenin/Tcf4 activity (TOP/FOP) with the r = 0.967 by linear regression. Also, the DNA-binding activities of β-catenin/Tcf4 were determined by gel-shift assay as described previously (Bottom) (4). Lanes 1–8 show the β-catenin/Tcf4-binding activity in indicated cell lines. Lanes 9–12 are the controls to demonstrate the specific binding. Lane 9 and 10, the same as lane 8 except 60-fold excess of wild-type (lane 9) or the mutant (lane 10) cold oligonucleotide was added; lane 11, nuclear extract was from the 293 vector control line; lane 12, nuclear extract was from 293 β-catenin stable line. (B) MCF-7 cells (Top) or HBL100 cells (Bottom) were cotransfected with cyclin D1 reporter (−1745CD1LUC) with different negative β-catenin/Tcf regulators or with the control pcDNA3 plasmid. The absolute luciferase activity of cyclin D1 reporter alone in MCF-7 cells was ≈7-fold higher than that in HBL100 cells.

cells and was detected most strongly in MCF-7 cells as shown in Fig. 2A Bottom.

To further address whether cyclin D1 promoter activity is indeed regulated by β-catenin in these breast cancer cell lines, we cotransfected the cyclin D1 reporter with different negative regulators of the β-catenin/Tcf4 pathway in MCF-7 cells. As shown in Fig. 2B Top, the reporter activity of cyclin D1 promoter was significantly reduced. This reduction of activity could be reversed when β-catenin was coexpressed (data not shown). In contrast, cyclin D1 reporter activity was not affected by the expression of APC, GSK-3β, or dnTcf4 in HBL100 cells in which both β-catenin activity and cyclin D1 expression were low (Fig. 2B Bottom). Our data, therefore, support a substantial role for β-catenin in activating cyclin D1 expression in breast cancer cells.

Correlation Between Activated β-Catenin and Cyclin D1 Overexpression and Their Association with Poor Patient Survival Rate. Because cyclin D1 overexpression has been well-documented in patients with breast cancer, we next sought to clinically verify whether β-catenin activity truly contributed to the cyclin D1 overexpression in breast cancer tissues. We determined both cyclin D1 expression and β-catenin activity in 123 primary human breast cancer tissues (age: 26–87 years old; medium: 48 years old) by immunohistochemical staining (Fig. 3A). We determined β-catenin activity by its subcellular localization (20, 21). It has been well documented that accumulated β-catenin in cytoplasm and/or the nucleus increased when cells had stabilized β-catenin and, consequently, the activated β-catenin/Tcf4 activity. In contrast, β-catenin was localized solely at the plasma membrane of cells when its transactivation activity was low. We also have confirmed the correlations between the β-catenin localization and its transactivation activity in various breast cell lines listed in Fig. 2 (data not shown).

As shown in Table 1, the subcellular localization of β-catenin and cyclin D1 was significantly correlated based on the analysis by Spearman rank correlation ($r = 0.6$, $P < 0.001$). The samples stained as either high β-catenin activity with high cyclin D1 expression (40%) or low β-catenin activity with negative cyclin D1 staining (37%). It is worthwhile to mention that, among the 53 cases staining positive for cyclin D1, 49 cases (92%) were positive for β-catenin activity (stained in cytoplasm/nucleus). Thus, the correlation between these two molecules in the primary tumor samples was consistent with our in vitro data from the breast cancer cell lines (Fig. 2A). Therefore, we believe that high β-catenin activity may significantly contribute to cyclin D1 overexpression in breast cancer. These results not only supported our molecular data described above but also further strengthened their clinical biological significance.

More importantly, when the prognostic significance was assessed by Kaplan-Meier analysis and log-rank test, we found that both cyclin D1 overexpression and activated β-catenin were associated with a poorer prognosis and were negatively correlated with patient survival rates ($P = 0.033$ and $P < 0.001$, respectively) (Fig. 3B).

To determine whether activated β-catenin was independent of other known prognostic factors in prognosis, multivariate analyses for survival rate were also performed. We found that activated β-catenin was a strong prognostic factor that provided additional and independent predictive information on the patient's survival rate even when other prognostic factors (lymph node metastasis, estrogen receptor and progesterone receptor status, and tumor size) were taken into account ($P = 0.001$). Cyclin D1 overexpression was also an independent prognostic factor. However, when multivariate analysis was performed including only cyclin D1 expression and β-catenin activity, cyclin D1 was no longer an independent prognostic factor (the P values for cyclin D1 and β-catenin activity were $P = 0.457$ and $P <$
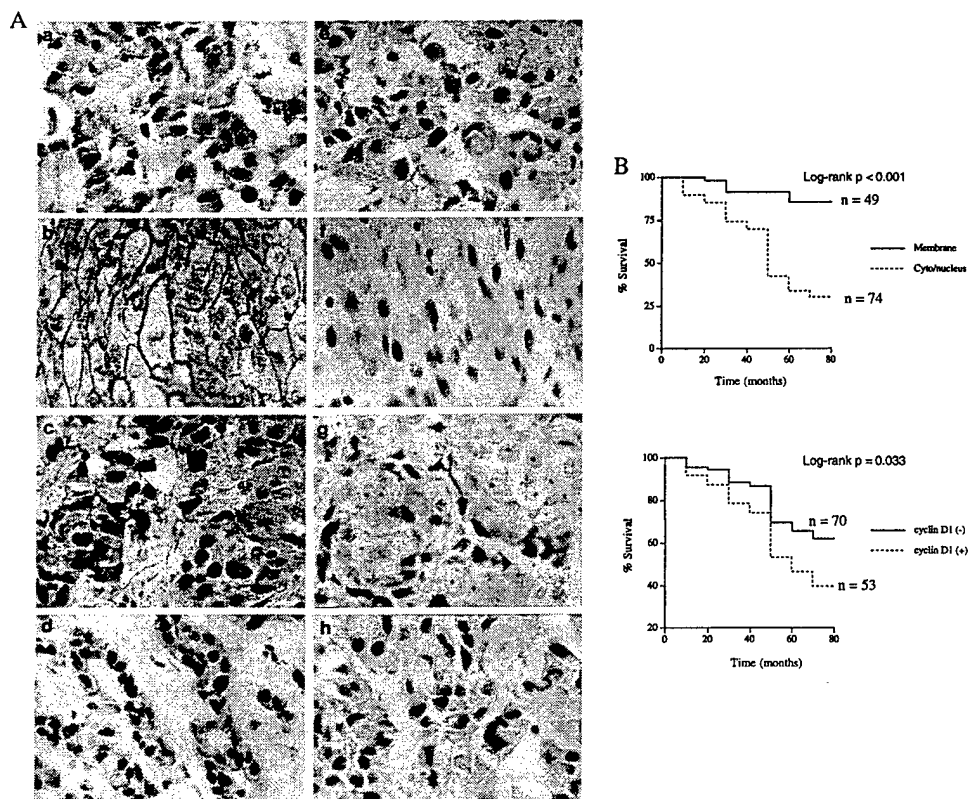
Fig. 3. Correlation between activated β-catenin and cyclin D1 overexpression and their association with poor patient survival rate. (*A*) Breast cancer tissue stained with β-catenin antibody (*a*, cytoplasm/nucleus; *b*, membrane) and cyclin D1 antibody (*c*, overexpression; *d*, negative). The right panels (*e–h*) showed the respective negative controls for a-d using PBS instead of primary antibodies. (*B*) Kaplan-Meier analysis for survival correlated with the subcellular localization of β-catenin (*Top*) and cyclin D1 expression (*Bottom*). The medium of follow-up of patients was 48 months.

0.001, respectively). These results were consistent with the model that cyclin D1 overexpression could be caused by activated β-catenin in breast cancer and consequently correlated to the prognosis.

Discussion

Our studies demonstrated that β-catenin was a poor prognostic marker in human cancer and was implicated in human breast cancer. How β-catenin activity is up-regulated in breast cancer is not clear at this moment. It is possible that activated Wnt

Table 1. Immunoreactivity of β-catenin and cyclin D1 in surgical specimens of breast cancer

	Cyclin D1 staining		
β-Catenin staining	Negative (*n* = 70)	Positive (*n* = 53)	Total
Cytoplasm/nucleus	25 (20%)	49 (40%)	74 (60%)
Membrane only	45 (37%)	4 (3%)	49 (40%)
Total	70 (57%)	53 (43%)	123 (100%)

The 123 surgical specimens of breast cancer were stained with either β-catenin antibodies (purchased from Transduction or Santa Cruz Biotechnology) or cyclin D1 antibody (NeoMarkers) as shown in Fig. 3*A*. The expression patterns of these two molecules in the samples from each patient were determined and summarized. Correlation of subcellular localization of β-catenin and cyclin D1 expression was analyzed by Spearman rank correlation (*r* = 0.6, *P* < 0.001).

pathway may contribute to this up-regulation (13–15). It requires further studies to elucidate the detail mechanisms.

In the past, β-catenin pathway has been studied mainly in colon carcinoma. Almost 100% of colon cancers have either mutated β-catenin or deleted APC, which is expectedly to activate the β-catenin pathway. In fact, during the time period of our studies, two groups identified cyclin D1 as the β-catenin target in colon carcinoma (22, 23). However, it is worthwhile to mention that cyclin D1 overexpression has been found in only ≈30% of colon cancer (24, 25), which might not be consistent with almost 100% deregulation of the β-catenin pathway, suggesting that the overexpression of cyclin D1 in colon cancer may be more complicated than purely up-regulation by β-catenin. Here, we showed that cyclin D1 was one of the targets for β-catenin in breast cancer. More importantly, we demonstrated the significant role of activated β-catenin in breast cancer both by molecular studies in cell culture and by clinical studies on breast tumor samples. Consistent with these findings, our studies provide strong evidence supporting the biological significance and clinical relevance of this pathway in human breast cancer. In contrast to colon carcinoma, the strong correlation between β-catenin activity and cyclin D1 expression was found in both breast cancer cell lines and breast patient tissue samples. Thus, the data presented in this study may open a new direction in the research of breast cancer involving both cancer formation and progression and provide an opportunity for development of potential therapy by blocking the β-catenin/Tcf4 pathway in breast cancer cells.

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Lin *et al.*

Transcriptional upregulation and activation of p55Cdc via p34^{cdc2} in Taxol-induced apoptosis

Keishi Makino, Dihua Yu, and Mien-Chie Hung[2]

Department of Molecular and Cellular Oncology [K. M., M-C. H.], and Department of Surgical Oncology [D. Y., M-C. H.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Runnning title: Role of p55Cdc in Taxol-mediated apoptosis

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[2]To whom requests for reprints should be addressed at The Department of Molecular and Cellular Oncology, Box 108, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030.

Phone: (713) 792-3668; Fax: (713) 794-0209; E-mail: mchung@odin.mdacc.tmc.edu.

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Paclitaxel (taxol) is a potent and highly effective antineoplastic agent for the treatment of advanced, drug-refractory, metastatic breast cancers. Taxol not only induces tubulin polymerization, stabilizes microtubules, blocks cell-cycle progression, and induces apoptosis, but it also alters gene expression. Here, we have identified that Taxol can upregulate expression of the gene encoding the cell-cycle protein p55Cdc by using cDNA microarray technique. Taxol induced p55Cdc mRNA expression through activation of the p55Cdc promoter, which led to increase p55Cdc protein expression. Taxol also activated p55Cdc-associated kinase. In addition, overexpression of the p55Cdc gene resulted in cell death in HeLa cells in a dose-dependent manner. A dominant-negative mutant of $p34^{cdc2}$ blocked Taxol-induced p55Cdc activation and inhibited p55Cdc-induced and Taxol-induced cell death. Our data suggest that transcriptional upregulation of p55Cdc and activation of p55Cdc by Taxol-mediated $p34^{cdc2}$ activation play a critical role in Taxol-induced cell death.

2.

INTRODUCTION

Paclitaxel (Taxol), a diterpene compound, was originally isolated from the stem bark of *Taxus brevifolia* and shown to have antiproliferative activity in various cultured cancer cells and antineoplastic activity in cancer patients (1). These effects appear to be related to Taxol's ability to bind tubulin, to promote microtubule assembly, and to stabilize microtubules by bundle formation (2-4). Taxol has also been shown to alter gene expression (5, 6). Thus, identification and characterization of changes in gene expression profiles in response to Taxol treatment may lead to a better understanding of mechanisms for Taxol-mediated activity and facilitate design of novel cancer therapies. The recently described cDNA microarray or DNA chip technology allows monitoring of expression of thousands of genes simultaneously and provides a format for identifying genes and changes in their activities (7-10). The p55Cdc gene product is a cell-cycle protein expressed in proliferating cells but not in differentiated, growth-arrested cells (11). This protein has homology to *Saccharomyces cerevisiae* proteins Cdc4 and Cdc20, which are believed to regulate DNA synthesis and mitosis, respectively (11, 12). Furthermore, overexpression of p55Cdc in a myeloid leukemic cell line resulted in increased serum- and factor-starved apoptosis, and inducible expression increased cell death in the same cell line (13, 14). Using the cDNA microarray technique in this study, we found that Taxol could induce p55Cdc gene expression in HeLa cells. Taxol also induced both p55Cdc mRNA and protein expression in a dose-dependent manner. Furthermore, p55Cdc expression resulted in apoptosis, but dominant-

negative p34[cdc2] could inhibit p55Cdc-induced and Taxol-induced cell death. These results suggest that p55Cdc may play a role in Taxol-mediated apoptosis.

Materials and Methods

Cell culture. HeLa cells (human epithelioid cervical cancer cell line) and human breast cancer cell line MCF-7 were maintained in DMEM/F12 medium containing 10% fetal bovine serum (37°C, 5% CO_2). MCF-10A cells (normal human breast epithelial cell line) were maintained in DMEM/F12 medium containing 5% horse serum with insulin (10 μg/ml), epidermal growth factor (20 ng/ml), cholera endotoxin (100 ng/ml), hydrocortisone (0.5 μg/ml), and calcium (1.05 mM). Cells were seeded at densities of 1 X 10^5 cells/ml so that they grew in log phase for the duration of each experiment. Two days after seeding, cells were grown in fresh medium with Taxol (0.1 μM) or without Taxol (Bristol-Meyers Squibb Co., Wallingford, CT) for 24 h.

Differential Hybridization of cDNA Expression Arrays. Total RNA was isolated from cells using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the protocol recommended by the manufacturer. Poly (A) RNA was purified from 250-300 μg of total RNA using the Oligotex purification (QIAGEN, Valencia, CA) according to the protocol recommended by the manufacturer. RNA was precipitated at -70°C for 1 h with 3 M sodium acetate (pH 5.2) and 100% ethanol. After drying, the RNA pellet was resuspended in 7.5 μl of DEPC-treated water and 2 μl of CDS primer (0.02 μM) (Clontech, Palo Alto, CA) and incubated at 70°C for 10 min. After placing the reaction mixture on ice, 2 μl of 10X reaction buffer, 2 μl of 25 mM $MgCl_2$, 1 μl of dNTPs (5mM dATP, dGTP, and dTTP, and 50 nM dCTP), 2 μl of 100 mM DTT, and 3.5 μl of [α– ^{32}P]dCTP (10 μCi) were added, and the mixture was incubated at 42°C for 5 min. One microliter of SuperScriptII reverse

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transcriptase (Life Technologies, Gaithersburg, MD) was added, and incubated at 42°C for 1 h. After purification using a spin column, cDNAs were hybridized to two Atlas human cDNA expression arrays (Clontech, Palo Alto, CA) in separate bags for 18 h at 50°C. After washing with 0.1X SSC and 0.1% SDS at room temperature for 10 min, and with 0.3X SSC and 0.1% SDS at 68°C for 30 min, the expression arrays were exposed to a phosphoimaging plate for 18 h. The data were analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The GAPDH gene was used as an internal control for hybridization efficiency and cDNA concentration.

RT-PCR. Reverse transcription was performed on 5 µg of total RNA using the SuperScript preamplification system (Life Technologies, Gaithersburg, MD). The full-length ORF of the p55Cdc cDNA was amplified by PCR from 5% of the RT products using forward primer (5'-GGGCTCTAGAATGGCACAGTTC-3') and reverse primer (5'-GGGTTCTAGATCAGCGGATGCCT-3'), both containing a XbaI site (underlined). Thirty cycles of RT-PCR were performed with denaturation 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Products were analyzed by electrophoresis through a 1% agarose gel.

Western blotting. Equal amounts of protein from cell lysates were separated on 8% polyacrylamide gels and transferred to nitrocellulose filters. Each filter was blocked at room temperature for 1 h with PBS containing 0.05% Tween 20 and 5% dry milk. They were then incubated at 4°C overnight in the same solution with anti-HA antibody (1 : 1000) (Boehringer-Mannheim Corp., Indianapolis, IN), anti-p55Cdc antibody (1 : 600) (Santa Cruz, Santa Cruz, CA), or anti-actin antibody (1 : 3000) (Sigma, St. Lous, MO). The filters were washed three times for 10 min each in PBS containing 0.05% Tween 20, and then incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody against

HA, anti-goat IgG antibody against p55Cdc, or anti-rabbit IgG antibody against actin (Boehringer-Mannheim Corp., Indianapolis, IN) for 40 min. Specific proteins were detected using an enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL).

Immunoprecipation and kinase assays. 293T cells were cotransfected with pCGN-p55Cdc or a pGEM control vector and either pCMVcdc2-dn (a dominant negative p34^{cdc2} expression vector) or a pCDNA3 control vector encapsulated in DC-Chol liposome. Forty-eight hours later, cells were lysed with immunoprecipitation (IP) buffer [0.5% NP-40, 150 mM NaCl, 50 mM Tris (pH 8.0)] containing 2% aprotinin, 5 mM PMSF, 100 mM NaF, 2 mM Na$_3$VO$_4$, and 1 mM EDTA. Five hundred micrograms of protein from each sample was incubated at 4°C with 1 µg of antibody overnight and for another 2 h after addition of protein G-agarose. The immunoprecipitates were washed three times with IP buffer and twice with kinase buffer [20 mM HEPES (pH7.4), 150 mM KCl, 5 mM MnCl$_2$, 5 mM NaF, 1 mM DTT] and then resuspended in 40 µl of kinase buffer containing 5 µg of myelin basic protein and 10 µCi of [γ-^{32}P]ATP. Following 30 min incubations at 30°C, the reactions were terminated with 40 µl of 2 X Laemmli SDS sample buffer. Samples were incubated for 5 min at 96°C and resolved by 15% SDS-PAGE.

Cloning of the p55Cdc promoter and generation of a p55Cdc promoter-luciferase reporter construct. Rat genomic DNA was isolated from the normal rat fibroblast cell line Rat1using Insta gane Matrix (Bio-Rad, Hercules, CA) according to the protocol recommended by the manufacturer. A 1000 bp region of the rat p55Cdc promoter was amplified by PCR using forward primer (5'-CCC<u>AAGCTT</u>GGGGCTTCCCTTCTTC-3') and reverse primer (5'-TA<u>AAGCTT</u>CGCCGAACAGTTATT-3'), both containing a HindIII site (underlined). Thirty-five cycles were performed with denaturation at 95°C for 1 min,

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annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR fragments were digested with HindIII, and ligated into a pGL2 vector (Promega, Madison, WI) containing a luciferase reporter gene.

Luciferase assays. HeLa cells were cotransfected with the pGL2-p55Cdc promoter vector and a β-actin-lacZ vector using the cationic liposome method. Cells were lysed with 1 X lysis buffer (Promega, Madison, WI) after 9, 24, 36, and 48 h. After centrifugation, half of the supernatant was used for a β-galactosidase (β-gal) assay to determine transfection efficiency, and one sixth was added to reconstituted luciferase assay reagent in a luciferase assay kit (Promega). Light emission was detected by a luminometer.

Construction of the p55Cdc expression vector. RT-PCR fragments from above were digested with XbaI and ligated into a pCGN expression vector containing an HA epitopetag. The pCGN-p55Cdc vector was transfected into HeLa cells by the liposome-mediated gene transfer method.

Results and Discussion

To elucidate Taxol-mediated gene expression profiles in cultured cells, we performed differential hybridization analysis of cDNAs from HeLa and MCF-10A cell lines grown with or without Taxol treatment and then hybridized to an Atlas cDNA expression array (Clontech). The expression of 8 genes in HeLa and 17 genes in MCF-10A cells were upregulated with Taxol treatment. Among these, p55Cdc expression was significantly upregulated in both HeLa (Fig. 1A) and MCF-10A cells (data not shown). To confirm upregulation of p55Cdc expression by Taxol, we performed RT-PCR in various cell lines. The upregulation was detected in HeLa and MCF-7 cells (Fig. 1B). To determine whether an increase in p55Cdc RNA resulted in an increase in p55Cdc protein, protein lysates were

prepared from HeLa and MCF-7 cells after Taxol treatment. As shown in Figure 2A, 0.1 µM Taxol clearly caused an increase in p55Cdc protein levels in these cell lines. Time course experiments further indicated that the Taxol-mediated increase in p55Cdc protein was detectable 8 hours after Taxol treatment in HeLa cells (data not shown).

The p55Cdc protein is known to play a role in mitosis (15, 16), Taxol induce mitotic block at the metaphase/anaphase transition in HeLa cells by stabilizing spindle microtubles (17). Activity of the p55Cdc-associated myelin basic protein kinase peaks in the G2/M phase in HeLa cells, paralleling Taxol-induced mitotic block (18). To examine whether Taxol could activate the p55Cdc-associated kinase, p55Cdc proteins were immunoprecipitated with anti-p55Cdc antibody from Taxol-treated or -untreated HeLa cell lysates and tested for the kinase activity of phosphorylating of myelin basic proteins. As shown in Figure 2B, Taxol not only induced expression of p55Cdc, but also enhanced activity of the p55Cdc-associated kinase.

Since Taxol induced p55Cdc RNA expression, we next investigated whether it did so by inducing transcriptional activity of the p55Cdc promoter. We subcloned 1000 bp of the rat p55Cdc promoter into the pGL2 vector, which contains the luciferase reporter gene. The construct was transiently transfected into HeLa cells with or without Taxol treatment, and then the transcriptional activity of the p55Cdc promoter was measured at various time. After 48 hours, the relative activity of the p55Cdc-driven luciferase reporter gene was 5.6 times greater in the treated cells (Fig. 3). Thus, Taxol can activate the p55Cdc promoter and enhance p55Cdc transcription, which contributes to increased p55Cdc RNA and protein expression and p55Cdc-associated kinase activity.

To address whether p55Cdc plays a functional role in Taxol-mediated responses, we examined the effect of increased p55Cdc expression on programmed cell death. We

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amplified the full-length ORF of the p55Cdc cDNA, which was subcloned into pCGN-p55Cdc and transiently transfected into HeLa cells. The pCGN-p55Cdc expression vector and a pCMV-luciferase reporter gene construct expressed in living cells but not in apoptotic cells were cotransfected into HeLa cells. Luciferase activity was quantitated and normalized by β-gal activity. Expression of p55Cdc resulted in decreased survival in a dose-dependent manner (Fig. 4A). Thus, upregulation of p55Cdc expression can lead to increased apoptosis.

Since Taxol can induce both cell death and p55Cdc expression, we ask whether Taxol-induced p55Cdc expression may contribute to Taxol-induced apoptosis. We had previously shown that activation of p34^{cdc2} is required for Taxol-induced apoptosis (19). Because p55Cdc is phosphorylated by cdc2-cyclinB, and this phosphorylation is required for p55Cdc-dependent APC/cyclosome activation (16), we therefore investigate whether Taxol-induced p34^{cdc2} activation is involved in Taxol-mediated p55Cdc activation and if a potential relationship between p34^{cdc2} and p55Cdc induces cell death. We cotransfected pCGN-p55Cdc and pCMV-cdc2-dn expression vectors into HeLa cells. The dominant-negative mutant of cdc2 inhibited p55Cdc-induced (Fig. 4B-1) and Taxol-induced cell death (Fig. 4B-2). Furthermore, forced expression of p55Cdc and Taxol treatment resulted in activation of the p55Cdc-associated kinase. Expression of dominant-negative cdc2 inhibited p55Cdc overexpression- and Taxol-induced p55Cdc-associated kinase activity (Fig. 4C).

As mentioned previously, p55Cdc-associated kinase activity is correlated with Taxol-induced mitotic block. This mitotic block is followed by an abnormal exit from mitosis into an interphase-like state with no accompanying cytokinesis. The block appears to be sufficient to inhibit further cell proliferation and to induce cell death by apoptosis (20). In

the present work, we showed that Taxol can induce p55Cdc, which is a known regulator of mitosis. The data suggest that Taxol induces cell death not only by regulating stabilization of microtubles but also by directly regulating mitotic regulators. In this regard, both transcriptional upregulation of p55Cdc and activation of p55Cdc by Taxol-mediated $p34^{cdc2}$ kinase activity may contribute to the Taxol-induced cell death (Fig. 4D).

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Legends

Fig. 1. Taxol can induce p55Cdc gene. A. HeLa cells were treated with 0.1 µM Taxol (+) or without Taxol (-), and Gene expression was analyzed using cDNA microarray. Arrows indicate p55Cdc gene expression. B. HeLa and MCF-7 cells were treated with (+) or without (-) Taxol for 24 h, and p55Cdc gene expression was analyzed by RT-PCR. GAPDH was used as a control.

Fig. 2. Taxol can induce p55Cdc protein expression and activate the p55Cdc-associated kinase. A. p55Cdc protein expression was analyzed in HeLa and MCF-7 cells by Western blot analysis after exposure to Taxol for 24h. Actin was used as a protein loading control. The numbers at the bottom show fold induction. B. Extracts were prepared from HeLa cells treated with 0.1 µM Taxol (+) or without Taxol (-) for 24 h and incubated with anti-p55Cdc antibody. Immunoprecipitates were assayed for associated kinase activity toward myelin basic protein (MBP). Samples were analyzed by SDS/PAGE followed by Phosphoimaging.

Fig. 3. Activation of the p55Cdc promoter with Taxol treatment. Five micrograms of the luciferase reporter gene driven by the p55 promoter were transfected into HeLa cells on 100 mm dishes. Twenty-fours after transfection, cells were split onto 6-well plates with (+) or without Taxol (-). Luciferase activity was measured at indicated time points and normalized by measurement of protein concentration and β-gal activity. Data represent the

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average of three independent experiments.

Fig. 4. Overexpression of p55Cdc can induce cell death, and dominant-negative cdc2 can rescue p55Cdc-induced cell death. A. HA-tagged p55Cdc expression vectors and control vectors were cotransfected with luciferase reporter plasmids and β-gal plasmids for normalizing transfection efficiencies. Cells were harvested, luciferase activity was measured, and survival ratios (HA-p55Cdc/vector only) were determined. Data represent the average of three independent experiments. HA-tagged p55Cdc protein expression was confirmed by Western blot analysis. B. Inhibition of p55Cdc-induced and Taxol-induced cell death by the dominant-negative mutant of p34^{cdc2} (DN-cdc2). (1) HA-tagged p55Cdc expression vectors or control vectors were cotransfected into HeLa cells with DN-cdc2 or mock vectors and with luciferase and β-gal plasmids normalizing transfection efficiencies. Luciferase activity was measured, and % cell death [(control - p55Cdc) /control] was determined and compaired between DN-cdc2 and mock transfections. (2) HeLa cells were cotransfected with DN-cdc2 or mock vectors and luciferase and β-gal plasmids. Luciferase activity was measured, and % cell death [(nontreatment - Taxol) /nontreatment] was determined and compared between the two effector plasmids. Data represent the average of three independent experiments. C. Inhibition of p55Cdc-induced and Taxol-induced p55Cdc associated kinase activity DN-cdc2. Cells were treated as in Figure 4B and were collected and assayed for p55Cdc-associated kinase activity as in Figure 2C. The protein levels of HA and p55Cdc were determined by Western blot analysis. Actin was

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used as a protein loading control. D. A model to demonstrate Taxol-mediated

cell death through p34^{cdc2} and p55Cdc.

A
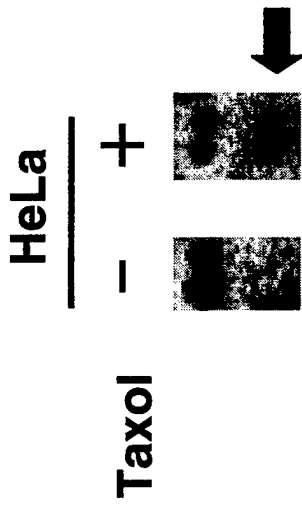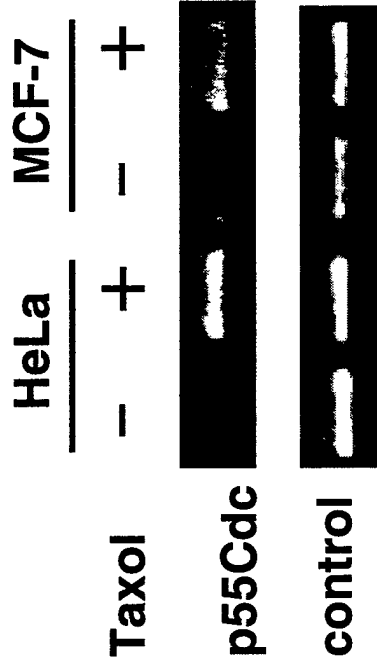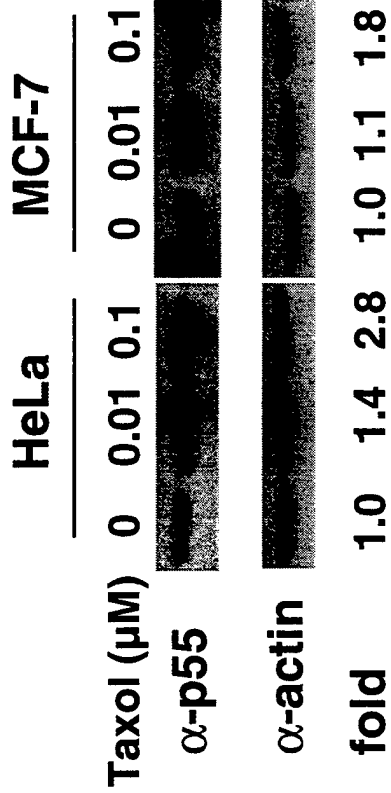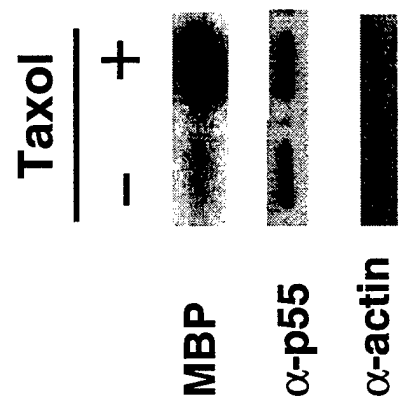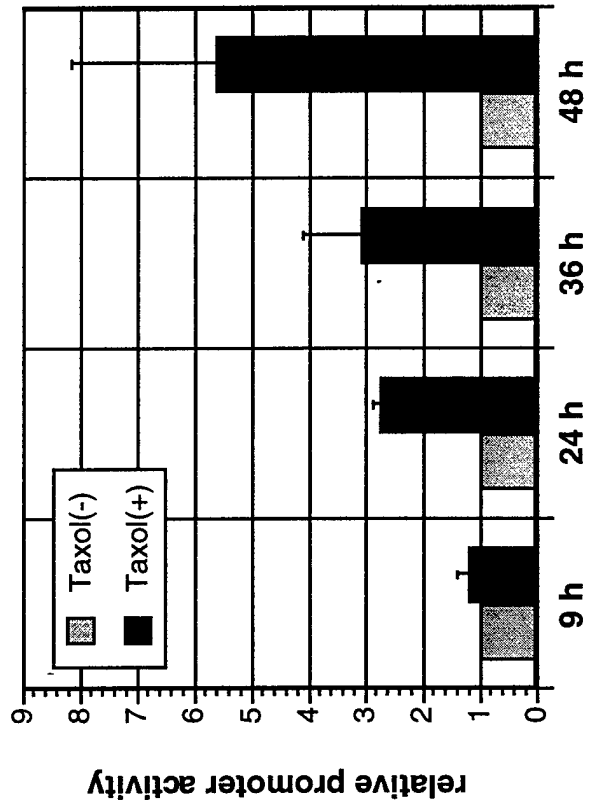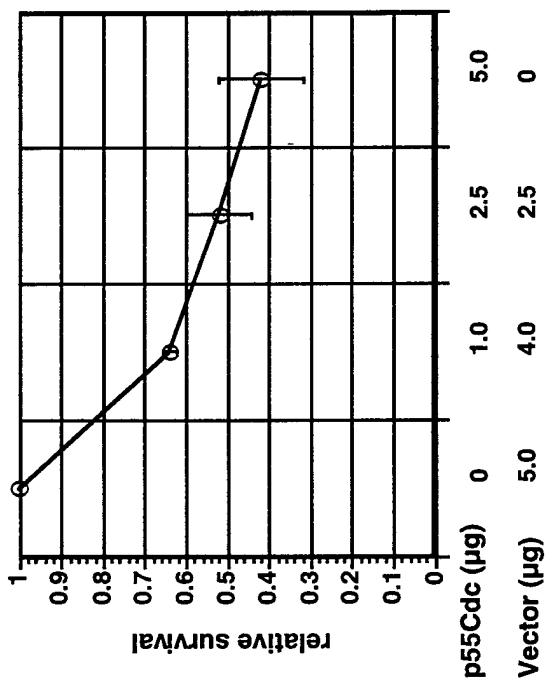
Taxol

HeLa
— +

B

Taxol

HeLa   MCF-7
— +   — +

p55Cdc

control

A

Taxol (μM)   HeLa   MCF-7
              0   0.01 0.1   0   0.01 0.1

α-p55

α-actin

fold   1.0 1.4 2.8   1.0 1.1 1.8

B

              Taxol
              —   +

MBP

α-p55

α-actin
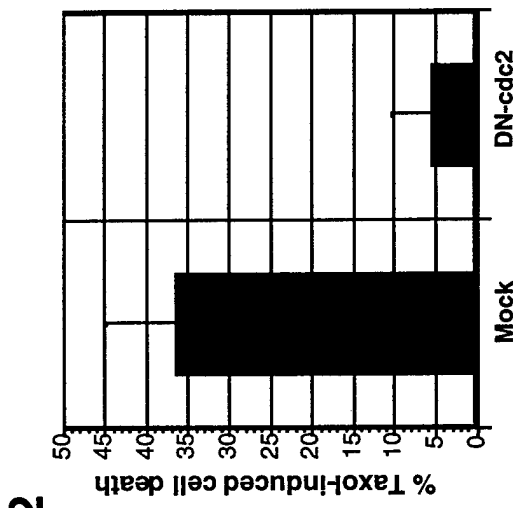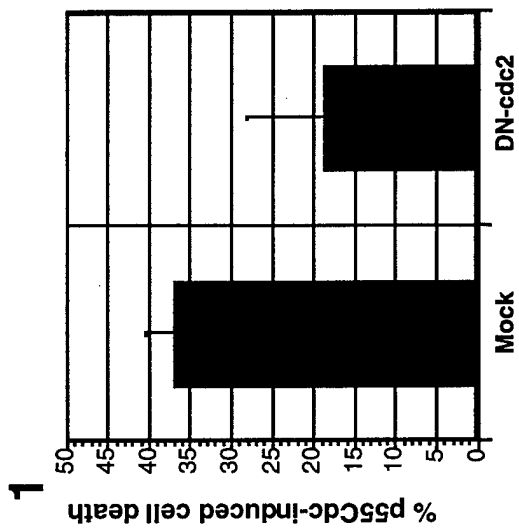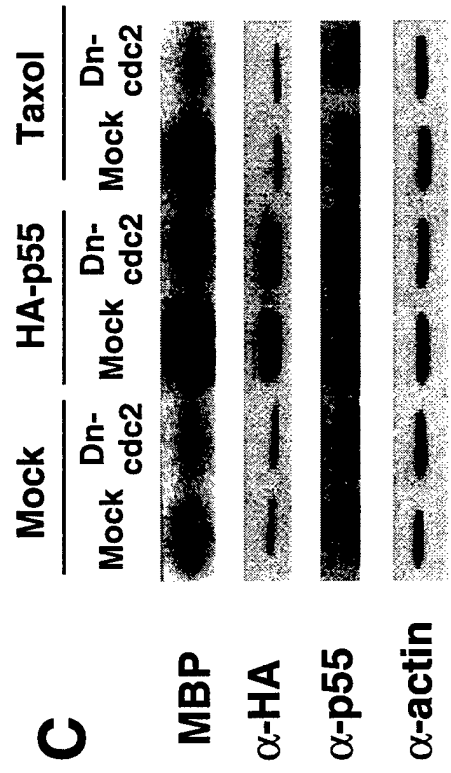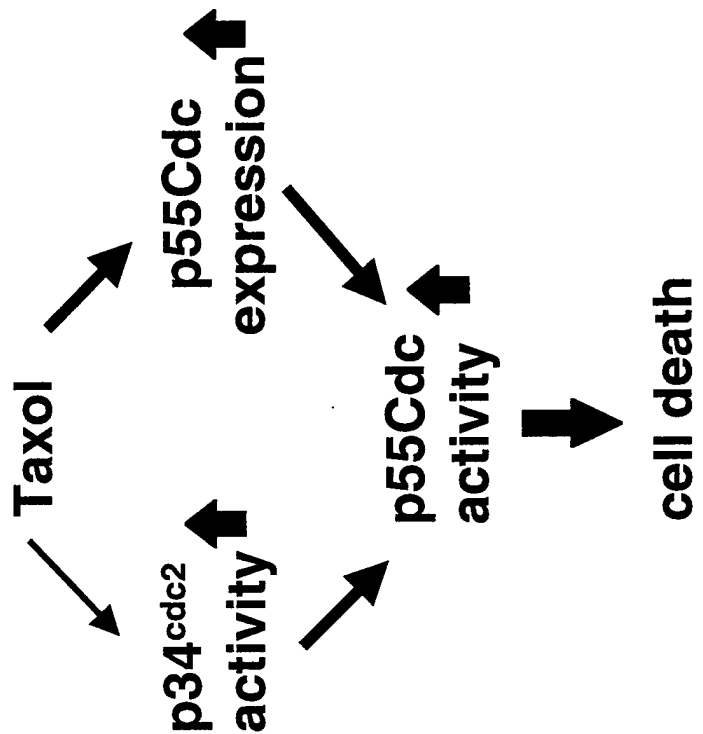
Transcriptional Repression of Estrogen Receptor by Metastasis-Associated Protein 1 Corepressor, a Component of Histone Deacetylase and Nucleosome Remodeling Complexes

Abhijit Mazumdar[+], Rui-An Wang[+], Sandip K. Mishra, Liana Adam, Rozita Bagheri-Yarmand, Mahitosh Mandal, Ratna K. Vadlamudi, and Rakesh Kumar*

Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA

Correspondence should be addressed to RK; +Contributed equally in this study

Abstract

Activation of heregulin/HER2 pathway in the estrogen receptor-positive breast cancer cells leads to the suppression of estrogen receptor element (ERE)-driven transcription, the disruption of estradiol responsiveness, and thus contributes to breast cancer tumor progression to more invasive phenotypes. We report the identification of a metastatic-associated protein 1 (MTA1), a component of histone deacetylase and nucleosome remodeling complexes, as a gene product induced by heregulin-β1 in both cultured cells and transgenic mice. Stimulation of cells with heregulin-β1 was accompanied by a suppression of histone acetylation and an enhancement of deacetylase activity. In addition, MTA1 was discovered to be a potent corepressor of ERE transcription as it blocked the ability of estradiol to stimulate the estrogen receptor-mediated transcription. Histone deacetylase inhibitor trichostatin A blocked the MTA1-mediated repression of ERE transcription. Furthermore, MTA1 directly interacted with the histone deacetylase-1 and -2 and with the activation domain AF2 of the estrogen receptor-α. Overexpression of MTA1 in breast cancer cells was accompanied by a significant enhancement in the ability of cells to invade, and grow in an anchorage-independent manner. In addition, heregulin-β1 promoted interaction of the MTA1 with endogenous ER, and also association of MTA1 or HDAC with the ERE-responsive target gene promoters chromatin in vivo. These results identify estrogen receptor-mediated transcription as a nuclear target of MTA1 and suggest that histone deacetylase complexes associated with the MTA1 corepressor mediates estrogen receptor transcriptional repression by heregulin-β1.

Text

Growth of human breast cells is closely regulated by steroid hormone and by peptide hormone receptor[1]. Members of both receptor classes are important prognostic factors in human breast cancer as well as determinants of endocrine therapy in breast cancer patients. After the initial stages of breast cancer progression, tumors frequently acquire steroid hormone resistance with concurrent amplification of growth factor receptors; this alteration is associated with a poor prognosis. For example, clinical and experimental data indicate that overexpression of c-*erb*B-2 (also known as human epidermal growth factor receptor [HER]2) in the estrogen receptor (ER)-positive breast cancer cells leads to reduced sensitivity of these cells to estrogen in vitro and in vivo[2-5].

Accumulating evidence suggests that the progression of breast cancer cells to a more invasive phenotype is regulated not only by HER2 overexpression but also by a mesenchymal growth factor, heregulin-β1 (HRG), which is a ligand for HER3 and HER4 and which transactivates HER2[6]. Recently, we and others have demonstrated that HRG activation of breast cancer cells in the absence of HER2 overexpression also promotes the development of aggressive phenotypes in breast cancer cells[7-10]. In spite of the widely acknowledged role of HRG in breast cancer, the mechanism through which it participates in breast cancer progression remains elusive.

There is also considerable evidence to suggest that the development of hormone-independence in breast cancer cells be influenced by cross-talk between steroid hormone and HER2/HRG pathways. For example, expression of the HER2 pathway in MCF-7 cells results in the downregulation of ER expression and inactivation of ER element (ERE)-driven reporter transcription[8, 9]. The finding that signaling by HRG promotes a ligand-independent suppression of ER transcript also supports a link between HER2 and ER receptor pathways[6-9]. These observations suggest that repression of ER-signaling by HER2 and HRG constitute one important mechanism of hormone independence. In spite

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of these observations, the molecular basis of hormone-independence and the potential role of the histone modifications remain unexplored.

The eukaryotic genome is compacted with histone and other proteins to form chromatin, which consists of repeating units of nucleosomes[11]. For transcription factors to access DNA, the repressive chromatin structure needs to be remodeled. Dynamic alterations in the chromatin structure can facilitate or suppress the access of the transcription factors to nucleosomal DNA, leading to transcriptional regulation. One way to achieve this is through alterations in the acetylation state of nucleosomal histones[11-13]. Acetylation of core histones occurs at lysine residues on the amino-terminal tails of the histones, thus neutralizing the positive charge of the histone tails and decreasing their affinity for DNA. Hyperacetylated chromatin is generally associated with transcriptional activation, whereas hypoacetylated chromatin is associated with transcriptional repression[14]. The acetylation state of histones is regulated by a dynamic interaction of two groups of recently identified enzymes - histone acetyltransferases (HATs) and histone deacetylases (HDACs). In brief, HATs and HDACs constitute important links between chromatin structure and transcription outcome.

A number of recent studies have raised the possibility of a close connection between HDACs and cancer. Since HDACs-mediated deacetylation of nucleosomal histones is known to be associated with transcriptional repression of some genes, it is being proposed that the deregulation of HDAC recruitment to specific promoters is a potential mechanism by which these HDACs contribute to tumorigenesis. Several recent findings[15-18], which demonstrated the identity of a polypeptide (NuRD-70) of nucleosome remodeling and HDAC complex with that of the metastatic associated protein 1 (MTA1) suggested a role of HDACs in tumor progression. The MTA1 also physically interacts with HDAC1[19]. The MTA1 gene was originally identified by differential expression in rat mammary adenocarcinoma metastatic cells and is now shown to correlate well with the metastatic potential of several human cell lines and tissues[20-22].

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Although MTA1 has been shown to be a part of the HDAC complex, the nature of its target or targets remains unidentified. Since one of the mechanisms by which hormone-independence by HRG is developed includes repression of the ER pathway, and since MTA1 is a component of the HDAC complex, we explored the hypothesis that HRG regulates the expression of MTA1, which, as a part of the HDAC complex, may antagonize the activation of the ER pathway by estradiol. We provide new evidence to demonstrate that the MTA1 is a target of HRG and represses the ER-transcription by recruiting HDACs.

Results

HRG Stimulation of MTA1 Expression in Breast Cancer Cells. Because deregulation of the HRG pathway into the ER-positive breast cancer cells leads to suppression of ER-driven transcription[9], and because MTA1 is a component of HDAC complex[15-18], we initially investigated whether HRG could upregulate the expression of MTA1. Using a pair of specific MTA1 primers[20], RT-PCR analysis of RNAs from breast cancer MCF-7 cells demonstrated a time-dependent stimulation of MTA1 mRNA by HRG (Fig.1a). The identity of the amplified MTA1 band was confirmed by sequencing and Southern analysis (data not shown). Using a 291 bp PCR probe for MTA1, the northern blot analysis showed a significant increase in the steady-state levels of 2.9-kb mRNA for MTA1 in a time-dependent manner (Fig. 1b). Western blot analysis with an anti-MTA1 antibody[23] also showed that the level of 80 kDa MTA1 significantly increased in HRG treated MCF-7 cells (Fig. 1c). There was no effect of HRG on the expression of MTA2 protein. Since there was no effect of 12 h HRG treatment on the levels of thymidine incorporation into DNA, and on the cell cycle distribution (data not shown), the observed effect of HRG on the MTA1 expression may not be reflective of the cellular proliferative status. To evaluate the HRG modulation of MTA1 in vivo, we used an MMTV-driven HRG transgenic mice which develop mammary adenocarcinomas and harderian tumors.[24] Since harderian tumors are usually detected by 3

weeks of age as opposed to 12-16 months of mammary gland tumors, we used the harderain tumors to establish the proof-of-principle of our hypothesis in vivo. Expression of HRG transgene in the Harderian gland tumors in a MMTV/HRG transgenic mouse model[24] was accompanied by an increased expression of MTA1 (Fig. 1d). Treatment of cultures with actinomycin D, an inhibitor of transcription, completely inhibited the HRG-mediated induction of MTA1 mRNA (Fig. 1e). Treatment of cells with cycloheximide stabilized the levels of MTA1 mRNA expression; treatment with HRG, however, superinduced the expression of MTA1 mRNA. The induction of MTA1 expression by HRG was also dose-dependent (Fig. 1f). To confirm that MTA1 is expressed in mammary epithelium cancer cells, we also cloned the full-length MTA1 cDNA from the human mammary gland cDNA library (data not shown).

Suppression of Histone Acetylation in HRG Treated Cells. Since MTA1, a component of the NuRD complex, was induced by HRG, we next examined the influence of HRG on the status of histone acetylation. MCF-7 cells were treated for 16 or 24 hr with or without HRG in the presence or absence of trichostatin-A (TSA), a specific HDAC inhibitor, and the status of histone acetylation was evaluated by acetic acid-urea-acrylamide gels. The addition of TSA to the control cells was accompanied by an expected increase in the acetylation of histone 4 (H4) (Fig. 2a). However, HRG treatment for longer than 16 hr suppressed the levels of H4 acetylation, particularly on bands 4 and 3. Similar pattern of results were obtained for cell lysates from the above experiment when immunoblotted with an anti-H4 antibody (Fig. 2b). However, this antibody predominantly recognized acetylated H4 bands 4, 3, and 2 in a decreasing order. This was a specific effect of HRG; another growth factor TGF-α demonstrated no such inhibitory effect (Fig. 2b). HRG treatment of MCF-7 cells was also accompanied by an enhancement of TSA-sensitive HDAC activity without any effect on the expression of HDAC2 protein (Fig. 2c). These findings suggest that HRG interfere with the acetylation of H4, presumably via stimulation of MTA1 expression.

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Repression of ERE-Mediated Transcription by MTA1. Because MTA1 has been shown to be

a component of the NuRD complex and to have HDAC activity[15-18], and because HRG induces MTA1

expression as well as histone deacetylation (this study), we hypothesized that MTA1 in conjunction

with HDAC complexes may repress ERE transcription and, thus, may provide a molecular explanation

for the earlier reported[7-8] suppression of the ER pathway by HRG. As shown in Fig. 3a, cotransfection

of ERE-luciferase with either HRG or activated (point-mutated) HER2, but not with control vector,

was accompanied by a significant suppression of ERE-reporter transcription, suggesting the existence

of cross talk between HER2/HRG and ER pathways.

To explore whether HRG-inducible MTA1 serves as specific corepressor of ERE-transcription,

the above experiment was repeated with MTA1 expression vector. Transient expression of the MTA1

effectively blocked the ability of estradiol to stimulate the ERE-transcription (Fig. 3b). The inhibitory

effect of MTA1 on ERE-transcription was antagonized by TSA, a specific inhibitor of HDAC enzyme,

suggesting that MTA1 potentially recruits HDAC to repress the ERE-transcription.

Next, we examined the recruitment of MTA1 complex(s) to ER elements by using a Gal4-

ER/Gal4-Luc assay system[25,26]. This system involves transient transfection of two plasmids Gal4-AF2

(ligand-binding domain of ERα) and Gal4-luciferase reporter, and luciferase activation depends on E2

stimulation of AF2 domain. Data in Fig. 4a demonstrated that E2-mediated activation of AF2 domain

could also be repressed by MTA1 expression, and this repression was relieved by TSA. These

observations suggested that the observed MTA1 regulation of AF2 function was independent of DNA

binding activity of ER receptor and that this involves HDACs. Earlier many repressors have been

shown to repress transcription of specific promoters when recruited by heterlogous DNA binding

domain. To further confirm the role of MTA1 in ERE transcription, we have constructed a reporter

gene containing 5X Gal4 sites placed in front of 3XERE sites and have utilized GAL4-DNA binding

domain fused MTA1 to recruit MTA to the ERE promoter. Expression of Gal4 –MTA1 repressed the

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E2 mediated ERE activity up to 80% (Fig. 4b). The noticed repressing effect of MTA1 on the ERE-transcription was not restricted to the ER, as MTA1 also effectively blocked the activation of the GRE-mediated transcription by progestin and dexamethozone in a TSA-sensitive manner (Fig. 5). In brief, these results establish a new function of MTA1 in the repression of the ERE and GRE-mediated transcription.

MTA1 Association with HDAC and Estrogen Receptor. To understand whether the observed repression of ERE transcription by MTA1 was associated with the recruitment of HDAC complexes *in vivo*, we next examined the association between T7-tagged MTA1 and the components of HDACs by coimmunoprecipitation-western blot assays. Results of a representative experiment are shown in Fig. 6a. Transient expression of T7-tagged MTA1, but not of control T7 vector, in MCF-7 cells was accompanied by interactions with HDAC1, HDAC2, and chromodomain protein 4 (CHD4). To investigate whether the observed association between MTA1 and HDAC1 or HDAC2 was direct or mediated via other proteins, we examined the binding ability of *in vitro* translated MTA1 protein with GST-HDAC1 and -2. As shown in Fig. 6b, MTA1 interacted with the GST-HDAC1 and GST-HDAC2, but not to GST alone in GST pull-down assays. While this study was in-progress, Toh *et al* also reported the physical interactions between MTA1 and HDAC1[19].

Since MTA1 repressed the ERE-mediated transcription by recruiting the HDAC, we hypothesized that MTA1 physically interacts with ER to influence its function. To explore this possibility, we examined the binding ability of *in vitro-* translated MTA1 protein with GST-AF1 and -AF2 domains of ER in GST pull-down assays. As shown in Fig. 6c, MTA1 protein effectively interacted with the GST-AF2 (ligand-binding domain of ER), but not to GST alone or to GST-AF1. Our results support the hypothesis that MTA1 may act as a transcription repressor by recruiting the HDAC complex.

Effect of MTA1 on the Biology of Breast Cancer Cells. To further delineate the potential contribution of MTA1 in breast cancer cells, we next established stable MCF-7 clones expressing T7-tagged MTA1 or control vector (Fig. 7a). For subsequent studies, we used MCF-7 clone 15 expressing T7-MTA1, and control vector transfected MCF-7 cells (clone V2). We next analyzed the influence of MTA1 expression on the invasion of MCF-7 cells using Boyden chamber assay. Vector-transfected cells showed low invasiveness (Fig. 7b). In contrast, expression of MTA1 resulted in a significant increase in cell invasiveness. To examine the potential influence of MTA1 expression on the growth characteristics of breast epithelial cancer cells, we measured the growth rate and ability of cells to grow in an anchorage-independent manner. Expression of MTA1 had very little or no significant effect on the growth-rate of MCF-7 cells on plastic (Fig. 7c). MTA1 expression however significantly enhanced the ability of MCF-7 cells to form colonies on soft agar (Fig. 7d). However, overexpression of MTA1 was accompanied by a significant reproducible enhancement of the ability of cells to form larger colonies in soft agar as compared to those formed by vector transfected control cells (Fig. 7d). Although there was no effect of MTA1 expression on the level of ER, MTA1 overexpressing breast cancer cells exhibited a reduction in the levels of estrogen receptor target genes, including pS2, and c-Myc (Fig.7e). Together, these observations suggested that cells expressing MTA1 may impact the status of ER-responsive genes and that these cells acquire more invasive phenotypes.

HRG Promotes MTA1 Interaction with Endogenous ER. Having shown a role of MTA1 in the invasiveness of breast cancer cells, we next sought to investigated the effect of HRG on potential interaction between the MTA1 and endogenous estrogen receptor in MCF-7 cells expressing T7-tagged MTA1 or control vector. Cells treated with or without HRG were fixed, co-stained with antibodies against ER or T7-tag, and resulted immunofluorescence was

quantitated by laser scanning confocal microscopy. The results in Fig. 8a show that the MTA1 protein (visualized in red) was abundantly present in nuclei of T7-MTA1 expressing cells in a fine granular pattern in untreated control cells (Fig. 8a, upper panel). In contrast, T7-MTA1 was expressed in larger nuclear domains in HRG-treated clones (left lower panel). No positive staining was detected in the empty vector-transfected cells (right column). Interestingly, within the larger nuclear domains, the MTA1 protein, partially colocalize with ERα protein only upon HRG treatment (represented by the yellow staining). Very fine serial confocal sectioning revealed that the ER staining was disposed as several smaller nuclear dots decorating the surface of the larger anti-MTA1-reactive nuclear domains. As shown in Fig. 8a, overexpression of T7-MTA1 was not sufficient to induce a significant increase in the interactions between the T7-MTA1 and ER, and HRG treatment could rapidly triggered this process. These observations raise the possibility that the observed MTA1-ER interaction may be an indirect effect of HRG, and may potentially involve unidentified component(s) of HRG pathways leading to MTA1.

HRG Treatment Induces MTA1-HDAC Association on the ERE-responsive Promoters In Vivo. To directly assess the potential significance of the physical interactions between the MTA1 and ER in HRG-activated cells, we next analyze whether MTA1-HDAC associate on chromatin of endogenous ERE-containing promoters by chromatin immunoprecipitation (ChIP) assay. T7-tagged MTA1 expressing MCF-7 cells were treated with or without HRG, and processed to formaldehyde-cross-link and sonicate chromatin for immunoprecipitation with specific antibodies against T7 or HDAC2. T7-MTA1- or HDAC2-bound genomic DNA fragments were analyzed by quantitative PCR using primers spanning ERE elements present in the promoter of pS2[27] and c-Myc[28] sequence, for potential HRG-triggered association of T7-MTA1 or HDAC2 with the promoter of two ERE target genes.

Results indicated that HRG treatment triggers a significant increase in the amount of both

pS2 and c-Myc (5.8 and 4.1 fold, respectively, over untreated cells) target gene promoter

chromatin associated with T7-tagged MTA1 (Fig. 8b). Alterations in the amount of HRG-

responsive increase of the pS2 promoter DNA with HDAC2 were also significant, but less

profound (1.8 fold increase over untreated cells) (Fig. 8b). These studies were repeated three

times with similar results, and thus, examplified the prevalence of potential complexicity with

promoter chromatin regulation by multi-protein complexes. Since the association of MTA1

with the ERE-responsive target gene promoters was dependent on HRG treatment, the

observed effects may involve participation from HRG-triggered cellular events, and hence,

may be indirect. Taken together, these findings strongly supported the notion that MTA1

interacts with endogenous estrogen receptor and that both MTA1 and HDAC associates with

the ERE-containing promoters in HRG treated cells.

Expression of MTA1 During Embryogenesis and Mammary Gland Development. During

embryonic development, HDAC may work constantly to control the on/off function of the genes that

regulate the cell proliferation and differentiation. However, little is known about the expression profile

of HDAC components. We investigated MTA1 expression in mouse embryonic development using *in

situ* hybridization. As shown in Fig 9a, MTA1 mRNA was expressed in most of tissues, with highest

levels demonstrated the rapidly proliferative tissues, i.e., liver, lung, thymus, etc. MTA1 mRNA

signals in the vertebral column, heart, and intestinal tract were significantly weaker. At high

magnification, the MTA1 signals were noticed in the cytoplasm of neuronal cells in the cervical

neuronal arch (Fig. 9a, *lower panels*). In accordance with the results from *in situ* hybridization, a high

levels of MTA1 mRNA expression was detected in the brain, lung, and testis; high level of MTA1

mTNA expression was detected also in mammary gland by northern blotting of RNAs from multiple

organs from female mice (Fig. 9b).

In situ hybridization analysis of mouse mammary gland demonstrated that MTA1 mRNA was in the cytoplasm of virgin ducts, in the growing-end buds of the pregnant mammary gland, and in the lactating alveoli (Fig. 9c). The signal was much stronger in the lactating and the virgin mammary gland than in the pregnant and the involuting mammary gland. Signals from the pregnant mammary gland was relatively weaker. Low levels of MTA1 expression were detected in the virgin fat pat tissues.

Since MTA1 regulates the function of ER in breast cancer cells (this study), we next examined the expression pattern of MTA1 during mammary gland development and the relationship MTA1 expression and the ER expression. Results from the northern blot analysis indicated that MTA1 was expressed expression during all stages of mammary gland development (Fig. 9d). The highest levels of MTA1 expression were observed during lactation, when the ER levels were lower. These results suggested that MTA1 regulate the expression of one or more ER target genes, and could influence ER function.

Discussion

The results of our study show that the MTA1 is a target of HRG in breast cancer cells and that MTA1 represses the ER transcription by recruiting HDACs. Our conclusion that ER is a nuclear target of MTA1 and that it utilizes HDACs to repress the ER transcription is based on the following evidence: 1) repression of ER-transcription by both HRG and MTA1 is relieved by a HDAC inhibitor; 2) MTA1 physically associates with a complex containing HDACs; 3) MTA1 directly interacts with HDAC1 and HCAD2 in the GST pull-down assays; 4) MTA1 represses ERE transcription in a TSA-sensitive manner in three independent assay systems, ERE-luciferase, ERE-Gal4, and ERE-Gal4-luciferase; 5) MTA1 physically interacts with the activation ligand-binding AF2 domain of ER; 6) HRG promotes MTA1 interaction with endogenous ER; and 7) HRG induces MTA1-HDAC association on the ERE-responsive target gene promoters chromatin in vivo.

The recruitment of HDAC to promoters has emerged as a general mechanism of transcription repression of target genes. The deregulation of HDAC recruitment to some target promoters may be a mechanism by which these enzymes contribute to tumorigenesis. For example, recruitment of HDAC complex to retinoic acid-receptor target genes represses transcription and prevents differentiation, and treatment with retionic acid induces differentiation by displacing HDAC complex from PML-RAR-alpha[29,30]. Similarly, the transcription repressor complexes containing HDAC have been discovered in the RB/E2F[31] and myc/mad [reviewed in 32] pathways. The recruitment of HDACs to specific promoters may be mediated either through direct interactions with regulatory proteins such as transcription factor YY1[33] or through interaction with the corepressors with HDAC-interacting domain such as Sin3A. Nuclear-hormone receptors have been shown to bind the corepressor N-CoR, which directly interacts with the Sin3A-HDAC corepressor complex[16].

Our observation that MTA1 can directly interacts with HDAC1/2 and the AF2 domain of ER is important because it suggests that the earlier reported[15-18] association between MTA1 and HDACs in the NuRD complex reflects direct MTA1 interactions. In addition, our findings have identified the specific nuclear targets and a corepressor function of MTA1, as it effectively suppressed the ligand-induced activation of ER.

The MTA1 was originally identified as a differentially expressed gene in the rat mammary metastatic adenomacarcinoma[20]. The expression of MTA1 correlates well with the metastasis potential of a number of human cancer cell lines or tissues[21,22]. However, direct evidence to implicate the enhanced MTA1 expression with metastasis is currently lacking. The MTA1 protein has been recognized to contain a domain (known as WFY domain) in similar to two regions of the N-CoR[17]. The WFY domain of MTA1 is less likely to be involved in recruiting HDACs in our system, because we detected no appreciable interaction between MTA1 and N-CoR (data not shown). However, specific domains of MTA1 may directly associate with both HDAC2 and AF-2 as shown in this study

(Fig. 6c). Additional studies involving a substantial amount of work are required to map the precise structural motif or motifs of ER that may be essential for its functional interaction with MTA1-HDAC co-repressor complex, and potential contribution of MTA1-interacting proteins. In summary, the present study has identified the ER transcription as a nuclear target of corepressor MTA1 and provided new evidence to support the idea that histone deacetylase complexes are involved in MTA1-mediated transcriptional repression of estrogen.

Methods

Cell Cultures, Transfection, Cell Extracts, and Reagents.

Breast cancer MCF-7 cells[10] were maintained in Dulbecco's modified Eagle's medium-F12 (1:1) supplemented with 10% fetal calf serum. Cell lysates were prepared as described[10] and were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel, transferred to nitro-cellulose, and probed with the appropriate antibodies. Antibodies against HDAC1, and HDAC2 were purchased from the Santa Cruz (Santa Cruz) Antibody against T7 were from the Novagen, Inc. Transfection was performed uing Fugene-6 kit (Roche Biochemical) as per the manufacture's instructions.

Northern Blot Analysis and Promoter-reporter Assays.

Total cytoplasmic RNA (20 µg) was analyzed by northern blot analysis[10]. For promoter assays, cells were transiently cotransfected with a reporter construct and β-galactosidase. Cells were harvested with passive-lysis buffer or processed for CAT assay (Promega).

Construction of Reporter Systems.

3X-ERE TATA luciferase plasmid was obtained from Donald McDonnell, Duke University, Durham, NC). 5XGAl4 plasmid was obtained from FX Claret, MD Anderson Cancer Center. Full length MTA1 cDNA was isolated from human mammary gland cDNA library (Invitrogen). MTA1 cDNA containing all reading frame of MTA1 was subcloned into pcDNA3.1-T7 tagged vector using restriction sites EcoR1 and XbaI to generate T7-taged MTA1. To construct 5XGal4-ERE luciferase construct, 5XGal4 sites were isolated by PCR using Gal4 5X luciferase as a back bone (SmaI and Kpn1 sites were added into primers) and subcloned into SmaI and Kpn1 site of 3XERE-TATA–luciferase plasmid. Construct was verified by sequencing.

In Vitro Transcription and Translation.

In vitro transcription and translation of the MTA1 proteins were performed using the TNT-transcription-translation system (Promega). One μg of MTA1 cDNA in pcDNA 3.1 vector was invitro translated in the presence of ^{35}S-methionine in a reaction volume of 50 μl using T7-TNT kit (Promega). The reaction was diluted to 1ml with NP40 lysis buffer and an aliquot (250 μl) was used for each GST pull down assay. Translation was verified by running 2 μl of reaction by SDS-PAGE and autoradigraphy.

Histone Urea Gels and Deacetylase Assays.

Histones were purified and resolved onto a 15% urea gel as described[34]. The HDAC activity was measured by scintillation counting of ^{3}H-acetic acid released from ^{3}H-acetylated histones as described[17].

GST Pull Down Assay.

The glutathione-S-transferase (GST) pull down assays were performed by incubating equal amount of GST, GST-AF1, GST-AF2, GST-HDAC1 or GST-HDAC2 proteins immobolized to glutathione beads-sepharose beads (Amersham) with *in vitro* translated [35]S -labeled MTA protein. The mixture was incubated for two hours at 4[0]C, washed with NP40 lysis buffer, bound proteins were eluted with 2XSDS buffer, separated on SDS PAGE and developed by flurograpy[35].

Production of Stable Cell Lines Expressing MTA1.

MCF-7 cells were transfected with pcDNA3.1 or pcDNA.T7-MTA1 using calcium phospahate method. Forty-eight hours post transfection, cells were selected in media containing 1000 μg/ml G418. Several individual clones were isolated and expended, and expression of exogenous MTA1 was verified by immunoblotting using anti-T7 mAb.

Cell Proliferation, Invasion, and Soft-Agar Assays.

The cell invasion assays were performed using the Boyden chambers as described[36]. Cells were plated on the upper well of a Boyden chamber at a concentration of 20,000 cells/well.

The lower side of the separating filter was coated with chemoattractant (thick layer of 1:2 diluted Matrigel (Life Technologies, Inc) in serum-free medium. The number of cells that successfully migrated through the filter and invaded the 2-mm Matrigel layer, as well as those that remained on the upper side of the filter, were counted after staining with propidium iodide (Sigma). The percentage of migrating cells compared with the total number of cells was recorded and represents the means ± S.E. of triplicate wells from three separate experiments.

Cell proliferation assays were performed using MTT dye method as described[8,10]. Soft-agar colony growth assays were performed as described[37]. Briefly 1 ml of solution of 0.6% DIFCO Agar in DMEM supplemented with 10 % FBS with insulin was layered onto 60 × 15 mm tissue culture plates. MCF-7 cell (10,000 cells) were mixed with 1 ml of 0.36% Bactoagar solution in DMEM prepared in a similar manner and layered on top of the 0.6% Bactoagar layer. Plates were incubated at 37°C in 5% CO_2 for 21 days.

Immunofluorescence Confocal Studies

Cellular localization of T7-MTA1 and ER was determined using indirect immunofluorescence as described[10]. Briefly, cells grown on glass coverslips were fixed (without permeabilization) in ethanol:methanol (1:1) at -20 'C for 3 min. Cells were treated with or without anti-T7 mAb or ER Ab followed by 546-Alexa-labeled goat anti-mouse Ab or 488-Alexa (Molecular Probes). For controls, cells were treated only with the secondary antibodies. Each image represents Z sections at the same cellular level and magnification. Confocal analysis was performed using a Zeiss laser scanning confocal microscope and the established methods, involving processing of the same section for each detector (the two excitations corresponding to 546 and 488) and comparing pixel by pixel. Co-localization of two proteins is demonstrated by the development of yellow color due to red and green overlapped pixels.

Chromatin Immunopreciptation Assays.

Quantitative ChIP aasay was done as described[38,39]. Cells were subject to 37% formaldehyde solution treatment (final concentration-1%) to cross-link T7-MTA1 or HDAC2 to DNA. The cells were washed twice with phosphate-buffered saline (pH 7.4) containing protease inhibitor cocktail (Boehringer Mannheim Corp) followed by lysis with lysis buffer containing 1% SDS and sonication as described[38]. Supernatant from sonicated lysates were diluted 10 fold by chromatin dilution buffer containing 0.01% SDS, 1.1% Triton-X100 and protease inhibitor cocktail. For input DNA 1% of the chromatin solution was kept aside before immunoprecipitation. Chromatin solutions were immunoprecipitated with either anti-T7 (Novagen) or anti-HDAC2 antibody (Santa-Cruz Biotech) at 4^{0}C overnight. Beads were washed as described (1) on a rotating platform before finally eluting the antibody bound chromatin by incubation with 400 μl of 1% SDS containing 0.1 M NaHCO$_3$. The elution as well as the input chromatin was heated to 65^{0}C for 6 h to reverse the formaldehyde cross-links followed by phenol chloroform extraction. The supernatant was ethanol precipitated and resuspended in 50 μl of TE. Quantitative PCR analysis was done with 10 μl of DNA sample restricted to 25 cycles. Our pS2 gene primers[27] amplify the region inclusive of ER-responsive element from −463 to −159, and c-Myc gene primers[28] from -65 to +192. PCR products were resolved on 1.5% agarose gel and visualized with ethidium bromide. Images were quantified using Sigma gel analysis software, version 1.

In-situ Hybridization.

For in-situ hybridization, mouse mammary gland tissues or 18 days old embryo (E18) were cut out and fixed with 10% neutral buffered formaldehyde and processed routinely to paraffin sections as described[40]. In situ hybridization was done in frozen sections by using the digoxigenin (Roche) labeled riboprobe. A 290 bp of mouse MTA1 cDNA corresponding to the human 1781-2070 MTA1 mRNA

region was amplified by RT-PCR, subcloned into TOPO II (Promega) and used for riboprobe synthesis. RNA probes were labeled with digoxigenin and hybridized for 16–20 hr in buffer containing 1 ug/ml riboprobes, 50% formamide, 300 mM NaCl, 10 mM Tris (pH 7.4), 10 mM NaH_2PO_4 (pH 6.8), 5 mM EDTA (pH 8.0), 0.2% Ficoll 400, 0.2% polyvinyl pyrolidone, 10% dextran sulfate, 200 μg/ml yeast total RNA, and 50 mM dithiothreitol. Alkaline phosphatase labeled sheep anti-digoxigenin antibody was applied and signals were visualized by NBT-BCIP. Sense-probe hybridization was used for background control.

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Figure legends

Fig. 1. HRG regulation of the MTA1 expression. (a) RT-PCR analysis of MTA1 expression. (b) Northern blot analysis of HRG regulation of MTA1 expression in MCF-7 cells. (c) Western blot analysis of HRG regulation of MTA1 or MTA2 expression in MCF-7 cells. (d) RT-PCR analysis of HRG and MTA1 expression in the lacriminal glands of HRG transgenic mice. TG1, TG2, and TG3, harderian glands from HRG transgenic mice. WT, wild-type mouse. (e) Regulation of MTA1 mRNA by HRG in MCF-7 cells. (f) Dose-dependent upregulation of MTA1 mRNA in MCF-7cells (lanes 1,2, and 3) and T47-D cells (lanes 4, and 5). (n=3)

Fig. 2. HRG regulation of histone deacetylase activity. (a) Urea-polyacrylamide gel analysis of histone H4 acetylation in TSA-treated MCF-7 cells in the presence or absence of HRG. (b) Western blot analysis of histone H4 acetylation using an anti-acetylated H4 antibody. (c) Regulation of TSA-sensitive deacetylase activity by HRG in MCF-7 cells. TSA, 300nM, HRG (1 nM). (n=4)

Fig. 3. MTA1 represses ERE-mediated transcription. (a) Repression of 17β–estradiol $(10^{-9}M)$-mediated stimulation of ERE-luciferase activity in MCF-7 cells by heregulin gene (pHRGβ1) and point mutated HER2 (pHER2NT). (b) MCF 7 cells were transfected with ERE-Luc in the presence or absence of MTA1 (T11). Schematic diagrams of the reporter constructs used is shown below. Some cultures were treated with TSA (300 nM). (n= 3)

Fig. 4. MTA1 regulation of AF2 function of ER. (a) MCF-7 cells were transfected with Gal4 AF2 and Gal4-Luc (contain 5X Gal4 binding domain) with or without MTA1. BRCA1 was used as a positive control to show its suppression. (b) E2-denpendent direct interactions between Gal4-MTA1 and (ERE)$_3$-Gal4-luc. (n=4)

Fig. 5. MTA1 act as a global steroidal repressor. MCF-7 cells were cotransfected with a GRE promoter-CAT reporter construct and MTA1 or vector control. Some cultures were treated with

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dexamethasone (500 nM) or progesterone (500 nM) for 16h, and cell extracts were assayed for CAT activity. (n=4)

Fig. 6. Direct association of MTA1 with HDACs and ER. (a) MTA1 interacts with the HDAC complexes in-vivo. MCF-7 cells were transfected with MTA1 or control vector. Immunoprecipitation was performed using T7-mAb, and western blotting was performed with the indicated antibodies. (b) MTA1 directly interacts with HDAC. GST pull down assays to show the association of HDAC with the *in vitro* translated MTA1. (c) GST pull-down assays to exhibit a direct interaction between MTA1 and ligand binding domian (AF2) of ER.(n=4).

Fig. 7. Characterization of MCF-7 cells overexpressing MTA1. (a) Western blot analysis of control and T7-MTA1 clones by anti-T7 mAb. The blot was reprobed with an anti-vinculin Ab, as a loading control. (b) Invasion of MCF-7 cells expressing T7-MTA1 (clone 15) or control vector pcDNA. (c) Effect of T7-MTA1 expression on the growth-rate of cells by MTT assay. (d) Effect of T7-MTA1 expression on anchorage independent growth of MCF-7 cells. (e) Representative photographs of soft agar colonies (f) Expression of ER protein, and pS2, c-Myc and GAPDH mRNAs in MCF-7 cells expressing vector or T7-MTA1. (n=3).

Fig. 8. HRG promotes MTA1 interaction with endogenous ER pathway. (A) T7-MTA1 and ERα partially colocalizes in the nucleus of MCF7 cells expressing T7-MTA1. Confocal single optical sections are shown. Double labeling was performed with a Rabbit antibody against human ERα (visualized in green) and a mouse monoclonal antibody against T7 for MTA-1 (visualized in red). HRG treatment induced colocalization of the ER with T7-MTA1 in large nuclear domains, as shown by the development of yellow color in the merged images. (b) Analysis of MTA1-HDAC2 association on the ERE-responsive promoters by chromatin immunoprecipitation assay. MCF-7 cells were treated with or without HRG (30 ng/ml for 16 h), and chromatin lysates were immunoprecipitated with antibodies against T7 or HDAC2,

and samples were processed as described in the "Methods". The lower panel shows the PCR

analysis of the input DNA. The upper panel demonstrates the PCR analysis of pS2 (304 bp)

and c-Myc (257 bp) promoter fragments associated with T7-MTA1 or HDAC2. Quantitation of

signals is presented as fold induction over control untreated cells. (n=3)

Fig. 9. MTA1 expression during embryogenesis and mammary gland development. (a) MTA1

expression in 18D embryo. MTA1 expression was detected by a specific anti-sense MTA1 riboprobe

(E18/AS). Sense probe hybridization was used to show the background staining (E18/S). Br, brain; Lu,

lung; Ty, thymus; Lv, liver. Lower panels are enlarged portion of the boxed areas in the upper panel.

(b) Northern blot analysis of the MTA1 expression in 14 mouse tissues. (c) In situ hybridization

analysis of MTA1 expression. V, virgin day 21 mammary gland; P, pregnant day 15 mammary gland;

and L, lactating day 12 gland. L/S, control in situ hybridization using the sense MTA1 probe in

lactating mammary gland. (d) Northern blot analysis for expression of MTA1 and ER in the above

samples. (n=2)

and samples were processed as described in the "Methods". The lower panel shows the PCR

analysis of the input DNA. The upper panel demonstrates the PCR analysis of pS2 (304 bp)

and c-Myc (257 bp) promoter fragments associated with T7-MTA1 or HDAC2. Quantitation of

signals is presented as fold induction over control untreated cells. (n=3)

Fig. 9. MTA1 expression during embryogenesis and mammary gland development. (a) MTA1

expression in 18D embryo. MTA1 expression was detected by a specific anti-sense MTA1 riboprobe

(E18/AS). Sense probe hybridization was used to show the background staining (E18/S). Br, brain; Lu,

lung; Ty, thymus; Lv, liver. Lower panels are enlarged portion of the boxed areas in the upper panel.

(b) Northern blot analysis of the MTA1 expression in 14 mouse tissues. (c) In situ hybridization

analysis of MTA1 expression. V, virgin day 21 mammary gland; P, pregnant day 15 mammary gland;

and L, lactating day 12 gland. L/S, control in situ hybridization using the sense MTA1 probe in

lactating mammary gland. (d) Northern blot analysis for expression of MTA1 and ER in the above

samples. (n=2)

Fig1

Fig 2

ERE-Luc [3XERE] —TATA—[Luc]

a

(ERE)$_3$-Luc	+	+	+
pHRG-β1	-	+	-
pHER2 (NT)	-	-	+
Vector	+	-	-

b

(ERE)3-Luc	+	+	+	+
ICI 182780	-	-	+	-
Vector	+	-	-	-
MTA1	-	+	-	+
TSA	-	-	-	+

Fig 3

Fig 4

Rakesh Kumar

Fig5

Fig 6

a

Parental Vector1 Vector2 clone15 clone25 clone27

T7-MTA1

Vinculin

b

Invasive Cell Number

250

150

50

pcDNA T7-MTA1 MCF-7 MCF-7 +HRG

c

Cell Number (X10⁻⁴)

■ pcDNA
▨ T7-MTA1

10

6

2

Day 0   Day 3   Day 7

d

Colony Number

250

150

50

pcDNA T7-MTA1 MCF-7 MCF-7 +HRG

e

pcDNA   T7-MTA1

f

pcDNA T7-MTA1

WB:ER

PS2

c-myc   mRNA

GAPDH

Fig 7

a

| MERGE | ER-α | T7 | MERGE |

CON

+HRG

T7-MTA1 Clone   pcDNA Clone

b

IP: T7   HDAC2
HRG -   +   -   +

PS2

Input

-463   -159   +1
304 bp

IP: T7
HRG -   +

cMyc

Input

+1
-65   192
257bp

Fig. 8

a

E18/AS   E18/S

b

lung, virgin mg, pregnant mg, intestine, stomach, colon, liver, cerebellum, uterus, ovary, kidney, cerebral cortex, spleen, testis

c

V   L/S

d

V   P   L

GAPDH

MTA1

ER

Fig 9

Advances in Brief

Tumor Suppression and Sensitization to Tumor Necrosis Factor α-induced Apoptosis by an Interferon-inducible Protein, p202, in Breast Cancer Cells[1]

Yong Wen,[2] Duen-Hwa Yan,[2] Bill Spohn, Jiong Deng, Shiaw-Yih Lin, and Mien-Chie Hung[3]

Department of Cancer Biology, Section of Molecular Cell Biology [Y. W., D-H. Y., B. S., J. D., S-Y. L., M-C. H.], Department of Surgical Oncology [D-H. Y., M-C. H.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

p202, an IFN-inducible protein, interacts with several important regulatory proteins, leading to growth arrest or differentiation. In this report, we demonstrate that, in addition to inhibiting *in vitro* cell growth, p202 can also suppress the tumorigenicity of breast cancer cells *in vivo*. Furthermore, we found that p202 expression could sensitize breast cancer cells to apoptosis induced by tumor necrosis factor α treatment. One possible mechanism contributing to this sensitization is the inactivation of nuclear factor-κB by its interaction with p202. These results provide a scientific basis for a novel therapeutic strategy that combines p202 and tumor necrosis factor α treatment against breast cancer.

Introduction

IFNs possess a wide variety of biological properties such as antivirus, antiproliferation, immunoregulation, antiangiogenesis, and antineoplasia and have been used in clinical treatment of certain cancers (1). Here, we examined the possibility of using an IFN-inducible protein, p202 (2), as a potential therapeutic substitute for IFNs. p202 is a M_r 52,000 nuclear phosphoprotein known to be a negative transcription modulator that, in most cases, inhibits transcription of its target genes by physically interacting with certain transcription activators (3–8). Like IFN treatment, constitutive expression of p202 causes G_1-S cell cycle arrest in murine fibroblast cells (9, 10). Consistent to that observation, we demonstrated previously that the enforced expression of p202 could significantly retard the *in vitro* growth of prostate cancer cells in both cell culture and soft agar (10). However, it is not known whether p202 expression could exert an antitumor effect on cancer cells. In this report, we demonstrated for the first time that p202 expression was able to inhibit tumorigenicity of human breast cancer cells *ex vivo*. Furthermore, p202 expression can sensitize breast cancer cells to apoptosis induced by TNF[4]-α and that correlates with inactivation of NF-κB by a NF-κB/p202 interaction. These results suggest a potential combined therapy using p202 and TNF-α against breast cancer.

Materials and Methods

Cell Culture, Transfection, and Colony-forming Assay. MDA-MB-453 and MCF-7 human breast cancer cell lines were obtained from the American Type Culture Collection. Cells were maintained in DMEM/F-12 (HyClone Laboratories, Inc.) supplemented with 10% (v/v) fetal bovine serum. Cells were transfected with a p202 expression vector (CMV-p202) or the control vector pcDNA3 (Invitrogen) using lipofectin (Life Technologies, Inc.) and selected in 500 μg/ml G418 (Geneticin; Life Technologies, Inc.). Western blotting using an anti-p202 polyclonal antibody (11) identified p202 stable transfectants.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay and FACS Analysis. These standard assays were done as described previously (12).

[³H]Thymidine Incorporation Assay and Soft-Agar Assay. These standard assays were done as described previously (12).

Tumorigenicity Assay. Female athymic nude mice (*nu/nu*), 4–5 weeks of age, were used in this *ex vivo* experiment. Briefly, MCF-7 cells were transfected with CMV-p202 (10 μg) using PEI. Twenty-four h after transfection, cells (3 × 10⁶) were harvested in 0.2 ml of PBS and injected into the mouse mammary fat pads. 17-β-Estradiol pellets (0.72 mg/pellet, 60-day release; Innovative Research of America, Inc.) were implanted s.c. into the mice 1-day before cell injection. The presence of estrogen is essential for MCF-7 cells to grow in mice. The size of the tumors was measured with a caliper every week, and the tumor volume was calculated using a formula: $V = 1/2 \times S^2 \times L$, where V = volume, S = the short length of the tumor, and L = the long length of the tumor in cm.

Immunoprecipitation and Immunoblotting. MDA-MB-453 (453) and 453-p202 cells were treated with 10 and 20 ng/ml of human TNF-α (R & D Systems, Inc., Minneapolis, MN) for 30 min. Cells with or without TNF-α treatment were extracted in RIPA lysis buffer without SDS on ice. Exacts were sonicated and cleared by centrifugation at 4°C. For immunoprecipitation, equivalent aliquots of cell lysates (1 mg of total protein) were incubated with 1 μg of anti-p65 antibody (Santa Cruz Biotechnology) for 4 h with gentle rotation at 4°C. Protein A-Sepharose beads (50 μl) was added for an additional 1 h. The beads were extensively washed with ice-cold RIPA buffer, and the precipitate was dissolved in a sample buffer for electrophoresis and Western blot.

Results and Discussion

To investigate a potential growth-inhibitory effect of p202 on breast cancer cells, we performed a colony-forming assay by transfecting a p202 expression plasmid driven by CMV promoter (CMV-p202) or a control vector (pcDNA3) containing neomycin-resistance gene into two human breast cancer cell lines, MDA-MB-453 (453) and MCF-7. After 3 weeks of G418 selection, the number of G418-resistant colonies was scored. A dramatic reduction in the number of G418-resistant colonies was seen in cells (MCF-7 and 453) transfected with p202 as compared with that with the control plasmid, pcDNA3 (Fig. 1a, *left panel*). There was at least a 75% reduction in colony number in both p202-transfected cell lines (Fig. 1a, *right panel*). These data suggest that p202 expression may be associated with antiproliferat-
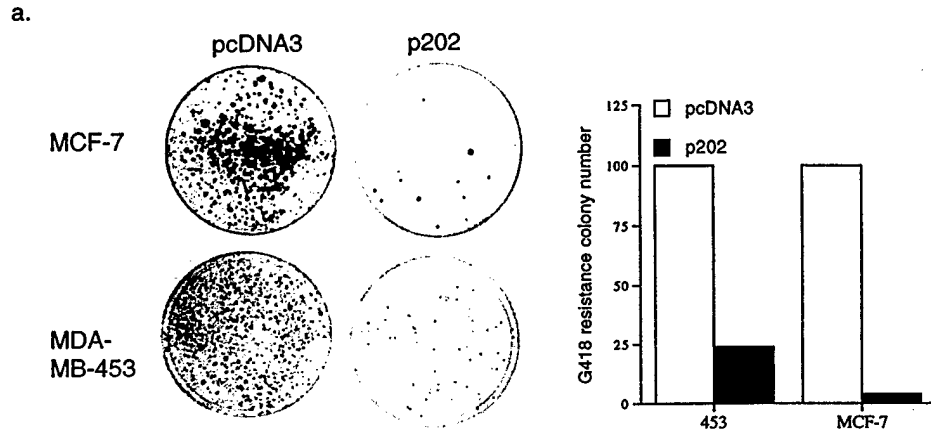
a.

b.

Fig. 1. Expression of p202 inhibits the proliferation of MDA-MB-453 and MCF-7 breast cancer cells. *a.* colony-forming assay. MDA-MB-453 and MCF-7 cells were transfected with either a control vector (pcDNA3) or a p202 expression vector. The colony number obtained from pcDNA3 transfection was set as 100%. *b,* Western blot analysis of the p202 stable transfectants. The M_r 52,000 protein represents p202, and the nonspecific M_r 68,000 protein cross-reacting with the antibody was used as an equal loading control. *c* and *d,* [^{3}H]thymidine incorporation assays. DNA synthesis rate was measured by the amount of [^{3}H]thymidine incorporated into the cells at each time point. The measurement was conducted in quadruplicates, and the variations within each quadruplicate are too small to be of any significance.

c.

d.
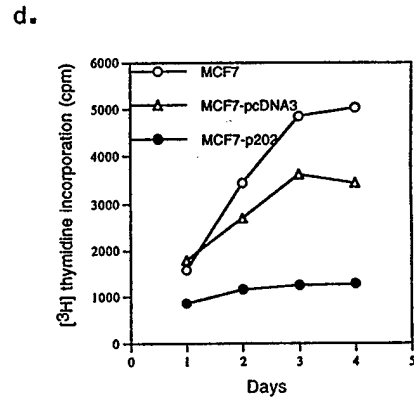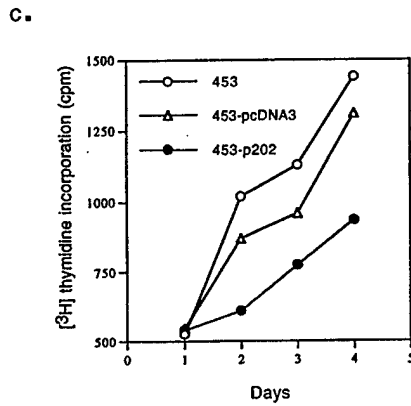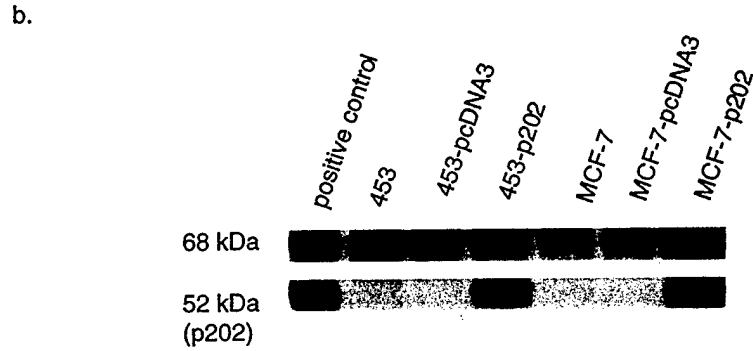
and/or proapoptotic activity in these breast cancer cells. To further characterize the biological effects of p202 expression on these cells, we attempted to isolate several lines of p202-expressing stable clones. Using Western blot with a p202-specific antibody (11), we were able to identify one p202-expressing stable clone (of 20) from each cell line, *i.e.,* MDA-MB-453-p202 (453-p202) and MCF-7-p202 (Fig. 1*b*). The low frequency of p202-expressing clones obtained from the G418-resistant colony supports the idea that p202 expression may cause an antiproliferation and/or proapoptotic effect on these cells. To assess these two p202-mediated biological effects, we first measured and compared the mitogenic activity between the p202 stable lines and the control cell lines using [^{3}H]thymidine incorporation assay. The p202-expressing cells (453-p202 and MCF-7-p202) exhibited a ːed DNA synthesis rate as compared with their respective control cₑₗₗ lines, *i.e.,* 453 and 453-pcDNA3; MCF-7 and MCF-7-pcDNA3 (Fig. 1, *c* and *d*). Similarly, the p202-expressing cells also showed a slower growth rate than the control cells as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data

not shown). Thus, our data strongly suggest that p202 functions as a growth inhibitor in breast cancer cells.

To test whether p202 expression in breast cancer cells may also suppress their *in vitro* transformation phenotype defined by the ability of these cells to grow in soft agar, we then measured the number of colonies formed in soft agar by the p202-expressing cells and the control cells. As shown in Fig. 2, *a* and *b,* both 453-p202 and MCF-7-p202 exhibited >60% reduction (after 3 weeks of incubation) in colony number than those of the parental and pcDNA3 transfectant. The difference in number was not attributable to the slower growth rate of the p202-expressing cells than that of the control cells (Fig. 1, *c* and *d*), because a prolonged (6 weeks) incubation of the same plates did not yield more colonies. Rather, it represents a real loss of anchorage-independent growth, *i.e.,* an *in vitro* transformation phenotype, of these p202-expressing cells.

One of the most critical biological properties determining the potential application of a tumor suppressor gene in cancer therapy is its ability to reduce tumorigenicity *in vivo*. To test a possible antitu-

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a.

parental   pcDNA3   p202
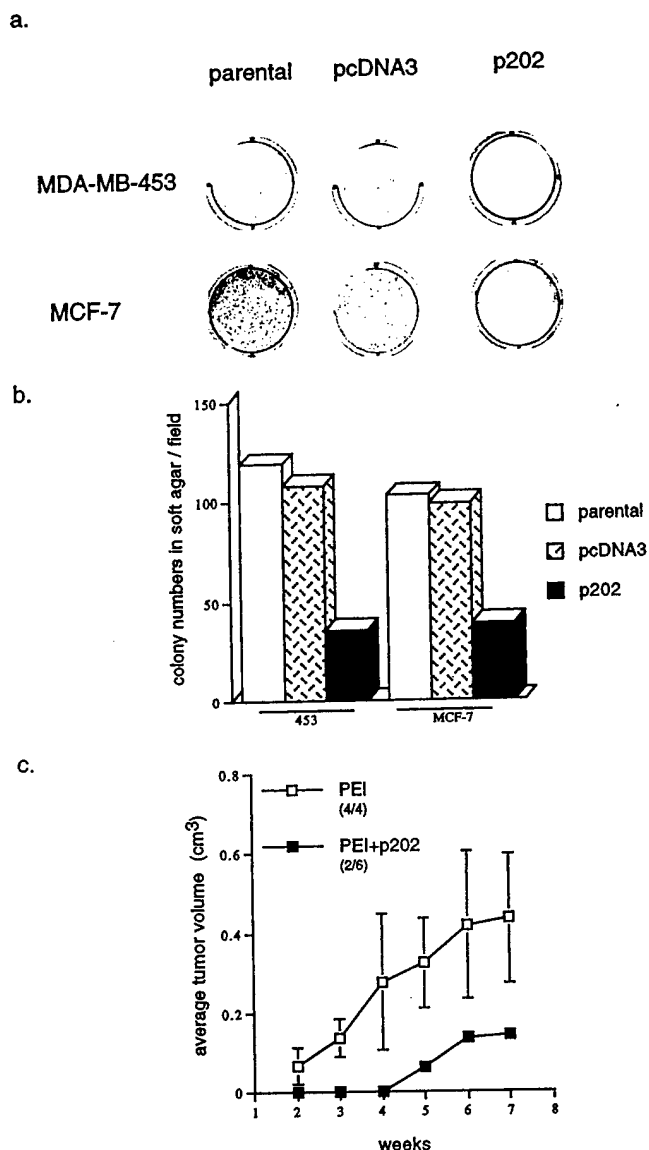
MDA-MB-453

MCF-7

b.

c.

Fig. 2. p202 inhibits the transformation phenotype of breast cancer cells. *a*, colony formation in soft agar. MDA-MB-453, 453-pcDNA3, 453-p202, MCF-7, MCF-7-pcDNA3, and MCF-7-p202 cell lines were subjected to anchorage-independent growth in soft agar. *b*, number of colonies formed in soft agar as shown in *a*. The number represents the average of five random microscopic fields from each cell line. *c*, p202 *ex vivo* experiment. MCF-7 cells were transfected with p202 expression vector using PEI. After 24 h, cells were harvested, and the p202 (*PEI+p202*) or mock (*PEI*) transfected cells (3×10^{6} cells/injection) were injected into the mammary fat pad of female nude mice. 17-β-Estradiol pellets were implanted s.c. into the mice 1-day before inoculation. Tumor formation was monitored every week. *Bars*, SE.

mor activity of p202, we performed an *ex vivo* tumorigenicity assay in an orthotopic breast cancer model. Briefly, CMV-p202/PEI or PEI alone (PEI is a polymer vector used for transfection) was transfected into MCF-7 cells before injection into the mammary fat pads of the estrogen-supplemented nude mice. The p202 transfection caused a drastic reduction of tumorigenesis of MCF-7 cells as compared with that of the mock transfection (PEI alone; Fig. 2c). Our data demonstrated, for the first time, that the p202 expression is associated with an antitumor activity in animals.

In an attempt to identify therapeutic agents that may cooperate with p202 to synergize the antitumor effect on breast cancer cells, we used FACS analysis (Fig. 3) to determine a potential synergism in inducing apoptosis. We found that the p202-expressing cells were more susceptible to TNF-α-induced apoptosis than the control cells, *i.e.*, after

treatment with TNF-α (0, 10, 20 ng/ml) for 48 h, more 453-p202 cells were undergoing apoptosis (sub-G_1 population) than the parental 453 cells and 453-pcDNA3 control cells in a dose-dependent manner (Fig. 3a). Likewise, MCF-7-p202 cells were also found to be more sensitive to TNF-α-induced apoptosis than the parental MCF-7 cells in a dose-dependent manner (Fig. 3b). These results suggested that p202 expression could sensitize cells to TNF-α-induced apoptosis.

One possible mechanism of the p202-mediated sensitization to TNF-α-induced apoptosis is that p202 could antagonize the antiapoptotic function of NF-κB (13–15). To test that hypothesis, we tested whether p202 expression could affect the NF-κB-mediated transcription activation in response to TNF-α treatment. We cotransfected CMV-p202 and a NF-κB-activatable promoter-reporter construct (κB-luc), *i.e.*, an IκB promoter-driven luciferase gene, into 453 cells in the presence of TNF-α (Fig. 4a). As expected, κB-luc was readily activated in the presence of TNF-α. However, this TNF-α-induced transcription activation was repressed by p202 in a dose-dependent manner. To test whether p202 acted on the NF-κB molecule to elicit such transcription repression, we cotransfected CMV-p202 with a Rel-A (a p65 subunit of NF-κB) cDNA expression vector and κB-luc. As shown in Fig. 4b, whereas p202 expression alone has no effect on κB-luc, it could greatly repress NF-κB (Rel-A)-activated IκB pro-

a

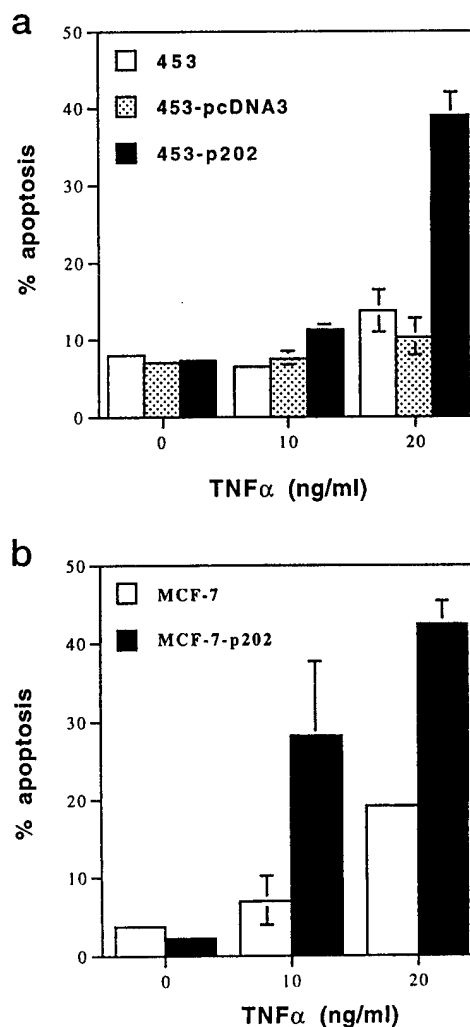TNFα (ng/ml)

b

TNFα (ng/ml)

Fig. 3. p202 sensitizes breast cancer cells to apoptosis induced by TNF-α in a dose-dependent manner. *a*, 453, 453-pcDNA3, and 453-p202 cell lines were treated with TNF-α (0, 10, and 20 ng/ml) for 48 h. *Bars*, SE. *b*, MCF-7 and MCF-7-p202 were treated with TNF-α (0, 10, and 20 ng/ml) for 48 h. Cells were fixed and stained with propidium iodide. Apoptosis was quantitated by FACScan cytometer. *Bars*, SE.

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Fig. 4. The interaction and inactivation of NF-κB by p202 is responsible for the p202-mediated sensitization to TNF-α-induced apoptosis. *a*, p202 expression represses NF-κB-mediated transcription activation in response to TNF-α. IκB-Luciferase reporter gene (0.2 μg) and CMV-p202 (0, 0.8, or 2 μg) were cotransfected into MDA-MB-453 cells. Thirty-six h after transfection, cells were either left untreated or stimulated with TNF-α (20 ng/ml) for 6 h. The fold difference in IκB-Luciferase expression was calculated with respect to IκB-Luciferase expression in the absence of TNF-α and p202. *b*, p202 expression represses Rel-A (p65)-activated transcription. MDA-MB-453 cells were cotransfected with κB-luc and ± NF-κB (p65) expression vector. The inhibitory activity of p202 on the induction of IκB promoter activity by p65 was assessed by cotransfection with p202 expression vector. Luciferase activity was measured 48 h after transfection. The data represent an average of two independent experiments after normalization: *bars*, SE. *c*, gel-shift assay. 453 and 453-p202 nuclear extracts, used in this assay, were isolated from TNF-α-treated cells (20 ng/ml of for 30 min). *Left panel*, the activated NF-κB (p65/p50) induced by TNF-α is indicated by an *arrow*. *Right panel*, competition assay was performed in the presence of a 70-fold excess of wild-type or mutant oligonucleotides containing NF-κB binding site. A polyclonal Rel-A antibody supershifted the NF-κB complex to a slower-migrating position, as indicated by an *arrow*. *d, top panel*, p202 is physically associated with p65. 453 and 453-p202 cells were treated with or without TNF-α (20 ng/ml for 30 min). Cell lysates (1 mg) were used in the subsequent immunoprecipitation with anti-p65 antibody. Immunoprecipitated complexes were analyzed by SDS-PAGE, followed by immunoblotting with p202 antibody. *Bottom panel*, immunoblots of p202 protein using untreated 453 and 453-p202 cells serve as negative and positive controls, respectively. ▲, p202 band. IgG band is also indicated.
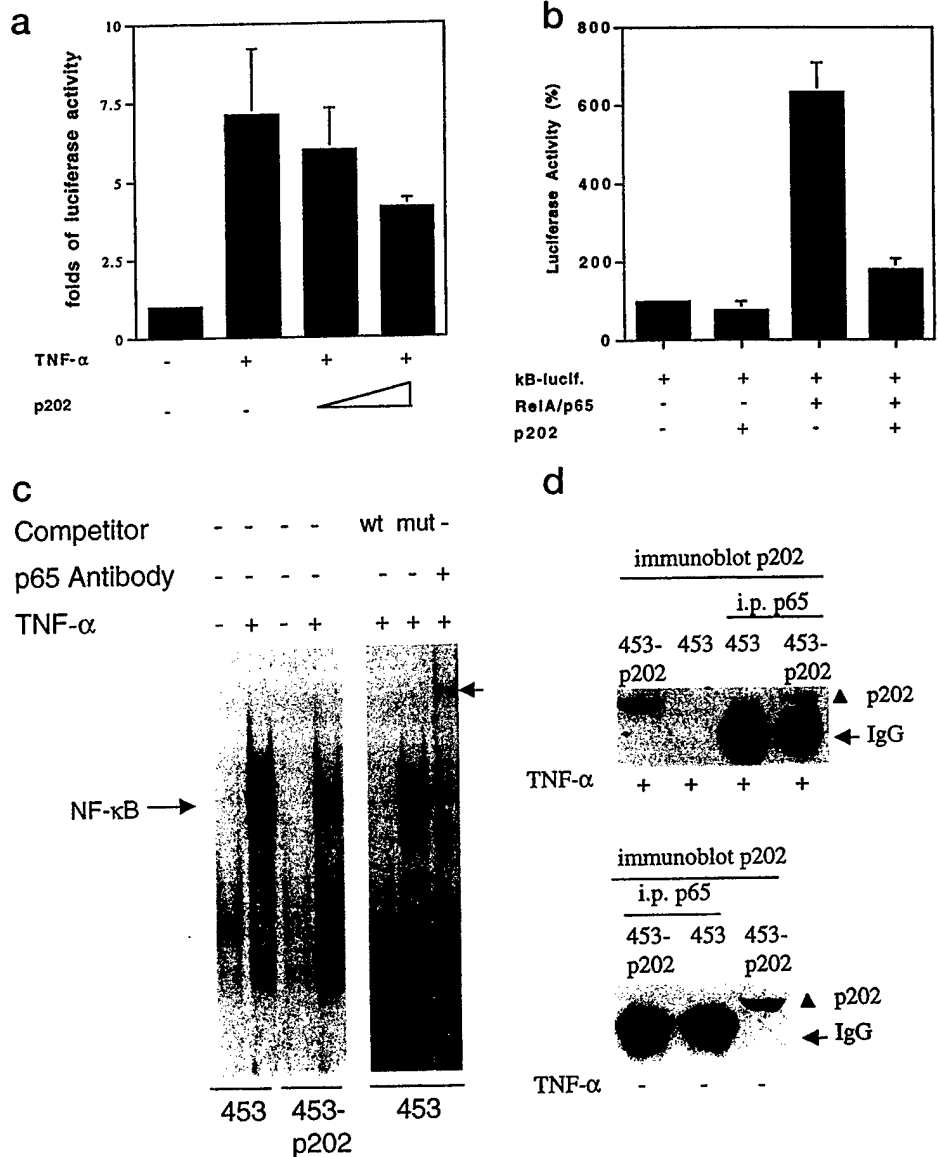
moter activity. These results suggest that the transcriptional repression of TNF-α-mediated gene expression by p202 may be attributable to the inactivation of NF-κB by p202.

This hypothesis was further supported by a subsequent observation that p202 expression was associated with a reduced level of the active NF-κB (p65/p50) molecule as measured by a gel-shift assay (Fig. 4*c*, *left panel*). As expected, the level of active NF-κB was found to be significantly increased in both the p202-expressing (435-p202) and the parental (453) cells treated with TNF-α (20 ng/ml). However, the level of activated NF-κB was greatly reduced in 453-p202. Using either a wild-type or mutant NF-κB DNA binding sequence as a competitor, we showed that the DNA/protein complex was indeed NF-κB specific in that only wild-type, but not mutant, sequence could compete with the NF-κB/DNA complex. Moreover, the fact that this complex could be supershifted in the presence of an anti-p65 antibody (Fig. 4*c, right panel*) further confirms the identity of this DNA/protein complex being NF-κB-specific. Thus, these data support the idea that p202 expression may impede the formation of active p65/p50 heterodimer. That, in turn, represses transcriptional activation induced by NF-κB.

It is possible that p202 may interact with p65, forming a p202/p65

complex, which may significantly reduce the concentration of free p65 in p202-expressing cells. To test that possibility, we performed a coimmunoprecipitation assay. As shown in Fig. 4*d, upper panel*, with TNF-α treatment, p202 could be coimmunoprecipitated with p65 by an anti-p65 antibody in 453-p202 nuclear extract but not 453 extract. As a control, no detectable p202 was observed in either cell line without TNF-α treatment (Fig. 4*d, lower panel*). These data strongly indicate that p202 and p65 are physically associated in the same complex upon TNF-α stimulation. The p65 protein level is comparable between 453 and 453-p202 cells with TNF-α treatment (data not shown), indicating that p202 may not regulate p65 expression.

The above observation presents a possible scenario that TNF-α-induced NF-κB activation could be antagonized by p202 via a p202/p65 interaction. That, in turn, causes subsequent transcriptional repression of genes, the activation of which requires active NF-κB. Although it has been reported previously that p202 could bind both p50 and p65 *in vitro* and p50 *in vivo* (6), our data are the first demonstration of an *in vivo* association between p202 and p65 upon TNF-α stimulation. Taken together, our results provide a possible mechanism that accounts for the p202-mediated sensitization to TNF-α-induced apoptosis in breast cancer cells.

Inflammatory cytokines, *e.g.*, TNF family members, can transduce apoptotic signals in certain tumor cells and have been tested in a number of clinical trials (16). Despite the promising data in animal models, unsatisfactory results have been observed in many clinical trials (17). It might be attributable to the resistance of many cancer cells to TNF-α-induced apoptosis, presumably, by the activation of NF-κB and the subsequent induction of survival factors that counteract apoptosis. In this report, we demonstrated that p202 expression not only exerted strong growth retardation and tumor suppression activities in breast cancer cells but also is able to sensitize these cells to TNF-α-induced apoptosis, and that sensitization is associated with inactivation of NF-κB via a p202/p65 interaction. Thus, our data implicate a potential therapeutic application of a combined treatment of TNF-α and p202 gene therapy for cancer patients.

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The Ets protein PEA3 suppresses *HER-2/neu* overexpression and inhibits tumorigenesis

XIANGMING XING[1], SHAO-CHUN WANG[1], WEIYA XIA[1], YIYU ZOU[1], RUPING SHAO[1], KA YIN KWONG[1], ZHENMING YU[1], SU ZHANG[1], SUSAN MILLER[1], LEAF HUANG[2] & MIEN-CHIE HUNG[1]

[1]*The University of Texas M.D. Anderson Cancer Center, Department of Cancer Biology, Section of Molecular Cell Biology, Houston, Texas 77030, USA* [2]*Department of Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, USA*

X.X. and S.-C.W. equally contributed to this study.

Correspondence should be addressed to M.-C.H.; email: mchung@odin.mdacc.tmc.edu

Because *HER-2/neu* overexpression is important in cancer development, we looked for a method of suppressing the cell transformation mediated by *HER-2/neu* overexpression. We have identified that the DNA-binding protein PEA3, which is encoded by a previously isolated gene of the *ets* family, specifically targeted a DNA sequence on the *HER-2/neu* promoter and downregulated the promoter activity. Expression of PEA3 resulted in preferential inhibition of cell growth and tumor development of *HER-2/neu*-overexpressing cancer cells. This is a new approach to targeting *HER-2/neu* overexpression and also provides a rationale to the design for repressors of diseases caused by overexpression of pathogenic genes.

Overexpression of the *HER-2/neu* gene (also known as *c-erbB2*) is frequently associated with many types of cancer including breast and ovarian cancers[1-7]. The overall survival rates of breast and ovarian cancer patients whose tumors overexpress *HER-2/neu* are significantly lower than those of patients whose tumors do not overexpress *HER-2/neu* (refs. 8–11). A large body of evidence indicates that *HER-2/neu* overexpression is associated with elevated tumorigenicity[12], enhanced metastatic potential[13-17], increased resistance to tumor necrosis factor (TNF)-α (ref. 18), and, in certain circumstances, resistance to chemotherapy[19-22].

On the other hand, downregulation of the *HER-2/neu* oncogene causes suppression of the cell-transforming phenotype induced by the oncogene[12,23-27]. We showed previously that the adenovirus type 5 *E1A* gene can indirectly downregulate *HER-2/neu* promoter activity through the coactivator p300 (ref. 28). Downregulation of *HER-2/neu* overexpression by *E1A* significantly mitigated tumorigenic activity of human breast and ovarian cancer cells in nude mice[12,26,29,30]. These results imply that transcriptional repressors that target the promoter of the oncogene may inhibit HER-2/neu mediated cell transformation.

One strategy to efficiently suppress oncogene-mediated cell transformation is to inhibit the activity of the oncogene promoter by DNA-binding protein that can directly bind to a specific DNA sequence on the promoter and inhibit transcription activity. A DNA motif (5′–AGGAAG–3′) containing the consensus binding site of PEA3 (ref. 31), a member of the *ets* transcription factor family, is present 26 nucleotides upstream from the main transcriptional start site on the *HER-2/neu* promoter[32]. The sequence and position of this motif is conserved among human, rat, and mouse *HER-2/neu* promoters[33]. In this study, we show that PEA3 binds directly to the consensus motif and prevents *HER-2/neu* gene overexpression by suppressing the promoter activity. Downregulation of *HER-2/neu* expression inhibited cell growth *in vitro*, and in a preclinical gene therapy setting, blocked tumor formation among *HER-2/neu*-overexpressing cancer cells

and prolonged the survival of treated animals. Thus, our study demonstrates a promising approach to the design of transcriptional repressors with which to target diseases caused by the overexpression of pathogenic genes.

PEA3 binds to the *HER-2/neu* promoter

A DNA motif with the sequence of 5′–AGGAAG–3′ has been identified on the *HER-2/neu* promoter[33]. To test whether PEA3 can recognize and bind to this putative PEA3 binding site, purified GST-PEA3 fusion protein was prepared and incubated with ^{32}P-labeled oligonucleotide probes containing either the wild-type PEA3 binding site sequence or the same sequence but with the core PEA3 binding motif mutated (5′–AGCTCG–3′). The DNA-protein association was investigated by means of an electrophoretic mobility shift assay (EMSA). Specific binding between the fusion protein and the wild-type probe was identified (Fig. 1), and the binding was diminished in the presence of unlabeled wild-type oligonucleotide. There was no detectable association between the fusion protein and the mutant oligonucleotide. GST alone did not bind to the probe (data not shown).
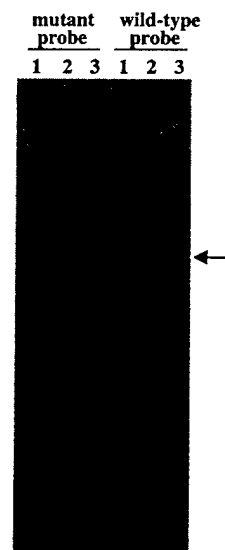

Fig. 1 PEA3 binds to the *HER-2/neu* promoter. GST-PEA3 fusion protein was incubated with ^{32}P-γ ATP end-labeled wild-type or mutated oligonucleotide containing the consensus PEA3 binding-site sequence on the *HER-2/neu* promoter (lane 1). The binding specificity was tested by competition with unlabeled wild-type (lane 2) or nonspecific oligonucleotide (lane 3). Arrow, probe–fusion protein binding complex.

Fig. 2 *PEA3* inhibits the *HER-2/neu* promoter activity in *HER-2/neu*-overexpressing cancer cell lines. Human ovarian cancer cells from the SKOV-3 cell line were co-transfected with 5 µg of pNulit or HER2-CAT with different amounts of *PEA3* plasmid DNA (pcDNA3-PEA3) as indicated. The activity of luciferase (**a**) or chloramphenicol acetyltransferase (CAT) reporter (**b**) was measured. For the CAT transient assay, the doses of co-transfected pcDNA3-PEA3 were 0, 5, 10 and 15 µg. The results of two independent experiments are shown.
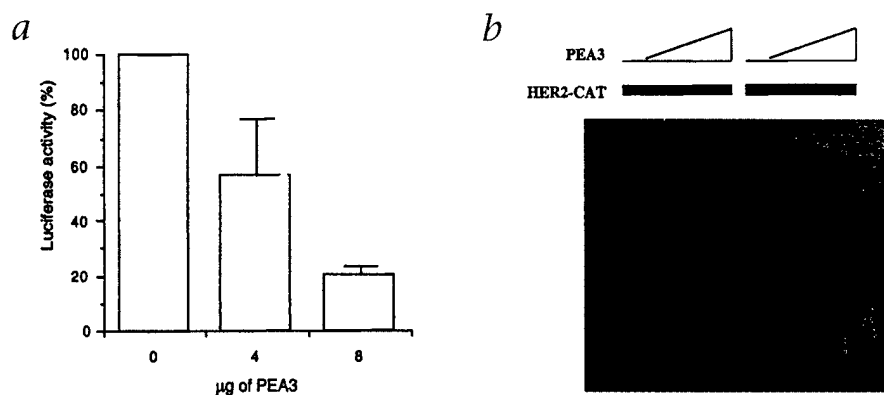
PEA3 represses the *HER-2/neu* through a positive regulatory motif

To examine how *PEA3* affected the promoter activity of *HER-2/neu*, a luciferase reporter gene driven by the *HER-2/neu* promoter (pNulit) was co-transfected with different amounts of *PEA3* cDNA into the SKOV-3 ovarian cancer cell line (Fig. 2a). The *PEA3* cDNA repressed the promoter activity of *HER-2/neu* in a dose-dependent manner. Similar results were observed in experiments using the breast cancer cell line MDA-MB-453. In these experiments, both the luciferase (Fig. 2a, 3a) and the chloramphenicol acetyltransferase (HER2-CAT; Fig. 2b) reporter genes consistently demonstrated that *PEA3* coexpression does repress the *HER-2/neu* promoter activity. These results indicate that *PEA3* is a potent transrepressor of the *HER-2/neu* gene promoter. We concluded that the intact PEA3 binding site on the *HER-2/neu* promoter is required for the *PEA3*-mediated transcriptional repression because the *HER-2/neu* promoter with a mutated PEA3 binding sequence (5'–AGGAAG–3' to 5'–AGCTCG–3') was not subject to negative regulation by PEA3 (Fig. 3a). The results indicate that PEA3-mediated *HER-2/neu* downregulation is through the PEA3 binding site on the *HER-2/neu* promoter. When the levels of wild-type and mutant promoter activities were compared in the absence of PEA3, the mutant promoter was significantly less active than the wild-type promoter (Fig. 3b), indicating that the PEA3 binding site on the *HER-2/neu* promoter actually acts as a positive regulatory element required for elevated expression of *HER-2/neu*.

PEA3 suppresses cell transformation and cell growth *in vitro*

To test whether expression of *PEA3* can repress *HER-2/neu*-in-

Table 1 PEA3 suppresses growth of cancer cells overexpressing HER-2/neu

HER-2/neu overexpressers Cell lines	pcDNA3	PEA3
SK-BR-3	100	0.7 (±0.9)
MDA-MB-453	100	19.7 (±3.0)
SKOV3-ip1	100	14.3 (±4.1)
HER-2/neu basal-concentration expressers Cell lines	pcDNA3	PEA3
MDA-MB-435	100	73.4 (±10.9)
2774 c-10	100	83.3 (±3.8)

Experiment for each cell line was independently repeated two to four times using different batches of plasmid DNA and cell culture. Inhibition of colony formation by PEA3 transfection is shown in percentage with the number of neomycin-resistant colony resulted from pcDNA3 transfection arbitrarily set as 100%.
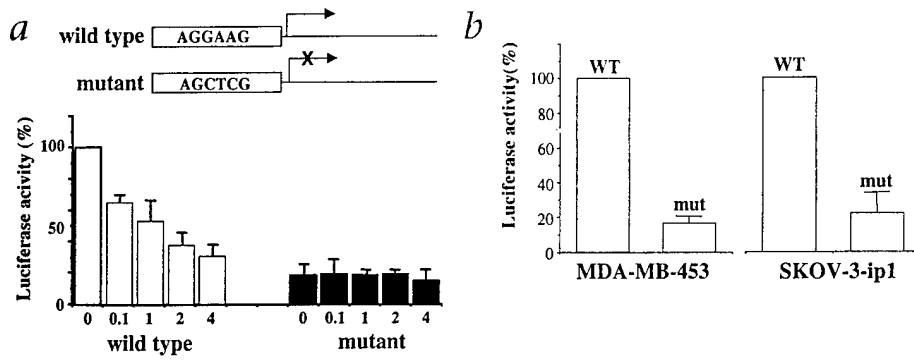
duced transforming phenotype, a cosmid DNA (cNu104) encoding the activated genomic rat *neu* oncogene that is able to transform mouse fibroblast cells[14] was subjected to a focus forming assay by cotransfecting the *PEA3* cDNA or the control vector into NIH3T3 cells. The results indicated that *PEA3* suppressed the focus-forming activity induced by activated *HER-2/neu* (Fig. 4a). This suppression is specific for the *HER-2/neu* gene because *PEA3* had no effect on the focus-forming activities of the oncogenes *fos* and *mos*.

To test if ectopic expression of *PEA3* can suppress the growth of cancer cells, breast and ovarian cancer cells with high or basal level of *HER-2/neu* expression were transfected with a plasmid carrying a neomycin-resistance gene, and the *PEA3* cDNA expression was controlled by a CMV promoter. Subsequent neomycin selection resulted in numerous resistant colonies from the cell lines with low *HER-2/neu* expression whereas only a few colonies of cell lines with *HER-2/neu* overexpression survived (Fig. 4b and Table 1). The surviving colonies were isolated and tested for ectopic expression of *PEA3* by reverse transcription-polymerase chain reaction (RT-PCR). Virtually all of the clones derived from the cell line with low *HER-2/neu* expression (MDA-MB-435) but only two clones derived from the cell line with *HER-2/neu* overexpression (MDA-MB-453) contained *PEA3* RNA expressed from the plasmid (Fig. 4c). One of the two clones from MDA-MB-453 (clone 2) did not express a detectable level of PEA3 protein. Another clone (clone 8) expressing ectopic PEA3 protein grew extremely slowly and was eventually lost during subsequent cell culturing (data not shown). On the other hand, ectopic expression of the PEA3 protein could be detected in the stable cell lines derived from MDA-MB-435 (Fig. 4d). The facts that transfection of PEA3 gene into *HER-2/neu*-overexpressing cells reduces the number of neomycin-resistant colonies and that the surviving clones either do not express PEA3 protein or are unable to grow in cell culture strongly suggest that PEA3 inhibits the growth of *HER-2/neu*-overexpressing cancer cells.

PEA3 inhibits tumor growth *in vivo*

The ability of *PEA3* to repress *HER-2/neu* overexpression *in vivo* was directly tested in xenograft tumor model in nude mice. Subcutaneous tumors derived from SKOV-3-ip1, a spontaneous *HER-2/neu*-overexpressing cell line, or 2774 c-10, a cell line with a basal level of *HER-2/neu* expression, were treated with the liposome-conjugated *PEA3* expression plasmid DNA or the backbone vector by intratumor injection. Expression of the endogenous HER-2/neu protein and the ectopic *PEA3* gene were determined by immunohistochemical staining of tissue sections of the tu-

Fig. 3 *PEA3* represses *HER-2/neu* promoter activity through a positive regulatory DNA motif. *a*, Luciferase reporter gene driven by either wild-type (□) or the PEA3 site-mutated (■) *HER-2/neu* promoter was measured after co-transfection with *PEA3* cDNA into MDA-MB-453 cells. The activity of the wild-type promoter in the absence of *PEA3* was defined as 100%. *b*, The luciferase reporter activity of the wild-type (WT) and the PEA3 site-mutated *HER-2/neu* (mut) promoters was compared by transfecting the reporter constructs into a breast cancer cell line (MDA-MB-453) and an ovarian cancer cell line (SKOV-3-ip1).

ing mice were treated with PEA3-expressing plasmid DNA or control vector delivered by intraperitoneal injection of a cationic liposome (DC-Chol) once a week. Fifty percent of mice with SKOV-3-ip1 tumors survived without detectable tumors for longer than one year after treatment with liposomal PEA3, whereas all of the mice in the control groups died of tumor growth within 6 months (Fig. 6*a*). On the other hand, no response to liposomal PEA3 treatment was observed in mice bearing 2774 10-c-derived tumors (Fig. 6*b*). The therapeutic effect of PEA3 combined with the cationic liposome was associated with downregulation of the *HER-2/neu* p185 protein product in the treated tumors of the SKOV-3-

mors (Fig 5). It is obvious that expression of PEA3 was concomitant with downregulation of the *HER-2/neu* gene in SKOV-3-ip1 xenografts.

To further investigate the tumor suppression potential of PEA3 in a preclinical setting, we used an established animal model to test whether PEA3 could suppress tumor development in animals and prolong animal survival. Ovarian cancer orthotopic tumor xenografts derived from SKOV-3-ip1 or from 2774 c-10 were developed intraperitoneally in nude mice[27-29]. Tumor-bear-

ip1 xenograft (Fig. 6*a*, insert). To rule out the possibility that the different responses of the two cell lines to PEA3-liposome treatment were due to differential gene transfer efficiency, SKOV-3-ip1 and 2774 c-10 cells were transiently transfected with PEA3 by liposome and the ectopic expression of PEA3 protein in both cell lines was compared with immunohistochemical analysis. Our results show that the PEA3 expression was readily delivered into SKOV-3-ip1 and 2774 c-10 cells at similar efficiencies (Fig. 6*c*).
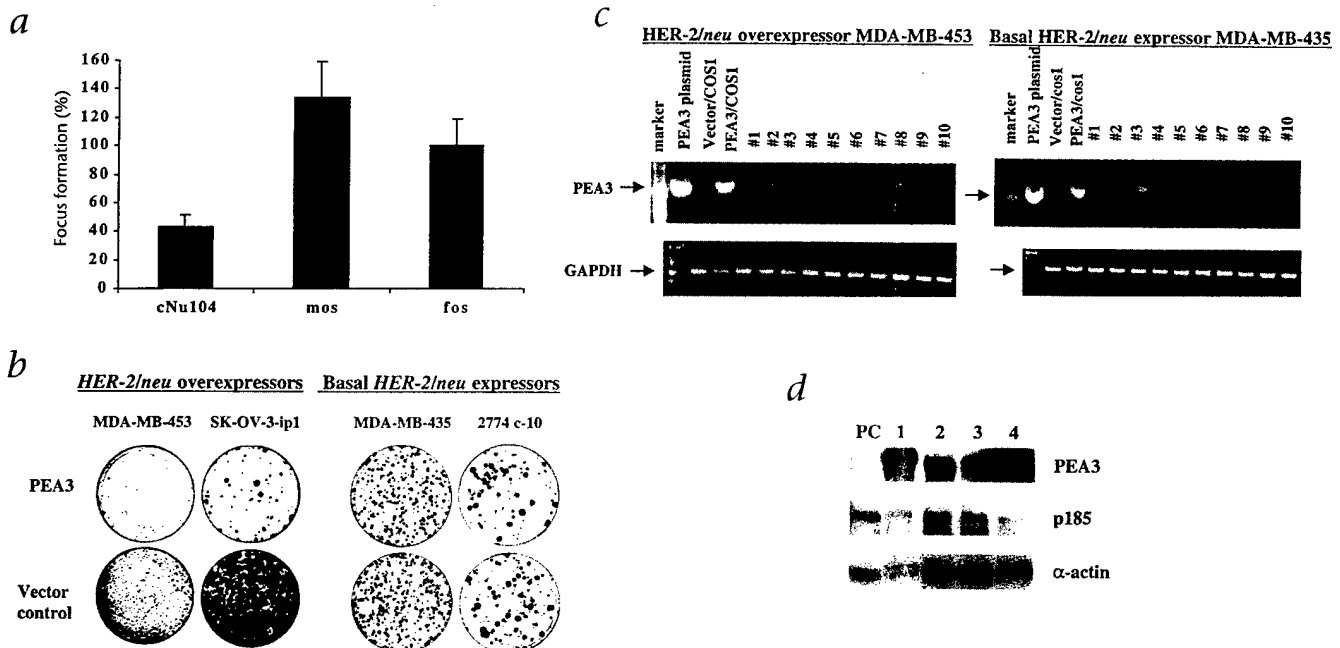
To test if the *PEA3*-mediated tumor suppression function can

Fig. 4 *PEA3* suppresses cell growth and transformation induced by *HER-2/neu* overexpression. *a*, *PEA3* specifically suppresses the transforming activity induced by *HER-2/neu*. NIH3T3 cells were co-transfected with a plasmid encoding PEA3 and cNu104, which expresses the activated rat *neu* gene, or expression plasmids of other oncogenes (*fos* or *mos*). For each oncogene tested, the number of foci obtained from co-transfection with *PEA3* and the control vector was defined as 100%. *b*, Cell growth of *HER-2/neu*-overexpressing breast and ovarian cancer cells was inhibited by *PEA3* transfection as indicated by the decrease of colony numbers; vector transfection did not inhibit growth of cancer cells. The growth inhibition effect was not seen in cell lines expressing basal level of *HER-2/neu* (MDA-MB-435 and 2774 c-10). *c*, Ectopic *PEA3* RNA expression was preferentially lost in the surviving neomycin-resistant clones derived from the *HER-2/neu*-overexpressing breast cancer cell line MDA-MB-453. *d*, Protein expression in representative *PEA3* stable cell lines derived from MDA-MB-435. PC, parental cells. Lanes 1-4, four independent *PEA3* stable cell lines derived from MDA-MB-435.

be extended to other *HER-2/neu*-overexpressing cancer cells, breast cancer cell lines with *HER-2/neu*-overexpression (SK-BR-3) and with basal level of *HER-2/neu* expression (MDA-MB-231) we transfected with plasmid encoding either wild-type *PEA3* (CMV-PEA3) or the luciferase gene (*Luc*) as a control (Fig 6*d*). Transfected cells were then inoculated subcutaneously in nude mice. Transfected MDA-MB-231 cells developed tumors of similar sizes regardless the plasmid used. There was no significant effect of *PEA3* to tumor growth of the cell line. On the other hand, cell growth of SK-BR-3 cells in nude mice was remarkably inhibited by *PEA3* transfection. Similar transfection efficiencies of the two cell lines were confirmed by transfections with a green fluorescence protein cDNA (data not shown). The absence of detectable growth of the *PEA3*-transfected SK-BR-3 cells might be the result of the requirement of a density threshold of cells that are *HER-2/neu*-overexpressing and growth-competent. Taken together, the data shown in the study indicate that *PEA3* preferentially suppresses both *in vitro* and *in vivo* growth of *HER-2/neu*-overexpressing cancer cells, and thus provides a basis to develop therapeutic applications using *PEA3* gene therapy for tumors with *HER-2/neu* overexpression.

Discussion

The PEA3 protein contains a domain of about 85 amino acids whose sequence is very similar to that of the ETS domain, a conserved region shared by all members of the *ets* family that characteristically bind to the cognate DNA binding site as monomers through their ETS DNA-binding domains. The consensus DNA binding site of PEA3 seems to occur on the promoters of a cohort of genes and to have a positive role in their expression. Examples include the stromelysine gene[34] and the tumor suppressor gene *maspin*[35,36]. In these cases, usually, there are other *ets* proteins besides the PEA3 protein itself that can be shown to target the consensus-binding site and regulate gene expression. The biological effects of the PEA3 protein on these genes remain to be determined. This study, to our knowledge for the first time, shows the tumor suppression function of an *ets* protein through suppression of oncogenic activity of the *HER-2/neu* oncogene.

We identified a DNA motif on the *HER-2/neu* promoter that directly binds to the *ets*-related transcription factor PEA3 resulting in downregulation of the *HER-2/neu* gene and thus inhibition of cell growth *in vitro* and tumor development *in vivo*. The fact that the PEA3 binding motif on the *HER-2/neu* promoter functions as a positive regulatory element for *HER-2/neu* gene transcription (Fig. 3*b*) implies the presence of an as yet unidentified transactivating factor(s) that recognizes the same positive regulatory element. PEA3 may compete with the transactivating factor(s) for the same DNA motif and repress *HER-2/neu* transcription. Consistent with this hypothesis, a DNA-binding activity to the PEA3 motif has been detected in the *HER-2/neu*-overexpressing cell line MDA-MB-453 and this DNA-binding activity can not be attributed to PEA3 itself (X.X. and M.-C.H., unpublished results). In addition, expression of the DNA-binding domain of PEA3 was enough to repress *HER-2/neu* promoter activity (data not shown). It is known that some *ets* family members, such as ERF and Net, act as repressors of gene expression[37,38]. On the other hand, promoters negatively regulated by the binding sites of the *ets* family members have also been reported[39,40].

The effect of PEA3 on the *HER-2/neu* promoter in this study is observed mainly in human breast and ovarian cancer cell lines, which are biologically relevant to cases of human disease. Other groups have previously reported transactivation of *HER-2/neu* promoter by PEA3 in COS-1 cells[41]. Under our experimental conditions, in COS-1 cells we did notice a very weak PEA3-mediated induction of *HER-2/neu* promoter. The potential species- or cell type-specific transactivating effect of PEA3 is an interesting phenomenon. It might be due to the disparity of regulatory mechanisms among different cell types, different species, or both. In this regard, it is worth noting that the COS cells were derived from monkey kidney cells transformed by *SV40* T antigen[42].

Targeting the protein product of *HER-2/neu* p185 with a humanized monoclonal antibody to p185 (*Herceptin*) has recently shown encouraging therapeutic effects in patients with *HER-2/neu*-overexpressing breast cancer, although with significant cardiotoxicity[43,44]. In addition to the immunotherapy strategy, a gene therapy setting using either the adenovirus type 5 *E1A* or the *SV40* large T antigen gene to repress *HER-2/neu* overexpression has been reported[12,26,27,29,30,45]. In the case of *E1A*, a phase I clinical trial has just been completed in which *HER-2/neu* downregulation associated with *E1A* expression was observed[46]. However, both *E1A* and *SV40* large T antigen are viral proteins, and they very probably suppress *HER-2/neu* in an indirect manner, as none of these proteins have been shown to bind to the *HER-2/neu* promoter directly. Nevertheless, these results indicate that *HER-2/neu* overexpression is an excellent focus of study in the development of therapeutic strategies against cancer.
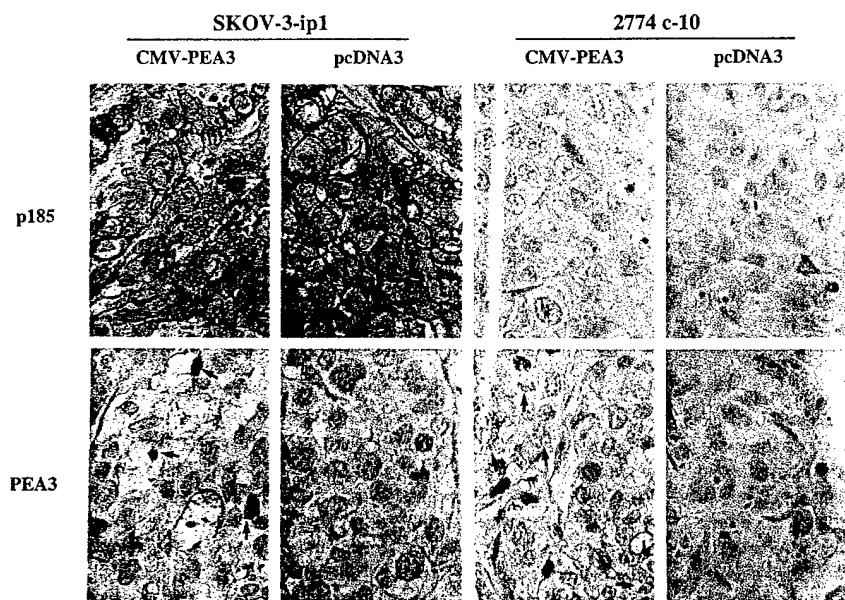

SKOV-3-ip1		2774 c-10	
CMV-PEA3	pcDNA3	CMV-PEA3	pcDNA3

Fig. 5 *PEA3* inhibits *HER-2/neu*-overexpression *in vivo*. Subcutaneous tumors derived from SKOV-3-ip1 and 2774 c-10 were treated with the *PEA3* cDNA plasmid or vector delivered by a cationic liposome (SN2; Y.Z. and M.-C.H., unpublished results). The DNA-liposome complex was injected into the tumors once a day for 2 d and the tumors were collected on day 3 and gene expression was monitored by immunohistochemical staining. SKOV-3-ip1 cells expressed high level of membrane-associated HER-2/neu protein, which decreased after *PEA3*-liposome injection. Arrows indicate examples of PEA3 nuclear expression in tumor sections after *PEA3* treatment.
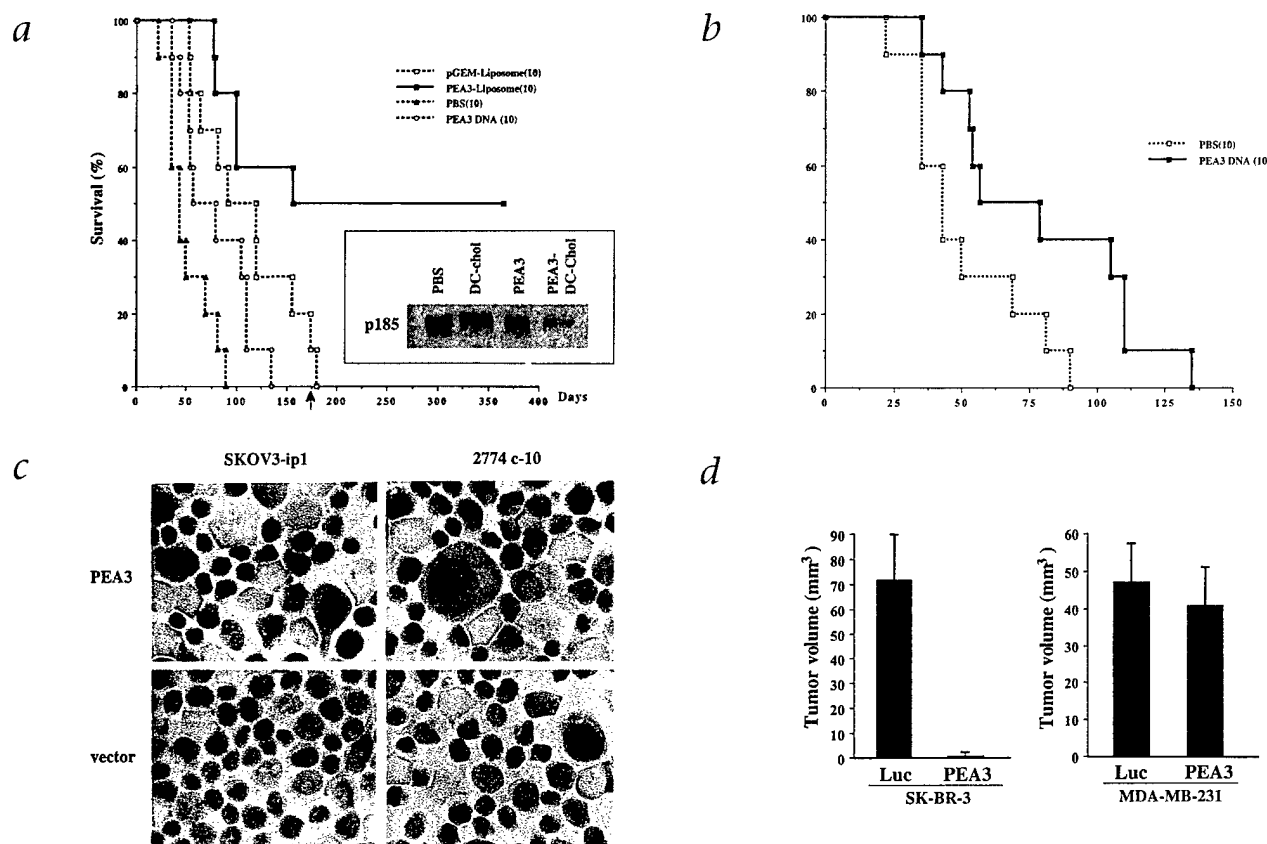
Fig. 6 *PEA3* suppresses tumor growth of *HER-2/neu*-overexpressing cancer cells. *a*, Nude mice with intraperitoneal tumors derived from SKOV-3-ip1 received weekly i.p. injections of a reagent containing *PEA3* plasmid DNA complexed with liposome (*PEA3*-liposome), control DNA (pGEM) complexed with the liposome (pGEM-Liposome), naked *PEA3* plasmid DNA, or PBS. Moribund mice were killed and the tumors in the peritoneal cavity were collected for further characterization. The number of mice in each group is shown in the parentheses. The arrow indicates the last injection. Tumor samples were retrieved from mice treated with PBS, the lipid vehicle DC-Chol, *PEA3* naked DNA, or *PEA3*-DC-Chol complex, and the levels of p185 were measured by immunoblotting as shown in the insert. Whole body pathological examination of the tumor-free mice showed no

evidence of tumor growth (data not shown). *b*, Nude mice with tumors derived from 2774 10-c were treated with PEA3-liposome complex (the solid line) or PBS alone (the dashed line) following the same procedure that was used with the SKOV-3-ip1 cells. *c*, Cationic liposome transduction of *PEA3* cDNA results in PEA3 protein expression in SKOV-3-ip1 and 2774 c-10 cells at similar efficiency. Cell cultures of SKOV-3-ip1 and 2774 c-10 cells were transfected with 10 μg *PEA3* cDNA plasmid. Transfected cells were trypsinized 36 h later and cells were transferred to slides and subjected to immunohistochemical detection for PEA3 expression. *d*, PEA3 treatment inhibited the tumor growth ability of *HER-2/neu*-overexpressing but not cells expressing basal level of *HER-/neu*. At least nine tumors were included for each treatment.

Because PEA3 directly binds to the *HER-2/neu* promoter, it may more strongly and specifically inhibit *HER-2/neu* promoter activity. This feature makes PEA3 an attractive candidate for further molecular manipulation in the development of the next generation of therapeutic molecules with higher binding affinity and enhanced specificity. It is worthwhile mentioning that low or undetectable *PEA3* expression in *HER-2/neu*-overexpressing breast cancer cell lines has been reported[47]. This inverse relationship between *PEA3* and *HER-2/neu* expression is certainly consistent to, although does not prove, our conclusion that *PEA3* expression can downregulate *HER-2/neu* overexpression. The study presented here shows a proof of concept in which the overexpression of *HER-2/neu* can be, at least, mitigated by a transcriptional repressor. Because *HER-2/neu* overexpression causes chemoresistance to certain anticancer agents including Taxol[21] and TNF-α (ref. 18), it will be interesting to test if downregulation of *HER-2/neu* by *PEA3* can enhance the therapeutic efficacy of Taxol or other anticancer drugs. In addition, the strategy described in this study provides a general approach to identifying potential transformation suppressors: a search for DNA-binding

proteins that recognize specific DNA sequence on the promoter of the targeted oncogenes or other pathogenic genes.

Methods

Plasmids. To construct the plasmid pNulit, the promoter region of human *HER-2/neu* was amplified with a pair of primers (primer A 5′-GATAGGATC-CGGGGGTCCTGGAAGCC-3′ and primer B 5′-GGGCAGATCTGGTTTTC-CGGTCCCAATGGA-3′). The amplified DNA fragment was digested with *BamH*I and *Bgl*II and ligated into the *BamH*I site of pGL2-Basic (Promega, Madison, Wisconsin). The sequence of this insert has been confirmed.

For site-directed mutagenesis of the PEA3 binding site, two more primers with sequences spanning the PEA3 binding site on the *HER-2/neu* promoter were synthesized; in these primers, the PEA3 motif AGGAAG was changed to AGCTCG (primer C 5′-GGAGGAGGAGGGCTGCTTG<u>AGCTCG</u>TATAA-GAATG-3′ and primer D 5′-CATTCTTATA<u>CGAGCT</u>CAAGCTCCTCC-3′). The PCR product amplified by primers A and D and the product amplified by primers B and C were both annealed followed by *Klenow* extension. The double-stranded DNA fragment was then cloned into the pGL2-Basic vector as the wild-type promoter. The full-length *PEA3* cDNA was cloned into pcDNA3 between the *Hind*III and *BamH*I sites (pcDNA3-PEA3). To test if PEA3 nonspecifically suppressed cell transformation, cDNA constructs of v-*mos* (driven by the M-MLV LTR promoter)[48], and c-*fos* (driven by the FBJ-

MSV LTR promoter) (gifts of Balraj Singh and Paul Chiao, M. D. Anderson Cancer Center, respectively) were used in co-transfection with PEA3 or the control vector as described.

Cells. Mouse fibroblast NIH3T3 and human cancer cells used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah) supplemented with 10% bovine calf serum and DMEM with10% fetal bovine serum, respectively.

Gel mobility shift assay. The annealed double-strand oligonucleotide derived from the *HER-2/neu* promoter containing either the wild type (5'–GGAGCTCGAGGGCTGCTTGAGGAAGTATAAGAATG–3' and the complementary strand) or a mutated PEA3 binding site (5'–GGAGCTC-GAGGGCTGCTTGAGCTCGTATAAGAATG–3' and the complementary strand) was end-labeled by ^{32}P-γATP. Binding of the protein factors to DNA sequences was achieved in a mixture containing 1× binding buffer (20 mM HEPES, pH 7.9, 5 mM MgCl$_2$, 5% glycerol, 0.1 M KCl, 0.2 mM EDTA, 2 mM dithiothreital (DTT)), 0.3 μg GST-PEA3 (or GST alone or 1 μg nuclear extract of MDA-MB-453), 2–8 μg poly d(I-C), 1 μg of bovine serum albumin, and 20,000 cpm of ^{32}P-labeled oligonucleotide. Unlabeled competitor oligonucleotide was added in an amount 100 times more than the labeled probe when it was necessary. In the protein binding competition assay, 0.3 μg or 0.6 μg of GST-PEA3 protein was included in the reaction mixture. The binding reaction was done at room temperature for 20 min. The samples were separated on a 5% polyacrymide gel; the gels were vacuum dried and autoradiographed.

Transient transfection and luciferase assay. Human cancer cells were seeded with 60–80% confluency and transfected by incubating with a DNA:DC-chol liposome complex (1 μg DNA:13 nmol lipid) in serum free medium for 2–5 h or by incubating with a DNA:polyethylenimine (PEI, 4.5 μg/μl, average MW 25,000; Aldrich, Milwaukee, Wisconsin) complex (1 μg DNA: 0.5 μl PEI) for 1–3 h. Cell lysates were prepared 48 h later for the luciferase activity assay by following the manufacturer's (Promega) instructions and using a monolight 2010 Luminometer (Turner Designs, Sunnyvale, California). The measured luciferase activity levels were normalized by measuring the co-transfected β-galactosidase activity. The β-galactosidase activity was measured by mixing equal amounts of cell extract and the 2× assay buffer (120 mM Na$_2$HPO$_4$, 80 mM NaH$_2$PO$_4$, 2 mM MgCl$_2$, 100 mM β-mercaptoethanol, 1.33 mg/ml orthonitrophenyl galactose). After incubating at 37 ˚C, enzyme activity was determined by absorbance at 420 nm.

Stable transfection, colony forming assay, and focus forming assay. Human cancer cells under 80% confluency were transfected with 1 μg of PEA3 expression plasmid or the pcDNA3 vector using DC-chol liposome as the carrier and subjected to neomycin selection. Neomycin-resistant colonies were stained with 1% crystal violet and counted. Transfections of each cell line were repeated two to three times using plasmid DNA from independent preparations. For focus forming assay, NIH3T3 cells in 35-mm wells were transfected with *PEA3* cDNA plasmid (or the backbone vector pcDNA3 as control) together with the cNu104 DNA (or the *fos* and *mos* cDNA plasmids) at a ratio of 5:1 (PEA3: oncogene). Forty-eight hours after transfection, cells were plated in 100-mm plates for culturing, and the resultant foci were stained by crystal violet for counting. Foci number was determined based on the counting of at least two independent persons.

Orthotopic ovarian cancer model and liposome-mediated *in vivo* gene transfer. Four- to six-week-old athymic female *nu/nu* mice were purchased from Harlan Sprague Dawley (Indianapolis, Indiana) and treated in accordance with institutional guidelines. To establish tumors, 2 × 10^6 SKOV-3-ip1 cells were injected into the intraperitoneal (i.p.) cavity as described[27,29]. Five days after i.p. injection of cancer cells, the mice were separated into five groups and received weekly i.p. injections of 200 μl of different reagents for a period of time. The responses and survival rates were observed for 1 year. Tumors from moribund mice were collected and analyzed. The subcutaneous tumors in nude mice were established by injecting 2 × 10^6 SKOV-3-ip1 or 2774 c-10 cells subcutaneously. When the tumor sizes reached 60–90 mm^3, which took about 3 weeks, treatment was started by intratumoral injection of the PEA3 expression plasmid or the vector DNA alone delivered by a cationic liposome. Each tumor received two doses of treatment. Sixteen hours after the final treatment the tumors were isolated and analyzed by immunohistochemistry.

Immunoblotting. Immunoblot analysis for PEA3 and the HER-2/neu protein expression in cells and tumor samples was done as described[26,45].

Immunohistochemical staining. Tumor tissue sections taken from the xenografts were fixed with formalin and paraffin-embedded. Monoclonal antibody against HER-2/neu (Ab-3; Calbiochem, La Jolla, California) or PEA3 (Santa Cruz Biotechnology, Santa Cruz, California) was used to detect the protein expression. After incubating with the primary antibody, a biotinylated antibody to mouse IgG was applied to the tissue section followed by incubating with the avidin biotinylated complex reagents (Vector, Burlingame, California). The staining was developed in the aminoethylcarbazole chromogen substrate solution. Mayer's hematoxylin was used as a counterstain.

RT-PCR. Total RNA was extracted from cell clones derived from stable transfection, and RT-PCR was done as per the manufacturer's instructions (SuperScript Preamplification system; Life Technologies, Inc.). The primers derived from the *PEA3* coding sequence (5'–TGAATTATGACAAGCT-GAGCCG–3') and from the expression vector pcDNA3 (5'–TCAGC-GAGCTCTAGCATTTAGG–3') were used to amplify the ectopically expressed *PEA3* transcript. Primers for GAPDH internal control were: 5'–AG-GTGAAGGTCGGAGTCAAC–3' and 5'–TCCATTGATGACAAGCTTCCC–3'. COS-1 cells transiently transfected by the *PEA3* cDNA were used as the positive control. Amplification was done on a Perkin Elmer DNA Cycler 480 for 35 cycles with denaturing at 94 °C for 30 s, annealing at 58 °C for 1.5 min, and extension at 72 °C for 1.5 min.

Matrigel assay. SK-BR-3 cells in tissue culture plates were transfected with either CMV-PEA3 or the backbone vector pcDNA3. Sixteen hours after transfection the cells were trypsinized and suspended in PBS with 2 × 10^7 cells/ml. Equal volume of Matrigel (Becton Dickinson Labware, Bedford, Massachusetts) was mixed with cell suspension at 4 °C. Nude mice were inoculated subcutaneously with 1 × 10^6 cells. Tumor volumes were measured 2 weeks later. The same procedure was applied to MDA-MB-231 except that Matrigel is not required for tumor formation from the cell line.

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Oncogenic Signals of HER-2/*neu* in Regulating the Stability of the Cyclin-dependent Kinase Inhibitor p27*

Heng-Yin Yang‡§, Binhua P. Zhou‡¶, Mien-Chie Hung‡∥**, and Mong-Hong Lee‡∥**‡‡

*From the Departments of ‡Molecular Cellular Oncology and ∥Surgical Oncology, **Breast Cancer Research Program, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030*

Overexpression and activation of HER-2/*neu*, a proto-oncogene, play a pivotal role in cancer formation. Strong expression of HER-2/*neu* in cancers has been associated with poor prognosis. Reduced expression of p27^{Kip1}, a cyclin-dependent kinase inhibitor, correlates with poor clinical outcome in many types of carcinomas. Because many cancers with the overexpression of HER-2/*neu* overlap with those affected by reduced p27 expression, we studied the link between HER-2/*neu* oncogenic signals and p27 regulation. We found that down-regulation of p27 correlates with HER-2/*neu* overexpression. To address the molecular mechanism of this inverse correlation, we found that reduction of p27 is caused by enhanced ubiquitin-mediated degradation, and the HER-2/Grb2/MAPK pathway is involved in the decrease of p27 stability. Also, HER-2/*neu* activity causes mislocation of p27 and Jun activation domain-binding protein 1 (JAB1), an exporter of p27, into the cytoplasm, thereby facilitating p27 degradation. These results reveal that HER-2/*neu* signals reduce p27 stability and thus present potential points for therapeutic intervention in HER-2/*neu*-associated cancers.

The HER-2/*neu* oncogene (also named c-erbB-2) encodes a growth receptor tyrosine kinase, and amplification/overexpression of the human HER-2/*neu* gene are frequently found in human cancers, including breast, ovarian, lung, gastric, and oral cancers (1–3). Also, HER-2/*neu* overexpression correlates with a shorter survival rate in breast cancer patients. The molecular mechanism underlying how oncogenic signals of HER-2/*neu* affect the cell cycle machinery in affecting tumorigenicity is not completely determined. It is possible that HER-2/*neu* overexpression may stimulate cell proliferation through tyrosine kinase signaling to mediate mitogenic signals in promoting cell cycle progression. The cell cycle is regulated by both the positive and negative regulators. Cyclin and cyclin-dependent kinase (CDK)[1] are positive regulators, whereas cyclin-dependent kinase inhibitors, including the Inhibitor of CDK4 family and the CIP/KIP family, are negative regulators (4). p27, a CIP/KIP member, encodes a cyclin-dependent kinase inhibitor that causes G_1 arrest by inhibiting the activities of G_1cyclin-CDKs. As a negative regulator of the cell cycle, p27 is a new class of tumor suppressor and is haplo-insufficient in tumor suppression (5, 6). In animal studies, the number of p27 gene copy can decide the rate of tumor formation, because p27 haplo-insufficient mice are hypersensitive to carcinogens (6).

Recently, reduced expression of p27 is frequently detected in human cancers, including breast (7, 8), prostate (9), gastric (10), lung (11), skin (12), colon (13), and ovarian cancers (14). Decreased expression of the p27 protein was shown to correlate with cancer development and poor survival, thus appearing as an important marker of cancer progression. Because p27 inhibits cyclin-CDK in a dosage-dependent manner to control cell cycle progression (15, 16), it is conceivable that decreased expression of p27 may result in abnormal cell proliferation in these cancers. However, the exact mechanism that underlies the decreased expression of p27 in cancer remains elusive. p27 is regulated post-transcriptionally through the ubiquitin-mediated proteasome degradation pathway (17); therefore, it is possible that reduced p27 in many types of cancer may be caused by the enhancement of ubiquitin-mediated p27 degradation.

Here, we assessed the roles of HER-2/*neu* signaling in regulating the stability of p27 and found that HER-2/*neu* activity specifically causes the decrease of p27 protein level by inducing the mislocation of p27 in the cytoplasm for ubiquitin-mediated degradation. Thus, this study provides an important mechanism link between two prognostic markers in cancers.

MATERIALS AND METHODS

Cell Culture—NIH3T3, 293T, B104–1-1 (18), B104–1-1/ΔN-Grb2, B104–1-1/ΔC-Grb2, SW3T3, SW3T3-X-1 (SW3T3 overexpressing HER2/*neu*), R1B/L17 cells (the mink lung epithelial cell line derivative) (15), human breast carcinoma cell lines MCF7 (does not overexpress HER-2/*neu*), and HER18 (MCF7 overexpressing HER2/*neu*) (19) were maintained in Dulbecco's minimal essential media containing high glucose levels and 10% fetal calf serum. The use of B104–1-1, B104–1-1/ΔN-Grb2, and B104–1-1/ΔC-Grb2 has been described previously (20). A DEAE-dextran method was used to transiently transfect R1B/L17 cells as described previously (15). PD98059 (Calbiochem), LLnL (Sigma), and MG132 (Sigma) were prepared in Me₂SO for use.

Western Blot Analysis—Total cell lysates were solubilized in lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, 1 μg each of aprotinin, leupeptin, and pepstatin per ml) and were processed as described previously (15). Analyses were performed on 12% polyacrylamide gels with a 5% polyacrylamide stacking gel. After electrophoretic transfer (Amersham Pharmacia Biotech) of protein from SDS polyacrylamide gels to

[1] The abbreviations used are: CDK, cyclin-dependent kinase; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; MAPK, mitogen-activated protein kinase; CIP, CDK interaction protein; KIP, kinase inhibitor protein; JAB, Jun activation domain-binding protein; CMV, cytomegalovirus.

polyvinylidene difluoride membranes (Millipore), the membranes were blocked with the buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 5% Blotto (Bio-Rad) for 1 h at room temperature and incubated for 1 h at room temperature with the following primary antibodies: polyclonal anti-p21 antibody (Santa Cruz), polyclonal anti-actin (sigma), polyclonal anti-p27 antibody, monoclonal anti-p27 antibody (Transduction Laboratories), and monoclonal anti-FLAG antibody (M2) (Sigma). Subsequently membranes were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies. Following several washes, membranes were incubated with a chemiluminescence (ECL) system (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Northern Blot Analysis—Total RNAs were isolated using Qiagen RNeasy kits. Each sample containing 20 μg of total RNAs was used for Northern blot analysis. RNAs were transferred to GeneScreen Plus membranes (NEN Life Science Products) using a Turboblotter system (Schleicher & Schuell). The p27 and glyceraldehyde-3-phosphate dehydrogenase cDNA probes were labeled by a random-primed DNA labeling kit (Roche Molecular Biochemicals). The glyceraldehyde-3-phosphate dehydrogenase probe was used to indicate the integrity and equal amounts of loading for each RNA sample.

The Cdk-associated Histone H1 Kinase Assay—The cells were lysed in lysis buffer as described above, and the protein concentration was quantified. 1 mg of cell lysate was immunoprecipitated with anti-Cdk2 antibody (Pharmingen). The immunoprecipitates were assayed for histone H1 kinase activity as described previously (15).

Metabolic Labeling, Immunoprecipitations, and Half-life Determination—Cells were maintained and grew to 70% confluency. The cellular proteins were pulse-labeled with ^{35}S methionine (100 μCi/ml) for 3 h in methionine-free media and chased with cold Met for 0, 2, or 5 h; p27 was then immunoprecipitated from each lysate with 10 μg/ml of p27 antibody, separated by SDS-PAGE, and analyzed by phosphorimaging (Molecular Dynamics) or autoradiography. The intensity of radiolabeled p27 was quantitated by the Imagequant program of the PhosphorImager. The half-life of the protein was determined graphically according to procedures described previously (21).

In Vitro Degradation Assays—A polymerase chain reaction-generated *Nde*I-*Bam*HI fragment of the p27 cDNA containing the full-length coding region was subcloned into pET21a (Novagen) to yield a construct that encodes p27 with a FLAG-tag sequence. The protein was expressed in BL21 (DE3), and FLAG-tagged proteins were prepared as described previously (15). 100 μg of cell lysate prepared from NIH3T3 or B104-1-1 cells was incubated with 100 ng of affinity-purified FLAG-tagged p27 protein at 37 °C for 0, 0.5, 3, and 19 h in a buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 μg of ubiquitin, 2 mM ATP, 1 mM dithiothreitol, 15 mM phosphocreatine, 1 μg of creatine phosphokinase according to the procedure described previously (13).

Immunofluorescence—Endogenous p27 or JAB1 subcellular localization was detected in NIH3T3 or B104-1-1 cells. Cells were prepared and seeded onto chamber slides with 2×10^5 cells per well 1 day prior to staining. Cells were then fixed with methanol/acetone (1:1, v/v) at room temperature for 2 min and stained for 1 h with rabbit anti-p27 (Santa Cruz) or goat anti-JAB1 (Santa Cruz) antibody followed by a 1-h incubation with Cy3-conjugated anti-rabbit antibody (Zymed Laboratories Inc.) or Texas red-conjugated anti-goat antibody (Jackson Research Laboratories). For studying subcellular localization of exogenous p27 or JAB1, R1b/L17 cells were transiently co-transfected with pCMV-HER-2/neu (a point mutation at the transmembrane domain results in constitutive activation of HER-2/neu) (18, 22) and pCMV-FLAG-p27, or pCMV-FLAG-JAB1. 24 h after transfection, 2×10^5 cells were seeded onto tissue culture chamber slides (Nunc). 2 days later, cells were fixed and stained as mentioned above. Monoclonal anti-FLAG antibody (M2) (Sigma) was used in detecting p27 or JAB1 expression. In this case, the fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (Jackson Research Laboratories) was used. For all staining, cells were incubated with 0.1 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) (Sigma) to stain the nuclei. Immunofluorescence was detected using a BX50 fluorescent microscope (Olympus).

RESULTS

HER-2/neu Overexpression/Activation Results in Reduced p27 Expression—To investigate whether HER-2/neu affects the expression of p27, we used NIH3T3 cells, B104-1-1 cells (derived from NIH3T3 overexpressing constitutively active HER-2/neu) (18), MCF7, and HER18 cells (derived from MCF7 overexpressing HER-2/neu) (19) to examine the role of HER-2/neu signaling in regulating the protein level of p27 by immunoblot-
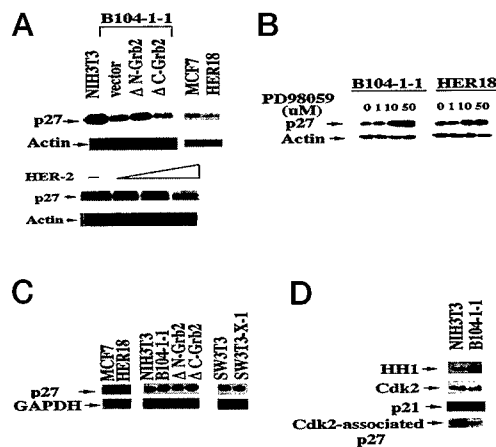

FIG. 1. **Expression of p27 in HER-2/*neu*-activating or HER-2/ *neu* non-activating cell lines.** *A*, levels of p27 protein in HER-2/*neu*-activating cells. Equal amounts (50 μg) of indicated cell lysates were separated by SDS-PAGE and then immunoblotted with p27 antibody. HER-2/*neu* in B104-1-1 cells is constitutively active. ΔN-Grb2, ΔC-Grb2, and vector control are stable transfectants of B104-1-1 cells. HER18 cells derived from MCF7 overexpressing HER-2/*neu*. 293T cells were co-transfected with p27 (1 μg) and increasing amounts of CMV-HER2/*neu* (0, 4, 8, or 18 μg). Levels of actin were shown as equal loading control. *B*, effect of MAPK inhibitor on p27 expression. B104-1-1 and HER18 cells were treated with MAPK kinase inhibitor PD98059 for 24 h at the indicated concentrations. Equal amounts of cell lysates were separated by SDS-PAGE and then immunoblotted with p27 antibody. Equal amounts of protein used for assay were indicated by equal anti-actin immunoblotting. *C*, expression of p27 transcripts in HER-2/*neu*-activating cell lines. Northern blot analysis was performed on total RNAs of indicated cell lines to examine the expression of p27 gene. Signals of glyceraldehyde-3-phosphate dehydrogenase were shown to indicate the integrity and quantity of the RNA. *D*, Cdk2 kinase activity in various cell lines. Equal amounts of indicated cell lysates were immunoprecipitated with Cdk2 antibody to assay for the Cdk2-associated histone H1 kinase activity or Cdk2-associated p27. The level of p27 or p21 protein was detected by immunoblotting with anti-p27 or anti-p21. Equal amounts of immunoprecipitated Cdk2 were indicated.

ting. As demonstrated in Fig. 1*A*, the constitutive activation (B104-1-1) or overexpression (HER-18) of HER-2/*neu* results in the decreased expression of p27. Interestingly, ΔN-Grb2, which is an amino-terminal deletion mutant of Grb2 and acts as a dominant mutant of Grb2 to block HER-2/*neu* signaling through the Grb2 pathway (20), can alleviate the decreased protein expression of p27 in B104-1-1 cells. On the other hand, ΔC-Grb2 (20), which has minimum effect in blocking HER-2/ *neu* signaling, does not block the down-regulation of p27 in B104-1-1 cells. In addition, when cells were co-transfected with p27 and increasing amounts of HER2/*neu*, the level of p27 was diminished as detected by immunoblotting (Fig. 1*A*). To determine whether the HER-2/Grb2/MAPK pathway is involved in regulating p27 protein level, we further examined the effect of MAPK inhibitor on p27 protein level in B104-1-1 and HER18 cells. The level of p27 protein is rescued when these cells are treated with MAPK inhibitor PD98059 (Fig. 1*B*), suggesting that the MAPK pathway of HER-2/*neu* signals is responsible for down-regulation of p27 protein. The decreased level of p27 protein is not due to down-regulation of mRNA, because each cell line contains the same amount of p27 transcripts (Fig. 1*C*). To study the consequence of decreased p27 in regulating Cdk activity, we measured the Cdk2-associated histone H1 kinase activity in cells. We found that Cdk2 kinase activity was markedly increased in B104-1-1 compared with NIH3T3, which reflects the reduction of both Cdk2-associated p27 (Fig. 1*C*) and total p27 level (Fig. 1*D*) in B104-1-1 cells. Interestingly, the level of p21, another CIP/KIP member, is up-regulated in B104-1-1 cells (Fig. 1*D*), indicating that
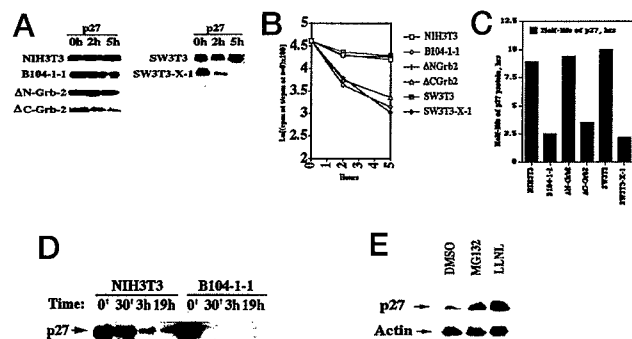
FIG. 2. **Effect of HER-2/neu activity on the stability of p27 protein.** A, pulse-chase analysis of p27 protein in various cell lines. Indicated cells were pulse-labeled with [35]S Met for 3 h and chased with unlabeled Met for the indicated time period. Radiolabeled p27 was immunoprecipitated by using p27 antibodies. B, effect of HER-2/neu activity on stability of the p27 protein. Images of the labeled p27 protein from the results of the experiment represented in A were analyzed and quantitated by the Imagequant program of the PhosphorImager (Molecular Dynamics). The ratio of labeled p27 protein at a given time point over the labeled p27 protein at time zero was plotted as a function of time. The slopes of the resulting curve (decided by Delta Graph) are used to calculate the half-life of the p27 protein according to procedures described previously (21). C, half-life of p27 in indicated cell lines with different HER-2/neu status. The half-life of p27 was calculated using the methods described in B. D, in vitro ubiquitin-mediated degradation of p27. 100 μg of cell lysate prepared from NIH3T3 or B104–1-1 cells was incubated with 100 ng of affinity-purified FLAG-tagged p27 protein at 37 °C for 0 min, 30 min, 3 h, or 19 h. The level of p27 in each reaction was immunoblotted with anti-M2. E, p27 is increased in the presence of 26 S proteasome inhibitors. B104–1-1 cells were treated with Me₂SO, LLnL (200 μM), or MG132 (50 μM) for 16 h. Me₂SO treatment was used as a control. 100 μg of each cell lysates were immunoblotted with anti-p27. Levels of actin were shown as equal loading control.

HER2/neu signals affect the expression of p27 or p21 (23) in a different manner. In conclusion, our results indicate that HER-2/neu signals cause the down-regulation of p27 specifically.

HER-2/neu Overexpression/Activation Accelerates the Turnover Rate of p27—To study whether HER2-mediated downregulation of p27 results from increased turnover rate of p27, we determined the half-life of p27 by pulse-chase experiment in cell lines with different HER-2/neu statuses, including HER-2/neu overexpression/activation cell lines (B104–1-1, B104–1-1/ΔC-Grb2, and SW3T3-X-1 cells) and non HER-2/neu overexpression/activation cells (NIH3T3, B104–1-1/ΔN-Grb2, and SW3T3 cells). A pulse-chase analysis of p27 protein clearly indicated that radiolabeled p27 protein decreased faster in HER-2/neu overexpression/activation cells than that in non HER-2/neu overexpression/activation cells (Fig. 2A). Images of the radiolabeled p27 protein were analyzed and quantitated by a PhosphorImager (Molecular Dynamics) to calculate the half-life of the p27 protein according to procedures described previously by Yang and Evans (Fig. 2B) (21). As shown in Fig. 2C, the half-life of p27 is dramatically decreased in HER-2/neu overexpression/activation cell lines, such as B104–1-1, B104–1-1/ΔC-Grb2, and SW3T3-X-1 cells. These results indicate that the status of HER-2/neu can affect the half-life of p27 and that overexpression and activation of HER-2/neu clearly decrease the half-life of p27. Because p27 is regulated by ubiquitin-mediated degradation, we determined whether HER-2/neu signaling can enhance this process to cause the decrease of p27 half-life. We prepared cell lysates from B104–1-1 and NIH3T3 cells for a substrate degradation assay. The in vitro ubiquitin-mediated degradation assay was performed in a buffer containing Tris, MgCl₂, ubiquitin, and the ATP regenerating system. As shown in Fig. 2D, cell lysates prepared from B104–1-1 cells can degrade the affinity-purified FLAG-tagged p27 protein much more quickly than the lysates from NIH3T3 cells. Furthermore, we showed that p27 were accumulated when B104–
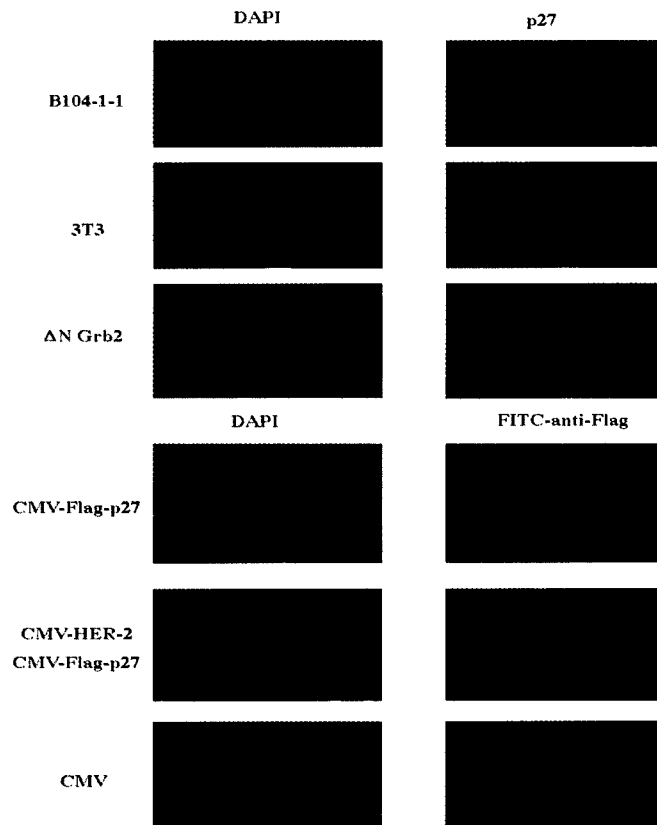

FIG. 3. **Activity of HER-2/neu inhibits the nuclear accumulation of p27 Kip1.** B104–1-1 cells, ΔN-Grb2 cells, and NIH3T3 cells were fixed, and p27 was immunodetected with anti-p27. The localization of p27 was detected by indirect immunofluorescence staining using polyclonal anti-p27 antibodies followed by Cy3-conjugated anti-rabbit immunoglobulin (red, top panels). R1B/L17 cells were transfected to examine the subcellular localization of p27 in the presence or absence of HER-2/neu as detected by immunofluorescence (green, bottom panels). Empty vector transfection (CMV) was used as a control. Cells that received indicated plasmids were seeded at 2 × 10³ in chamber slide. Cells were fixed, and p27 were immunodetected with monoclonal anti-FLAG antibody followed by FITC-conjugated anti-mouse immunoglobulin. DAPI staining was used to show the localization of nuclei.

1-1 cells were treated with specific 26 S proteasome inhibitors LLnL or MG132 (Fig. 2E). These results indicate that HER-2/neu activity can accelerate the ubiquitin-mediated degradation process of p27 and illustrated that the increased turnover rate of p27 in B104–1-1 cells is due to enhanced ubiquitin-mediated degradation activity imposed by HER-2/neu signaling.

HER-2/neu Signaling Is Involved in the Regulation of p27 Nuclear Localization—p27 was shown to be transported to the cytoplasm for degradation (24). To determine whether HER-2/neu signals are involved in the degradation of p27 by affecting its localization, we analyzed the subcompartmental localization of p27 in the presence of activating HER-2/neu by immunostaining. As shown in Fig. 3, p27 was detected in the nucleus of the NIH3T3 cells, whereas p27 was detected mainly in the cytoplasm of the B104–1-1 cells that contain active HER-2/neu. Blockade of HER2/neu signals with a ΔN-Grb2 also causes the retention of p27 in the nucleus (Fig. 3). To further define the involvement of activating HER-2/neu in the above observation, we transiently transfected cells with a mutated HER-2/neu, which has resulted in constitutive activation of HER-2/neu in terms of foci formation and transformation efficiency (18, 20), to investigate the effect of HER-2/neu on p27 localization. Cotransfection of a mutated HER-2/neu with a FLAG-tagged p27 into R1B/L17 cells also resulted in the detection of p27 in the cytoplasm, whereas the transfection of FLAG-tagged p27 alone
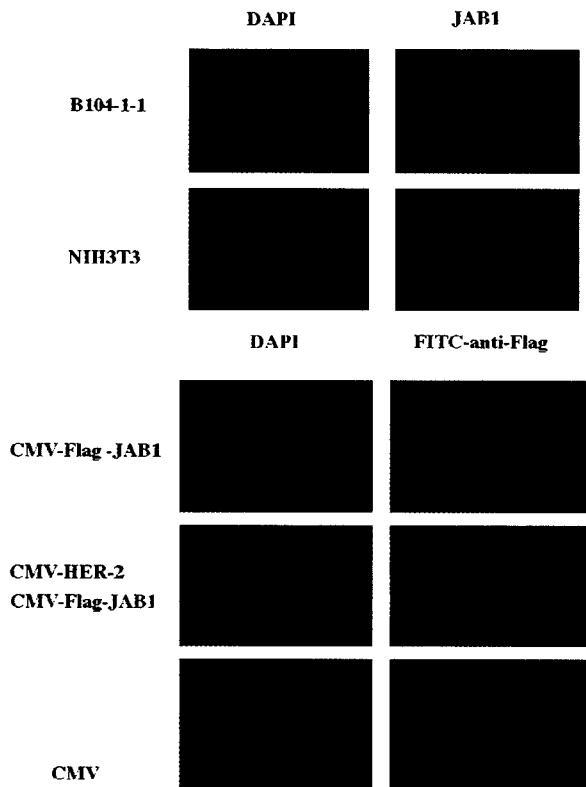
FIG. 4. **Constitutively active HER-2/*neu* can stimulate the accumulation of JAB1 in the cytoplasm.** B104–1-1 and NIH3T3 cells were fixed and analyzed for the localization of JAB1. The localization of JAB1 was analyzed by indirect immunofluorescence staining using polyclonal anti-JAB1 antibodies followed by Texas red-conjugated anti-goat immunoglobulin (*red, top panels*). R1B/L17 cells were transfected with plasmid-encoding FLAG-tagged-JAB1 (*CMV-FLAG-JAB1*) in the presence or absence of plasmid-encoding activating HER-2/*neu* (*CMV-HER-2/neu*). Empty vector transfection (*CMV*) was used as a control. Cells were fixed and analyzed for immunofluorescence using monoclonal anti-FLAG antibody followed by FITC-conjugated anti-mouse immunoglobulin (*green, bottom panels*). Nuclei were visualized by DAPI staining of DNA.

demonstrated the nucleus location of p27 (Fig. 3, *bottom panels*). Thus, activating HER-2/*neu* can cause the translocation of p27 into cytoplasm, thereby accelerating p27 degradation.

HER-2/neu Signaling Can Prevent Nuclear Localization of JAB1—JAB1, a Jun activation domain-binding protein (25), can bind p27 and is involved in the transportation of p27 into the cytoplasm for degradation. To determine whether HER-2/*neu* signal-mediated exportation of p27 involves JAB1 regulation, we investigated the effect of HER-2/*neu* activity on the localization of JAB1. As shown in Fig. 4, JAB1 is affected by HER-2/*neu* activity and is located in the cytoplasm of B104–1-1 cells, whereas JAB1 is in the nucleus of NIH3T3 cells. To further confirm the involvement of HER-2/*neu* signals in affecting JAB1 location, we co-expressed JAB1 with activated HER-2/*neu* in a transient co-transfection assay. Ectopic FLAG-JAB1 expressed in R1B/L17 cells was located in the nucleus. Co-expression of an activated HER-2/*neu* markedly increased the JAB1-specific immunofluorescent signals in the cytoplasm (Fig. 4, *bottom panels*). Consequently, HER-2/*neu* activation can affect the exportation of JAB1 to the cytoplasm, which may in turn facilitate p27 degradation.

DISCUSSION

HER-2/*neu* overexpression plays an important role in promoting tumorigenicity in colon, gastric, breast, lung, and ovarian cancers. Interestingly, these cancers overlap with those affected by reduced p27 expression. Now both are significant
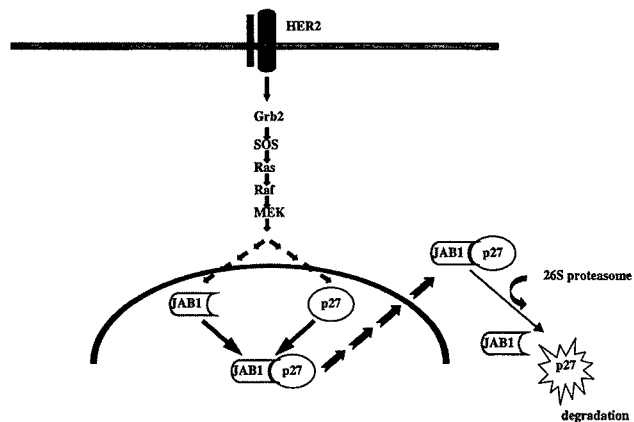

FIG. 5. **A model for p27 down-regulation induced by HER-2/*neu* oncogenic signals.**

prognostic markers for breast cancer. Accordingly, we screened the protein expression of HER-2/*neu* and p27 in primary breast tumor samples by immunohistochemistry. Among the 60 cases we have studied, we have found that there was a trend for a decreasing degree of p27 staining with increasing HER-2/*neu* expression,[2] suggesting a link between HER-2/*neu* expression and p27 regulation. These results are consistent with the biochemical studies described here in HER2/*neu* overexpressing cell lines. This study showed that HER-2/*neu* oncogenic signals can cause reduced expression of the haplo-insufficient tumor suppressor p27 (6). We have investigated the molecular mechanism of HER-2/*neu* signals in down-regulating p27. First, we used isogenic cell lines that only differ in HER-2/*neu* statuses to investigate the regulation of p27. We found that p27 protein is down-regulated by HER-2/*neu* activity. However, the mRNA levels of p27 are equally expressed in these cell lines, which is supported by the notion that p27 is mainly regulated post-transcriptionally (17). Also, the half-lives of p27 protein are significantly reduced in these cell lines that have overexpressed HER-2/*neu* activity. Furthermore, *in vitro* degradation assay indicates that HER-2/*neu* signals potentiate the ubiquitin-mediated proteasome degradation system that regulates p27 stability. Second, using a HER-2/*neu* signal mutant that blocks the Grb2/Ras pathway, we observed that the down-regulation of p27 is rescued. Interestingly, it was shown that Ras can integrate mitogenic signals with cell cycle progression by down-regulating p27 (26), although the mechanism is not characterized, yet this observation supports our studies in defining the activity of Ras of HER-2/*neu* signals in regulating p27 protein level. Third, by blocking the MAPK kinase activity in the HER-2/*neu* pathway, we found that p27 down-regulation is reversed. MAPK kinase is involved in cell proliferation and transformation. Several studies have shown that overexpression of MEK1 alone can lead to up-regulation of cyclin D1 and down-regulation of p27 through uncharacterized mechanism (27, 28), so these studies are consistent with our observation about the activity of MAPK of HER-2/*neu* signals can regulate p27 expression. Together, our studies characterized how the HER-2/Grb2/Ras/MAPK pathway of HER-2/*neu* oncogenic signals is involved in the down-regulation of p27 through enhanced ubiquitin-mediated degradation as presented in our model (Fig. 5).

p27 is a nuclear protein, and its subcompartmentation is important for its function. p27 has been shown to be transported to the cytoplasm for degradation (24). Several diseases have abnormal phenotypes because of mislocation of p27 in the

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[2] L. Newman, W. Xia, H.-Y. Yang, A. Sahn, M. Bondy, F. Lukmanji, M.-C. Hung, and M. H. Lee, unpublished results.

cytoplasm, including tuberous sclerosis complex (30) and Barrett's adenocarcinoma (31). Moreover, several anchorage-transformed cells have misplacement of p27 into cytoplasm (32). The mechanism of mislocation of p27 in these observations remains elusive. It is possible that mislocation of p27 in the cytoplasm could trigger degradation and loss of function. Because p27 is degraded faster in HER-2/neu-overexpressing cells, we investigated its subcellular localization and found that p27 is excluded from the nucleus in the presence of constitutively active HER-2/neu. It could be argued that cytoplasmic mislocation *per se* may not be sufficient to account for p27 degradation. We then further examined whether JAB1, a p27 exporter, can be affected by HER-2/neu activity to facilitate p27 degradation. It was shown that overexpression of JAB1 resulted in reduced expression of p27 (24). In addition, JAB1 was shown to mediate p27 degradation in a proteasome-dependent manner, because proteasome inhibitor LLnL can interfere with JAB1 to export p27 from the nucleus to the cytoplasm (24). We found that HER-2/neu signals enhance p27 degradation through ubiquitination. Also, we found that JAB1 is co-localized with p27 when HER-2/neu activity is high. Thus, HER-2/neu signals are directed to regulate the JAB1 activity, thereby affecting the localization and stability of p27. Investigating the link between HER-2/neu signals and JAB1 regulation will shed light on how HER-2/neu signals influence the turnover rate of p27.

Recent studies have shown that anti-HER-2/neu monoclonal antibody has an effect in growth inhibition and up-regulating p27 in HER-2/neu-overexpressing cancer cell lines (33), which supports our studies that HER-2/neu signals can cause reduction of p27. The inhibitory effect of HER-2/neu antibody was successfully used in the treatment of human breast cancers (29, 34). It is conceivable that regulating the level of p27, at least in part, accounts for the treatment outcome of these diseases. In conclusion, our studies suggest that p27 and JAB1 are the downstream targets of HER-2/neu oncogenic signals, and they may be useful targets for therapeutic intervention in HER-2/neu-associated tumors.

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HER-2/neu Blocks Tumor Necrosis Factor-induced Apoptosis via the Akt/NF-κB Pathway*

Binhua P. Zhou‡, Mickey C.-T. Hu, Stephanie A. Miller, Zhenming Yu‡, Weiya Xia, Shiaw-Yih Lin§, and Mien-Chie Hung¶

From the Department of Molecular and Cellular Oncology, Breast Cancer Basic Research Program, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Overexpression of *HER-2/neu* correlates with poor survival of breast and ovarian cancer patients and induces resistance to tumor necrosis factor (TNF), which causes cancer cells to escape from host immune defenses. The mechanism of *HER-2/neu*-induced TNF resistance is unknown. Here we report that *HER-2/neu* activates Akt and NF-κB without extracellular stimulation. Blocking of the Akt pathway by a dominant-negative Akt sensitizes the *HER-2/neu*-overexpressing cells to TNF-induced apoptosis and inhibits IκB kinases, IκB phosphorylation, and NF-κB activation. Our results suggested that *HER-2/neu* constitutively activates the Akt/NF-κB anti-apoptotic cascade to confer resistance to TNF on cancer cells and reduce host defenses against neoplasia.

Overexpression of the *HER-2/neu* (*ErbB2*) oncogene correlates with poor prognosis in breast and ovarian cancer patients because it enhances the metastatic potential of cancer cells and induces resistance to Taxol and TNF[1] (1–5). Cancer cells that overexpress *HER-2/neu* are therefore an excellent target for the development of anticancer therapies. For instance, an anti-*HER-2/neu* antibody (Herceptin™) has been used clinically as a potent growth inhibitor of such breast cancer cells (6), and previous research has shown that overexpression of *HER-2/neu* up-regulates p21^Waf1 and leads to resistance by these cancer cells to Taxol (3). Still, the mechanism of *HER-2/neu*-mediated TNF resistance in cancer cells remains unclear. The *HER-2/neu* gene encodes a 185-kDa transmembrane receptor tyrosine kinase with homology to members of the EGF receptor family. Unlike the other EGF receptors, *HER-2/neu* has an intrinsic tyrosine kinase activity that activates receptor-medi-

ated signal transduction in the absence of ligand. Although EGF can bind to EGF receptor to induce receptor dimerization and activate phosphatidylinositol 3-kinase (PI3K) (7), it is not known whether *HER-2/neu* homodimer can activate the PI3K pathway without extracellular stimulation. Activation of PI3K generates PtdIns-3,4-P2, which in turn recruits and activates a downstream serine/threonine kinase, Akt. Activated Akt phosphorylates specific targets such as Bad (8), pro-caspase-9 (9), and transcription factor FKHRL1 (10, 11), with the result of promoting cell survival. Thus, the Akt signaling pathway has a critical role in anti-apoptosis that may contribute to the pathogenesis of cancer (12, 13).

In this study, we examined the activation of Akt in breast tumor specimens and breast cancer cell lines for its anti-apoptotic roles in *HER-2/neu*-overexpressing breast cancer cells. We found that Akt was constitutively activated in *HER-2/neu*-overexpressing breast cancer cells and that Akt activity was required for these cells resistance to TNF-induced apoptosis. We showed that *HER-2/neu*-overexpressing cancer cells became sensitive to apoptosis when the Akt pathway was blocked by the dominant-negative Akt. Furthermore, we found that Akt activity was required for the activation of both IKK-α and -β, for IκB phosphorylation, and for NF-κB activation. Our results provide a molecular explanation for the finding that *HER-2/neu*-overexpressing breast cancer cells are more resistant to TNF-induced apoptosis, leading to poor prognosis and shortened survival of patients.

EXPERIMENTAL PROCEDURES

Cell Lines and Cultures—All breast cancer cell lines and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium/F12 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. *HER-2/neu*-transformed NIH3T3 cells were generated by transfecting the cells with membrane point-mutated human *HER-2/neu* cDNA. Transformed cells were cloned from the transformed foci in three rounds of selection. The DN-Akt transfectants in MDA-MB453 and *HER-2/neu*-transformed 3T3 cells were established by transfecting these cells with HA-tagged Akt (K179M) cDNA. The transfectants were grown under the same conditions, except that 600 μg/ml of G418 was added to the culture medium.

Apoptosis Assay—Cells treated with or without TNF were collected at the time interval as indicated and washed once with ice-cold PBS, and apoptosis was analyzed by either a flow cytometry assay or DNA fragmentation, as described previously (14, 15).

Electrophoretic Mobility Shift Assay—Cell nuclear extracts from samples treated with or without TNF for 30 min were prepared as described previously (14, 15). The nuclear extract (5 μg) was incubated with 1 μg of poly(dI-dC) (Amersham Pharmacia Biotech) on ice for 20 min, and a ³²P-labeled double-stranded oligonucleotide containing the κB site of the human immunodeficiency virus was added. Binding of the probe was carried out at room temperature for 20 min. The resulting complexes were resolved in 4% nondenaturing polyacrylamide gel.

Immunoprecipitation—Cells were washed twice with PBS, scraped into 500 μl of lysis buffer, and incubated on ice for 20 min. After centrifugation at 14,000 × g for 10 min, 500 μg of each supernatant was preincubated with 2 μg of rabbit immunoglobulin G and 50 μl of protein

[1] The abbreviations used are: TNF, tumor necrosis factor; EGF, epidermal growth factor; IKK, IκB kinase; HA, hemagglutinin; luc, luciferase; PBS, phosphate-buffered saline; DN-Akt, dominant-negative Akt; p-Akt, phosphorylated Akt; ER, estrogen receptor; NF-κB, nuclear factor-κB; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; 3T3 cells, NIH3T3 cells.
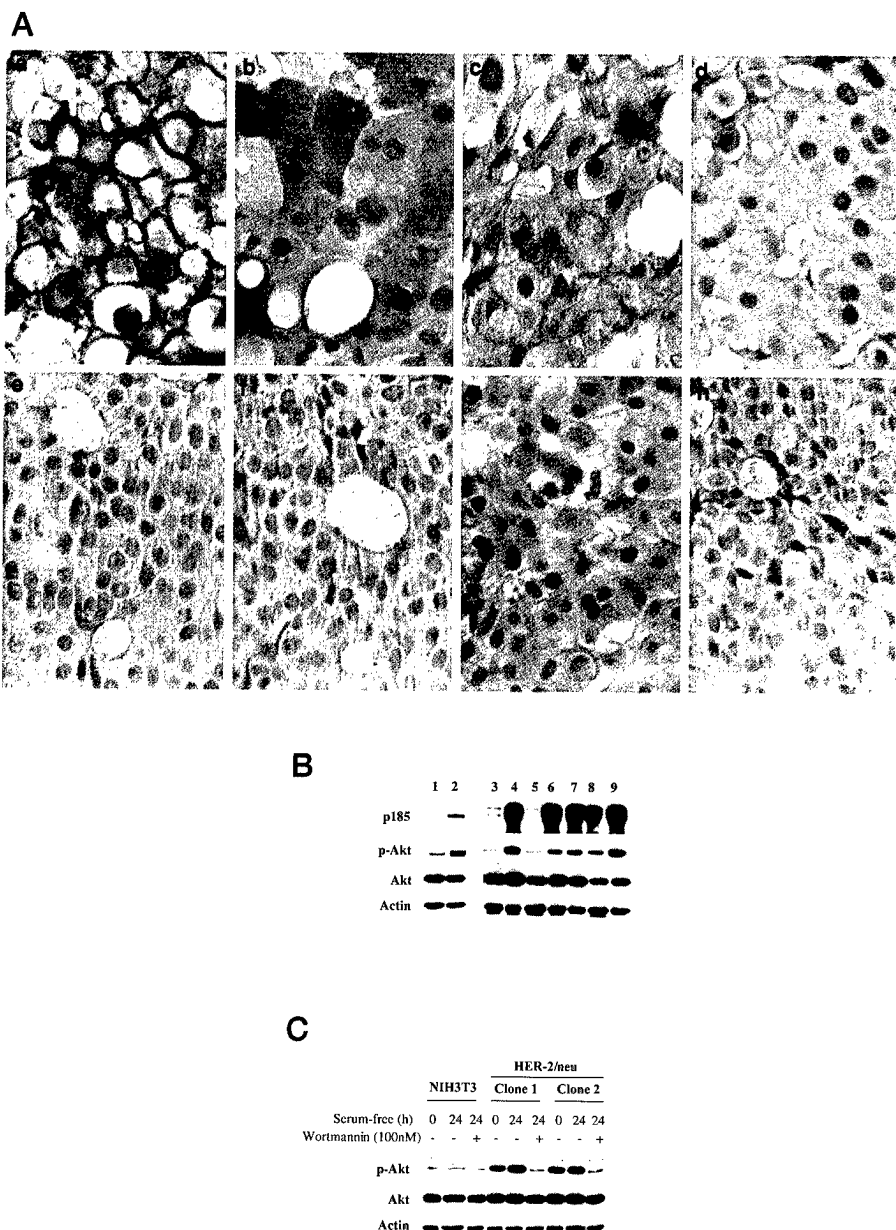
A

FIG. 1. **HER-2/neu activates Akt.** *A,* ten tissue sections from the *HER-2/neu*⁺ adenocarcinoma (*a–d*) and 10 sections from the *HER-2/neu*⁻ adenocarcinoma (*e–h*) were stained with antibodies specific to *HER-2/neu* (*a, e*), p-Akt (*b, f*), Akt (*c, g*), or normal rabbit serum (*d, h*) followed by immunostaining with an anti-rabbit IgG antibody conjugated with peroxidase. Antibodies were obtained from DAKO and New England Biolabs. *B,* nine human breast cancer cell lines were starved for 24 h without serum. Whole-cell lysates (50 μg each) were subjected to Western blot analyses using antibodies specific to *HER-2/neu*, Akt, p-Akt, and actin (Roche Molecular Biochemicals). *Lanes 1–9,* respectively: MCF-7, MCF-7/ HER-2, MDA-MB435, BT483, MDA-MB231, MDA-MB453, MDA-MB361, SKBR3, and BT474. *C, HER-2/neu*-transformed NIH3T3 cells were established by transfecting human *HER-2/neu* cDNA into NIH3T3 cells. After being cultured for 3 weeks, the *HER-2/neu*-transformed clones (foci) were isolated and characterized by the transformed phenotypes and overexpression of *HER-2/neu*. Two *HER-2/neu*-transformed clones and parental cells were cultured in medium containing 10% fetal bovine serum or serum-free medium for 24 h, with or without wortmannin (100 nM), a PI3K inhibitor, before harvest. Whole-cell lysates were analyzed by Western blots using antibodies against Akt, p-Akt, and actin.

B

C

G for 1 h at 4 °C. Endogenous IKK-α was immunoprecipitated overnight with 2 μg of anti-IKK-α antibody (Santa Cruz) and 50 μl of protein G. The immunocomplex was washed five times with lysis buffer, dissolved in loading buffer, and subjected to SDS-PAGE.

Western Blot—The protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's instructions. The immunoblots were visualized by an enhanced chemiluminescence (ECL) kit obtained from Amersham Pharmacia Biotech.

Immunocomplex Kinase Assay—Cell extracts were prepared from samples treated with or without TNF, and immunocomplex kinase assays were performed as described previously (15).

Transient Transfections—Approximately 0.2 × 10⁶ cells of either MDA-MB453 or its DN-Akt transfectants were cotransfected in 6-well plates with pcDNA3-*lacZ* and either wild-type NF-κB luciferase (κB-luc) or mutant NF-κB luciferase (mut/κB-luc). After 40 h of transfection, TNF was added to the culture medium as indicated, and both TNF-treated and untreated cultures were continued to incubate for another 8 h. The luciferase activity of each sample was measured with the luciferase assay kit (Promega) and normalized with a β-galactosidase assay.

RESULTS AND DISCUSSION

Because overexpression of *HER-2/neu* induced resistance to TNF (4, 5), and the Akt pathway is known to enhance cell survival, we examined whether expression of *HER-2/neu* correlated with activation of Akt in breast cancers. We compared the levels of activated Akt (phosphorylated Akt) (p-Akt) of 10 *HER-2/neu*-positive and 10 *HER-2/neu*-negative human breast tumors by immunostaining them with an antibody specific to p-Akt. Although no p-Akt signal was detected in the 10 *HER-2/neu*-negative tumors, 7 of 10 *HER-2/neu*-positive tumors showed strong p-Akt staining, suggesting that expression of *HER-2/neu* correlates significantly with Akt activation (*p* < 0.01). As control, all samples were Akt-positive when they were stained with an anti-Akt antibody. Representative stainings of p-Akt are shown in Fig. 1*A*. To confirm our observation of a correlation between *HER-2/neu* expression and Akt activation in the clinical samples, we used Western blotting with an anti-p-Akt antibody to analyze p-Akt in nine breast cancer cell lines that showed various expression levels of *HER-2/neu*. The level of p-Akt paralleled the cell's *HER-2/neu* expression (Fig.
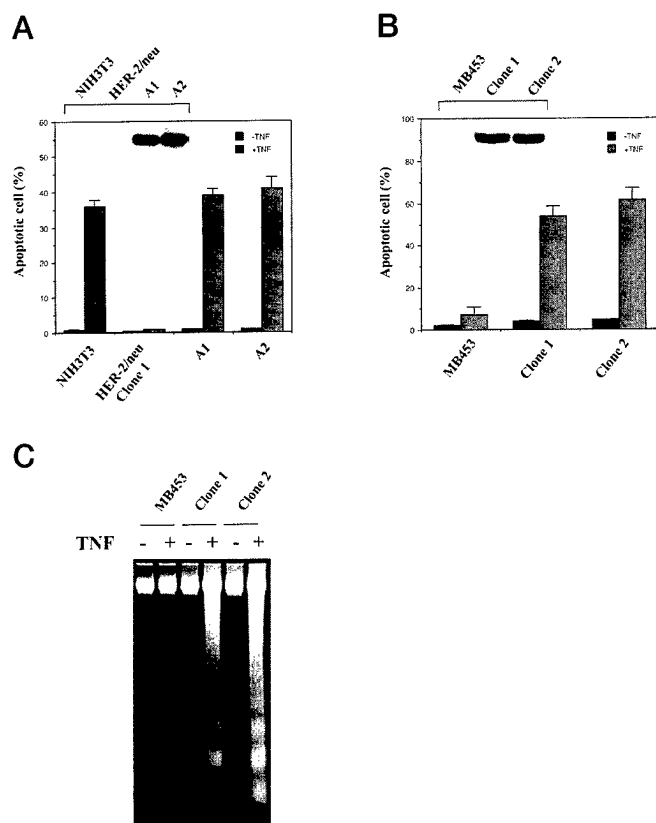
FIG. 2. *HER-2/neu* **inhibits TNF-induced apoptosis through Akt.** DN-Akt stable transfectants were generated by transfecting *HER-2/neu*-transformed NIH3T3 and the MDA-MB453 cells with DN-Akt cDNA (HA-tagged Akt, K179M). Several stable cell clones were isolated after G418 selection (600 μg/ml). *A*, NIH3T3, *HER-2/neu*-transformed 3T3 and its DN-Akt-transfected cell lines (clones *A1* and *A2*) were cultured in low-serum (1% serum) medium with TNF (40 ng/ml) or without it for 48 h. Apoptotic cells were measured by flow cytometry using a fluorescence-activated cell sorter (FACS) with propidium iodide staining (mean ± S.E. in three separate experiments). Equal amounts of cell lysates were analyzed by Western blot for expression of DN-Akt in these cell clones (*insert*). *B*, MDA-MB453 cells and their DN-Akt transfectants (*Clone 1* and *Clone 2*) were cultured in low-serum medium with (20 ng/ml) or without TNF for 48 h. Expression of DN-Akt in these clones was analyzed as described for *A* (*insert*). The apoptotic cells were quantitated by FACS (mean ± S.E. in three separate experiments) or determined by DNA fragmentation (*C*).

1B), indicating that activation of Akt correlates well with expression of *HER-2/neu* in breast cancer cells. Moreover, this correlation remained the same in the absence of serum, suggesting that activation of Akt corresponds to the level of *HER-2/neu*, independent of stimulation of growth factors or cytokines in the serum (*i.e.* constitutive activation). To create a model system, and to rule out the possibility that some other mechanisms might contribute to concurrent activation of Akt and overexpression of *HER-2/neu*, we compared Akt activation between the *HER-2/neu*-transformed NIH3T3 cell clones with that of their parental cells. In the absence of serum, Akt was activated constitutively in the *HER-2/neu*-transformed cells but not in the parental cells (Fig. 1C), which confirmed that Akt was activated by the intrinsic tyrosine kinase activity of *HER-2/neu* in the absence of extracellular stimulation. Furthermore, activation of Akt was blocked by wortmannin, an inhibitor of PI3K, suggesting that this *HER-2/neu*-mediated Akt activation occurs through PI3K.

If the *HER-2/neu*–induced resistance to TNF is caused primarily by activation of Akt and not by other mechanisms, blocking this Akt pathway should render the cells sensitive to TNF-induced apoptosis. Therefore, to inhibit the Akt pathway,

we transfected a DN-Akt (kinase-dead) DNA into the *HER-2/neu*-transformed NIH3T3 (*HER-2/neu*-3T3) cells. Upon TNF treatment, the DN-Akt transfectants of *HER-2/neu*-3T3 and NIH3T3 cells were about 20-fold more sensitive to apoptosis than the *HER-2/neu*-3T3 cells (Fig. 2A). Expression levels of DN-Akt in these cell clones are indicated in the *insert* to Fig. 2A. To confirm the Akt anti-apoptotic effect in the *HER-2/neu*-overexpressed human breast cancer cells, we transfected DN-Akt DNA into *HER-2/neu*-overexpressing MDA-MB453 cells and obtained several independent DN-Akt-overexpressing cell clones (Fig. 2B, *insert*). Similarly, the DN-Akt transfectants (clones 1 and 2) of MDA-MB453 cells became about 10-fold more sensitive to TNF-induced apoptosis than the parental cells (Fig. 2B). Apoptosis induced by TNF was further verified by DNA fragmentation assay (Fig. 2C). Thus, *HER-2/neu* was found to block TNF-induced apoptosis via the PI3K/Akt pathway.

PI3K has recently been shown to be involved in the activation of transcription factor NF-κB (16, 17), which is a p50/p65 (RelA) heterodimer regulated by its inhibitory protein, IκB (18, 19). Clinical evidence indicates that loss of estrogen receptor (ER) correlates strongly with overexpression of *HER-2/neu* (20), which is consistent with our previous finding that ER down-regulates *HER-2/neu* expression (21). Analogously, NF-κB is often activated constitutively in ER-negative breast cancer cells (22). Thus, we hypothesized that activation of Akt by *HER-2/neu* may turn on NF-κB, which inhibits TNF-induced apoptosis (23–25). To test whether overexpression of *HER-2/neu* can activate NF-κB, we assayed the NF-κB DNA binding and transcriptional activation activities in *HER-2/neu*-3T3 and NIH3T3 cells and found NF-κB DNA binding activity higher in the *HER-2/neu*-3T3 cells than in the NIH3T3 cells, in a serum-independent manner (Fig. 3A, *lanes 4* and *5*). As controls, NF-κB DNA binding activities were strongly activated by TNF treatment (Fig. 3A); these activities were abrogated by the competing wild-type κB oligonucleotides (data not shown; see below). Furthermore, activation of the transcriptional activity of NF-κB in the *HER-2/neu*-3T3 cells without serum was confirmed by luciferase assay (Fig. 3B). Similar results were obtained in rat *HER-2/neu*-transformed NIH3T3 and SW3T3 cells (data not shown). These data strongly suggested that overexpression of *HER-2/neu* activates NF-κB constitutively. To determine whether sensitization of TNF-induced apoptosis in the DN-Akt transfectants occurs through inhibition of NF-κB, we measured NF-κB activities in the transfectants and MDA-MB453 cells. As shown in Fig. 3, *C* and *D*, TNF-induced NF-κB DNA binding and transcription activities in the DN-Akt transfectants were significantly inhibited (3–5-fold). That these inhibitions were not caused by down-regulation of p65 or p50 by TNF is demonstrated by the finding of no change in the p65 and p50 levels of these cells in the absence or presence of TNF (Fig. 3C, *bottom panel*).

To investigate whether DN-Akt inhibits IκB phosphorylation and degradation, we analyzed the expression and phosphorylation patterns of IκB-α in the DN-Akt transfectants and MDA-MB453 cells before and after TNF treatment. As shown in Fig. 4A, only one IκB-α band was observed in the DN-Akt transfectants before or after the TNF treatment, whereas two bands were detected in the TNF-treated parental cells. The upper band may be the phosphorylated form of IκB-α (p-IκB-α), because it disappeared after treatment with calf intestine phosphatase (*CIP*, Fig. 4B). TNF has been demonstrated to activate IκB kinases (IKKs), which in turn phosphorylate IκB, which is then degraded and activates NF-κB (26, 27). To examine whether DN-Akt blocks activation of IKKs, we compared the kinase activities of IKK-α and -β in the DN-Akt transfectants
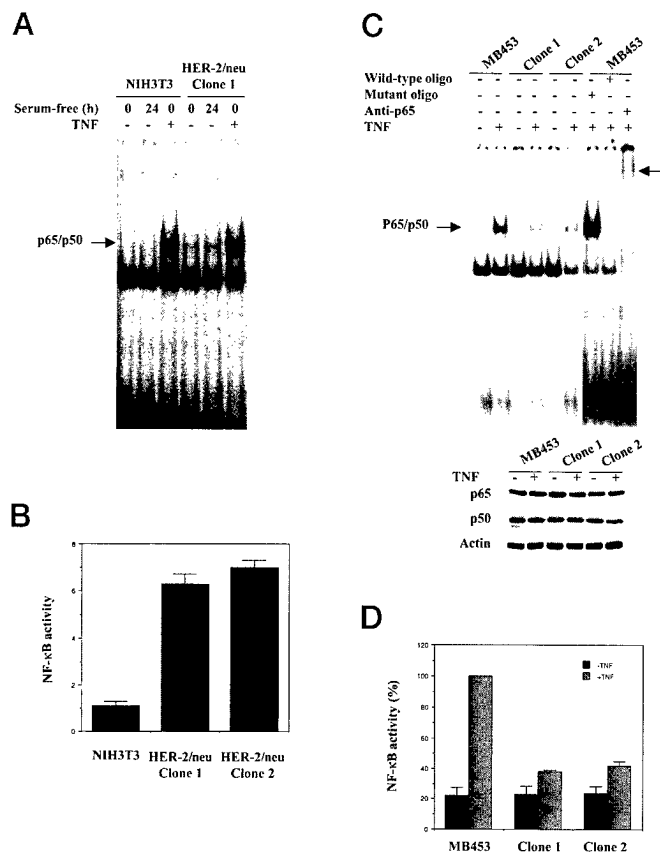
A

B

C

D

FIG. 3. **HER-2/neu activates NF-κB.** *A*, NIH3T3 cells and *HER-2/neu*-transformed 3T3 cells were cultured in serum-containing or serum-free medium for 24 h. Nuclear extracts (5 μg each) were used to determine NF-κB DNA binding activities by an electrophoretic mobility shift assay using an oligonucleotide probe containing the κB binding site. Nuclear extracts from the TNF-stimulated cells were included as positive controls. *B*, NIH3T3 and *HER-2/neu*-transformed 3T3 cells were cotransfected with 0.2 μg of pcDNA3-*lacZ* plus 1.8 μg of either wild-type or mutant NF-κB luciferase (κB-luc or mut/κB-luc) plasmids. Forty-eight h post-transfection, luciferase activities were determined and normalized by β-galactosidase activities. NF-κB activities were calculated by the luciferase activities of κB-luc *versus* mut/κB-luc (mean ± S.E. in three separate experiments). *C*, DN-Akt blocks activation of NF-κB induced by TNF. The MDA-MB453 cells and DN-Akt transfectants (*Clone 1* and *Clone 2*) were treated with (20 ng/ml) or without TNF for 5 min, and NF-κB DNA binding activities were determined as described above for *panel A*. Cold wild-type or mutant NF-κB oligonucleotides were included as controls in the TNF-induced MDA-MB453 cells. An anti-p65 antibody (Santa Cruz) was also included in the assay; the supershifted complex is indicated by an *arrow*. As control, 50 μg of each cell lysate was assayed for the expression of p65 or p50 by Western blots using anti-p65 or anti-p50 antibody (*bottom panel*). *D*, MDA-MB453 cells and DN-Akt transfectants were cotransfected with pcDNA3-*lacZ* plus κB-luc or mut/κB-luc plasmids as described above. After 40 h of transfection, the cells were treated with or without TNF (20 ng/ml) for 8 h and then harvested. NF-κB activities were determined as above.

with those in the parental cells after TNF treatment, using immunocomplex kinase assays. The endogenous IKK-α and -β kinase activities were readily detected in the MDA-MB453 cells, whereas their activities were inhibited in the DN-Akt transfectants (Fig. 4*C*), suggesting that Akt activity is required for activation of IKKs by TNF. Furthermore, we showed that in the DN-Akt transfectants, DN-Akt and the endogenous Akt associate specifically with IKK-α *in vivo* regardless of TNF treatment (Fig. 4*D*). To further confirm that Akt is an activator upstream of IKKs, we transfected the DNA of p65 (RelA), IKK-α or -β, or a constitutively active Akt into the DN-Akt transfectants to restore TNF-induced NF-κB activities in these cells. Overexpression of each of these proteins significantly
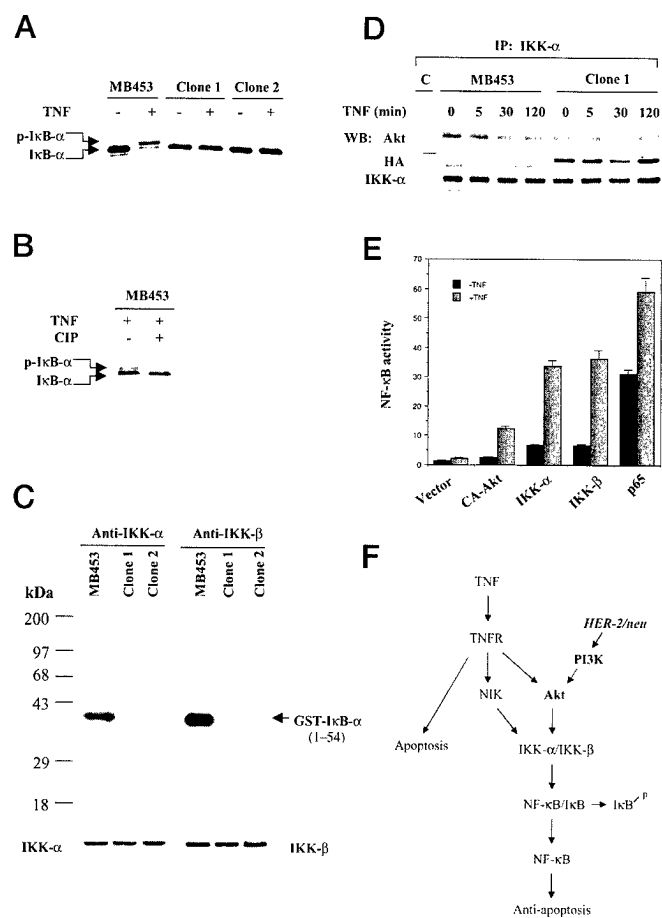
A

B

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E

F

FIG. 4. **HER-2/neu activates the Akt/NF-κB signaling cascade.** *A*, MDA-MB453 and DN-Akt transfectants were starved for 12 h without serum and treated with TNF (20 ng/ml) or without it for 5 min before harvesting. Equal amounts of whole-cell lysates were subjected to SDS-PAGE (12%) and transferred to nitrocellulose membranes. IκB-α was detected by Western blot using an anti-IκB-α antibody (Santa Cruz). *B*, phosphorylation of IκB-α induced by TNF could be dephosphorylated by a phosphatase. Cell lysates (500 μg each) from the TNF-treated MDA-MB453 cells were immunoprecipitated with anti-IκB-α, and the precipitates were then incubated with or without 20 units of calf intestine phosphatase (*CIP*) at 37 °C for 30 min before Western blot analysis with anti-IκB-α. *C*, MDA-MB453 and DN-Akt transfectants were starved and treated with TNF (10 min). Endogenous IKK-α and -β were immunoprecipitated with anti-IKK-α and anti-IKK-β antibodies, respectively, and their kinase activities were determined by immunocomplex kinase assays using GST-IκB-α (amino acid 1–54) as a substrate as described previously (15). *D*, MDA-MB453 and DN-Akt transfectants (*Clone 1*) were treated with TNF (20 ng/ml) for different time periods. The endogenous IKK-α was immunoprecipitated with an anti-IKK-α antibody, then analyzed by Western blot (*WB*) using an anti-Akt, anti-HA, or anti-IKK-α antibody. *E*, NF-κB activity in the DN-Akt transfectants could be partially restored by overexpression of p65, IKK-β, IKK-α, or a constitutively active Akt (*CA-Akt*). The DN-Akt transfectants (clone 1) were cotransfected with pcDNA3-*lacZ* (0.2 μg) and either κB-luc or mut/κB-luc (0.6 μg each) plus p65, IKK-β, IKK-α, or CA-Akt plasmid (1.2 μg each). Forty h post-transfection, the cells were treated with or without TNF, and the NF-κB activities were determined as described above for *panel B* in Fig. 3 (mean ± S.E. in four separate experiments). *F*, model of *HER-2/neu* activation of the Akt/NF-κB pathway that blocks TNF-induced apoptosis. *TNFR*, TNF receptor; *NIK*, NF-κB-inducing kinase.

overrode the inhibitory effect of DN-Akt and restored activation of NF-κB by TNF (Fig. 4*E*), indicating that Akt is indeed upstream of both IKKs. Taken together, our results suggested that Akt activity is essential for NF-κB activation by *HER-2/neu* and TNF. A model we propose to illustrate the parallel *HER-2/neu*- and TNF-induced anti-apoptotic pathways is shown in Fig. 4*F*. While we were preparing this manuscript, NF-κB was reported to be a target of Akt (28, 29), confirming

our finding that *HER-2/neu* activates the NF-κB anti-apoptotic pathway through Akt.

In general, activation of the Akt signaling pathway requires extracellular survival factors (mitogenic stimuli) such as EGF, insulin, platelet-derived growth factor, thrombin, heregulin, and nerve growth factor. To our knowledge, this is the first evidence that *HER-2/neu* activates the Akt/NF-κB pathway without extracellular stimulation. Our study also details a molecular mechanism of TNF resistance that may provide an interpretation for the *HER-2/neu*-overexpressing cancer cells, escape from host immune defenses, and the contribution of this mechanism to the poor survival of cancer patients with *HER-2/neu* overexpression. Understanding the *HER-2/neu*-mediated anti-apoptotic pathway may open an avenue for developing novel anticancer therapies for *HER-2/neu*-overexpressing breast and ovarian cancers.

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Cytoplasmic Localization of p21$^{Cip1/WAF1}$ by Akt-Induced Phosphorylation in *HER-2/neu*-Overexpressing Cells

Binhua P. Zhou, Yong Liao, Weiya Xia, Bill Spohn, Mong-Hong Lee, and Mien-Chie Hung[*]

Department of Molecular and Cellular Oncology, Breast Cancer Basic Research Program, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

[*]To whom correspondence should be addressed. Tel.: 713-792-3668; Fax: 713-794-0209; E-mail: mhung@notes.mdacc.tmc.edu.

SUMMARY

Amplification/overexpression of *HER-2/neu* in cancer cells confers resistance to apoptosis and promotes cell growth. Cellular localization of p21$^{\text{Cip1/WAF1}}$ has been proposed to be critical in either promoting cell survival or inhibiting cell growth. In this study, we showed that *HER-2/neu*-mediated cell growth required the activation of Akt, which associated with p21$^{\text{Cip1/WAF1}}$ and phosphorylated it at threonine 145, then resulted in cytoplasmic localization of p21$^{\text{Cip1/WAF1}}$. Furthermore, blocking the Akt pathway with a dominant-negative mutant of Akt restored the nuclear localization and the cell growth inhibition activity of p21$^{\text{Cip1/WAF1}}$. Our results indicated that *HER-2/neu* induces cytoplasmic localization of p21$^{\text{Cip1/WAF1}}$ via the activation of Akt to promote cell growth, which may have implications in the oncogenic activity of *HER-2/neu* and Akt.

Introduction

The *HER-2/neu* gene (also known as c-erbB2) encodes a 185-kDa transmembrane receptor tyrosine kinase that has partial homology with the other members of the epidermal growth factor receptor family. Amplification/overexpression of *HER-2/neu* occurs in approximately 30% of human breast and ovarian cancers and is a marker of poor prognosis (1-3). We have previously shown that *HER-2/neu* activates the phosphatidylinositol-3 kinase (PI-3K)/Akt pathway and confers resistance to apoptosis induced by tumor necrosis factor (4). The PI-3K/Akt pathway plays an important role in preventing cells from undergoing apoptosis and contributes to the pathogenesis of malignancy (5, 6). For example, activated Akt phosphorylates specific targets such as Bad (7), caspase-9 (8), forkhead transcription factors (9, 10), and IKK-α (11, 12) and so promotes cell survival. However, in addition to its anti-apoptotic function, Akt is also involved in cell proliferation (13-15). Furthermore, Akt detachs from the inner surface of the plasma membrane, where it is initially activated, and relocalizes to nucleus within 30 min of activation by growth factors (16, 17). These findings suggest that some critical Akt targets that control cell cycle progression are located within the nucleus.

Cell-cycle progression is tightly regulated by the family of cyclin-dependent kinase (CDK) inhibitors. p21$^{Cip1/WAF1}$ was identified through its interaction with cyclin-dependent kinase Cdk2 (18), and its expression is induced by activation of wild-type p53 (19), and during cellular senescence (20). The cell growth inhibitory activity of p21$^{Cip1/WAF1}$ is strongly correlated with its nuclear localization (21, 22). However, recent evidence has shown that p21$^{Cip1/WAF1}$ can also localize in the cytoplasm and plays an important role in protecting the cells against apoptosis. For instance, nuclear p21$^{Cip1/WAF1}$

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becomes cytoplasmic after differentiation of U937 cells into monocytes, and this translocation event is accompanied by resistance to various apoptotic stimuli (23). Furthermore, cytoplasmic p21$^{Cip1/WAF1}$ forms a complex with apoptosis signal-regulating kinase 1 (ASK1) that inhibits the stress-induced mitogen-activated protein (MAP) kinase cascade and results in resistance to apoptosis in these cells (23). However, the mechanism that regulates the cytoplasmic or nuclear localization of p21$^{Cip1/WAF1}$ is still unknown.

In this study, we found that blocking the Akt pathway by using a dominant-negative Akt (DN-Akt) inhibited cell growth. This growth inhibition was correlated with nuclear localization of p21$^{Cip1/WAF1}$. We demonstrated that Akt could associate with p21$^{Cip1/WAF1}$ and phosphorylated a consensus threonine residue (T145) in the nuclear localization signal (NLS) of p21$^{Cip1/WAF1}$ which resulted in the cytoplasmic localization of p21$^{Cip1/WAF1}$. We have thus identified a novel signal pathway and shown that overexpression of HER-2/*neu* may enhance cell proliferation by inducing cytoplasmic localization of p21$^{Cip1/WAF1}$ through the serine/threonine kinase Akt.

Results

To study the effect of Akt on *HER-2/neu*-mediated cell proliferation, we used a model system that consists of NIH3T3 cells, *HER-2/neu* 3T3 cells (*HER-2/neu* transformed NIH3T3 cells), and DN-Akt 3T3 cells (*HER-2/neu* 3T3 cells transfected with DN-Akt, a kinase-dead mutant of Akt) (4). As expected, the *HER-2/neu* 3T3 cells grew much faster than did the parental NIH3T3 cells (Fig. 1A). The Akt pathway is known to be constitutively activated in *HER-2/neu* 3T3 cells (4), and when this pathway was blocked by DN-Akt in DN-Akt 3T3 cells, cell growth became slower (Fig. 1A). This was not due to the heterogeneity of the cell clones, because a specific PI-3K inhibitor, wortmannin, also produced a similar slowing of growth in *HER-2/neu* 3T3 cells (Fig. 1A). When the DNA synthesis rate was determined by measuring [^{3}H]thymidine incorporation, *HER-2/neu* 3T3 cells also had greater DNA synthesis than did the parental NIH3T3 cells (Fig. 1B). *HER-2/neu*-induced DNA synthesis was significantly inhibited by blocking Akt pathway with either wortmannin or DN-Akt. As the net cell growth rate depends on a fine balance between the cell proliferation rate and the cell death rate, we also examined whether apoptosis contributes to the difference in growth in these cells. There was no significant difference in apoptosis among these cells as measured by FACS analysis (Fig. 1C). Therefore, the reduction in cell growth in DN-Akt 3T3 cells was most likely due to the decrease in cell proliferation. To extend our findings, we also performed the same experiments in another *HER-2/neu*-overexpressing breast cancer cell line, MDA-MB453, and stable DN-Akt transfectants of it (4). Like the *HER-2/neu* 3T3 and DN-Akt 3T3 cell lines, three independent clones of DN-Akt transfectants showed reductions in cell growth and DNA synthesis (Fig. 1D and E), but no difference was

observed in apoptosis assayed by FACS analysis (data not shown). A revertant that had lost DN-Akt during culture had cell growth and DNA synthesis rates almost identical to those of parental MDA-MB453 cells (Fig. 1D and E). Taken together, our results indicated that the Akt pathway was required for *HER-2/neu*-mediated cell proliferation and that inhibition of the Akt pathway by either a PI-3K inhibitor or DN-Akt significantly reduced cell proliferation.

Cell proliferation is tightly regulated by the family of cyclin-dependent kinase (CDK) inhibitors. p21$^{Cip1/WAF1}$ was shown to play a critical role in regulating cell proliferation (18-20). Therefore, we measured the expression of p21$^{Cip1/WAF1}$ in these cells. The expression of p21$^{Cip1/WAF1}$ was significantly higher in *HER-2/neu* 3T3 cells than in the parental cells (Fig. 1F), consistent to the previous report (24). Inhibition of the Akt pathway by DN-Akt in DN-Akt 3T3 cells did not significantly change p21$^{Cip1/WAF1}$ expression (Fig. 1F). This result could not account for the HER-2/*neu*-induced cell proliferation and suggested that there may be an unknown mechanism that overrides the enhanced expression of p21$^{Cip1/WAF1}$ in *HER-2/neu* 3T3 cells.

Since protein phosphorylation plays a major role in the regulation of protein function, we examined the phosphorylation pattern of endogenous p21$^{Cip1/WAF1}$ using in vivo [^{32}P]-orthophosphate labeling and two-dimension phosphopeptide analysis (Fig. 2A). A strong phosphorylated peptide dot was observed after the induction of insulin which is known to activated PI-3K/Akt, and this phosphorylation dot was greatly inhibited when cells were incubated with PI-3K inhibitor, LY294002. This insulin induced phosphorylation spot is most likely due to the phosphorylation of serine or threonine instead of tyrosine residues in p21$^{Cip1/WAF1}$, since no tyrosine phosphorylation

was detected when endogenous p21$^{Cip1/WAF1}$ was immunoprecipitated and examined in western blot using antibody specific against tyrosine-phosphorylation (Fig. 2B). That no signal was detected on tyrosine phosphorylation of endogenous p21$^{Cip1/WAF1}$ was not due to the quality of the antibody, because the same antibody was able to detect the tyrosine phosphorylation of HER-2/neu in the same cell lysate (Figure 2B). The insulin-induced phosphorylation site in p21$^{Cip1/WAF1}$ was further analyzed by in vivo [^{32}P]-orthophosphate labeling and the trypsin-digested phosphopeptides were then subjected to amino acid sequencing by Edman's degradation. The activity of radioisotope was released after the second cycle of Edman's degradation (Figure 2C), and this second-cycle activity of radioisotope was greatly reduced when the cells were pretreated with PI-3K inhibitor, LY294002 (data not shown). Judging from the amino acid sequence of p21$^{Cip1/WAF1}$, the only trypsin-digested peptide that contains threonine or serine at the second amino acid residue from the N-terminus is, ^{144}QTSMTDFYHSK^{154}. Thus, our results indicated that threonine 145 of p21$^{Cip1/WAF1}$ was phosphorylated in vivo, and this phosphorylation can be induced by insulin and inhibited by PI-3K inhibitor.

The nuclear localization of p21$^{Cip1/WAF1}$ appears to be responsible for its cell-growth inhibition and is controlled by the NLS at the C-terminus of the molecule (25). We noticed that there was a putative Akt phosphorylation motif in the NLS of p21$^{Cip1/WAF1}$ and that this Akt phosphorylation motif was highly conserved among different species (Fig. 3A). The phosphorylated T145 residue of p21$^{Cip1/WAF1}$ detected in Fig. 2 was located in this motif. To test whether Akt interacts with and phosphorylates p21$^{Cip1/WAF1}$ and so regulates its cellular localization, we first investigated whether Akt associates with p21$^{Cip1/WAF1}$ by co-immunoprecipitation experiments. We cotransfected a

constitutively active Akt (CA-Akt) or DN-Akt with wild-type or mutant p21$^{Cip1/WAF1}$ (T145A) into 293T cells. After immunoprecipitating the p21$^{Cip1/WAF1}$, we detected Akt and visa versa (Fig. 3B and C), which suggests that these two molecules are associated. The association was dependent on the kinase status of Akt and the status of p21$^{Cip1/WAF1}$. p21$^{Cip1/WAF1}$ associated more strongly with CA-Akt than with DN-Akt, and un-phosphorylated p21$^{Cip1/WAF1}$ (T145A) associated much more weakly with Akt than did wild-type p21$^{Cip1/WAF1}$. Furthermore, Akt could phosphorylate p21$^{Cip1/WAF1}$ both *in vitro* and *in vivo*, whereas DN-Akt could not (Fig. 3D and E). That no phosphorylation was observed on the mutant p21$^{Cip1/WAF1}$ (T145A), in which threonine 145 in the putative Akt phosphorylation site was replaced with alanine, indicated that Akt interacts with p21$^{Cip1/WAF1}$ and phosphorylates it at threonine 145. This is further supported by the data presented in Fig. 2 that phosphorylation of threonine 145 is induced by insulin and inhibited by LY294002, since Akt is known to be activated by insulin and inhibited by LY294002.

We next tested whether the activation of Akt affects the cellular localization of p21$^{Cip1/WAF1}$, as Akt could phosphorylate a critical threonine residue in the NLS of p21$^{Cip1/WAF1}$. We cotransfected CA-Akt or DN-Akt with wild-type or mutant p21$^{Cip1/WAF1}$ (T145A and T145D) into p21$^{-/-}$ MEF cells and examined the cellular localization of p21$^{Cip1/WAF1}$ by immunofluorecense analysis. As seen in Fig. 4, wild-type p21$^{Cip1/WAF1}$ was found predominantly in the cytoplasm in the presence of CA-Akt but was predominantly in the nucleus when DN-Akt was introduced. Mutation in the critical threonine residue in T145A abolished cytoplasmic localization even in the presence of CA-Akt. Mutant p21$^{Cip1/WAF1}$ (T145D), in which threonine 145 was replaced with

asparate acid to mimic the phosphorylation of p21$^{Cip1/WAF1}$ by Akt, was found predominantly in the cytoplasm no matter whether the Akt pathway was turn on by CA-Akt or shut off by DN-Akt. These results strongly indicated that threonine 145 of p21$^{Cip1/WAF1}$ is critical in determining the cellular localization of p21$^{Cip1/WAF1}$, and phosphorylation of threonine 145 of p21$^{Cip1/WAF1}$ by Akt resulted in the cytoplasmic localization of p21$^{Cip1/WAF1}$. Taken together, our results indicated that activated Akt could interact with p21$^{Cip1/WAF1}$ and phosphorylated the threonine 145 residue in the NLS of p21$^{Cip1/WAF1}$, which led to predominant cytoplasmic localization of p21$^{Cip1/WAF1}$.

The above experiments were carried out in cells that were transiently transfected with exogenous gene. To examine whether endogenous p21$^{Cip1/WAF1}$ can be regulated in a similar way, we first performed biochemical cellular fractionation to determine the localization of endogenous p21$^{Cip1/WAF1}$ in *HER-2/neu*-overexpressing cells and their DN-Akt transfectants. We found that p21$^{Cip1/WAF1}$ was predominantly located in the cytoplasm in both *HER-2/neu* 3T3 cells and MDA-MB453 cells, in which Akt was constitutively activated (Fig. 5A). However, when the Akt pathway was blocked by DN-Akt, p21$^{Cip1/WAF1}$ was found exclusively in the nucleus in both DN-Akt 3T3 and DN-Akt/MB453 cells (Fig. 5A). As a control, we used actin and proliferating cell nuclear antigen (PCNA) as cytoplasmic and nuclear markers, respectively, to confirm that the cellular localization of p21$^{Cip1/WAF1}$ was not due to contamination. Similar results were obtained when we stained *HER-2/neu* 3T3 and its DN-Akt transfectants for endogenous p21$^{Cip1/WAF1}$ (data not shown). To further investigate whether the phosphorylation of endogenous p21$^{Cip1/WAF1}$ by Akt affects its subcellular localization, we treated the MDA-MB453 cells with insulin, which is known to be a potent Akt activator. We found that

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endogenous p21$^{Cip1/WAF1}$ was distributed in both nucleus and cytoplasm in MDA-MB453 cells, and this distribution was shifted more to cytoplasm when the cells were stimulated with insulin (Fig. 5B). However, this distribution shift was blocked by the addition of PI-3K inhibitor, LY294002 (Fig. 5B). In contrast to the parental cells, endogenous p21$^{Cip1/WAF1}$ of DN-Akt transfectants was mainly localized in the nucleus, and no distribution shift was observed when cells were treated with insulin. Taken together, these results indicate that the distribution of endogenous p21$^{Cip1/WAF1}$ can be regulated by the extracellular stimuli, such as insulin, through PI-3K/Akt pathway

The above results clearly establish the regulation of p21$^{Cip1/WAF1}$ by HER-2/neu-Akt pathway in cell culture. To examine whether this phenomena could also be observed in clinical tumor tissues, we compared the levels of activated Akt (phosphorylated Akt) and the cellular localization of p21$^{Cip1/WAF1}$ in 5 HER-2/neu-positive and 5 HER-2/neu-negative human breast tumors by immunostaining with antibodies specific to the phosphorylation of Akt and specific to p21$^{Cip1/WAF1}$. Consist with our previous study, Akt was activated in 5 out of 5 HER-2/neu-positive breast tumor tissues. In these 5 breast tumor tissues, we found that p21$^{Cip1/WAF1}$ was not only localized in nucleus but also localized in cytoplasm. In contrast, in all 5 HER-2/neu-negative breast tumor tissues examined, Akt was not activated, and the p21$^{Cip1/WAF1}$ was exclusively localized in the nucleus. One of the representative staining was shown in Fig. 6. The tumor staining data supports the observation in cell culture and further strengthens the notion that overexpression of HER-2/neu can regulate the cellular distribution of p21$^{Cip1/WAF1}$ via the activation of Akt.

Because Akt could phosphorylate p21$^{Cip1/WAF1}$ and caused its cytoplasmic localization, we next addressed whether the phosphorylation status of p21$^{Cip1/WAF1}$ at threonine 145 affected the cell growth inhibitory activity of p21$^{Cip1/WAF1}$. We transfected wild-type p21$^{Cip1/WAF1}$ and its mutants T145A (which could not be phosphorylated) and T145D (in which threonine 145 was mutated to aspartic acid to mimic the phosphorylation) into NIH3T3, *HER-2/neu* 3T3, and DN-Akt 3T3 cells, and measured their growth inhibitory activity by using the colony-formation assay. As seen in Fig. 7A, wild-type p21$^{Cip1/WAF1}$ did not effectively inhibit the growth of *HER-2/neu* 3T3 cells, in which Akt is constitutively activated, compared with NIH3T3 and DN-Akt 3T3 cells. However, T145A, which had lost the Akt phosphorylation site, had similar growth-inhibition activities in these three cell lines, and the activities were independent of the activation of Akt. In contrast, T145D, which mimicked the phosphorylation of p21$^{Cip1/WAF1}$, behaves similar to wild-type p21$^{Cip1/WAF1}$ in HER-2/neu$^+$ cells in all three cell lines. When DNA synthesis rate was measured by bromodeoxyuridine (BrdU) incorporation between wild-type and mutant p21$^{Cip1/WAF1}$ (Fig. 7B), similar to the results of colony-formation assay, the growth inhibition activity of wild-type p21$^{Cip1/WAF1}$ was regulated by the Akt status. T145A exhibited its inhibition activity independent of Akt activity whereas T145D is also independent of Akt status and its suppression effect is comparable to wild-type p21 in HER-2/neu$^+$ cells which has p21 predominantly located in cytoplasm. The mechanism that T145D only partially loses its ability to inhibit cell growth is not clear. This could due to that T145D still retains its ability to inhibit the function of cdk2, cdk4, and cyclin D, which are shuttle molecules between nucleus and cytoplasm. Alternatively, cytoplasmic p21$^{Cip1/WAF1}$ may also have unknown effects on

cell growth.   Further systemic study is required to elucidate the detailed mechanism.

Taken together, these results indicate that phosphorylation of threonine 145 in the NLS of

p21$^{\text{Cip1/WAF1}}$ by Akt triggers cellular localization and then regulate the growth-inhibitory

activity of p21$^{\text{Cip1/WAF1}}$.

Discussion

The cellular localization of $p21^{Cip1/WAF1}$ was recently suggested to be critical in the regulation of $p21^{Cip1/WAF1}$ function (26). However, how the cellular localization of $p21^{Cip1/WAF1}$ was controlled was not known. Here we identified the mechanism as Akt phosphorylation of $p21^{Cip1/WAF1}$ at threonine 145, which results in cytoplasmic localization and suppresses its growth inhibition activity. Our results, together with those of *Diehl et al.* (27), allowed us to propose a plausible model for how Akt simultaneously coordinates two functionally different proteins to achieve the harmonious effect on cell proliferation (Fig. 8). On the one hand, activation of Akt inhibits GSK-3β and stabilizes the growth-promoting factor cyclin D in the nucleus to stimulate cell growth (27), while on the other hand, Akt can also phosphorylate $p21^{Cip1/WAF1}$ to cause $p21^{Cip1/WAF1}$ cytoplasmic localization and so suppresses the growth-inhibitory activity of $p21^{Cip1/WAF1}$. In addition, our results, together with the findings of Asada *et al.* (23) also suggested an interesting mechanism for the anti-apoptotic effect of $p21^{Cip1/WAF1}$. Asada and colleagues showed that cytoplasmic $p21^{Cip1/WAF1}$ forms a complex with ASK1 to inhibit the stress-induced mitogen-activated protein kinase, which results in the resistance to apoptosis induced by many stimuli (23). Overexpression of *HER-2/neu* is known to activate the Akt pathway and induce resistance to the apoptosis induced by various stimuli (4, 24). It would be interesting to investigate whether this resistance is due to the formation of complexes between the cytoplasmic $p21^{Cip1/WAF1}$ and ASK1 in *HER-2/neu*-overexpressing cells. In general, protein translocation is recognized as a crucial mechanism for the regulation of protein function (26). Our results provide a clear-cut example in which Akt regulated the cellular location of $p21^{Cip1/WAF1}$ by phosphorylation

at threonine 145 and suggest that it may be involved in the HER-2/*neu*- or Akt- mediated

cell proliferation.

Methods

Materials

The PI-3K inhibitor wortmannin and the DNA dye Dapi were purchased from Roche Molecular Biochemicals. The anti-flag and anti-HA (12C5) antibodies were obtained from Sigma Chemical Co. and Roche Molecular Biochemicals, respectively. The Akt and p21$^{Cip1/WAF1}$ antibodies were obtained from New England Biolabs and Santa Cruz Biotech., respectively.

Constructs of p21$^{Cip1/WAF1}$

A BamHI site and an EcoRI site were generated near the start and termination codons, respectivly, in human wild-type p21$^{Cip1/WAF1}$ by PCR and subcloned into the expression vector pcDNA3. Site-directed mutagenesis was performed according to the manufacturer's protocol (Clontech Inc.). Threonine 145 in p21$^{Cip1/WAF1}$ was replaced by either Ala or Asp by using the following primers: for T145A, 5'-CGAAAACGGCGGCAG<u>G</u>CCAGCATGAC-3', and for T145D, 5'-CGAAAACGGCGG-CAG<u>GA</u>CAGCATGAC -3'. For the colony-formation assay in DN-Akt 3T3 cells, a BamHI and EcoRI fragment containing wild-type or mutant p21$^{Cip1/WAF1}$ was subcloned into the expression vector pcDNA3-hygromycin. The sequences of the wild-type and mutant p21$^{Cip1/WAF1}$ constructs were verified by automated sequencing. To generate a wild-type and mutant p21$^{Cip1/WAF1}$ GST-tagged bacterial expression constructs, the same fragments containing the wild-type and mutant p21$^{Cip1/WAF1}$ were subcloned into the bacterial expression vector pGEX4T-3 (Pharmacia Biotech.). Wild-type and mutant p21$^{Cip1/WAF1}$ proteins were inducible expressed in *E.coli* strain BL21 and purified by glutathione sepharose chromatography (Pharmacia Biotech.).

Cell Culture

NIH3T3, HER-2/*neu* 3T3, breast cancer cell MDA-MB453, p21$^{Cip1/WAF1}$-deficient MEF, and 293T cells were culture in DMEM/F12 supplemented with 10% fetal bovine serum. The DN-Akt transfectants of HER-2/*neu* 3T3 and MDA-MB453 cells were grown under the same conditions except that 600 μg/ml G418 was added to the culture medium (4). The 293T cells was transfected by the calcium phosphate technique and p21$^{Cip1/WAF1}$-deficient cells by the liposome method.

In vitro Growth Rate Analysis

The *in vitro* growth rates of the cell lines were assessed by counting the cells with a Coulter counter or by the MTT assay as described previously (28).

[^{3}H]Thymidine Incorporation Assay

The cell proliferation rates of the cell lines were analyzed by measuring [^{3}H]thymidine incorporation as described previously (28).

Endoproteinase Cleavage and Two-dimensional Phosphopeptide Analysis

Two-dimensional analysis of p21$^{Cip1/WAF1}$ phosphopeptides was carried out by using the HTLE-7000 electrophoresis system from CBS Scientific (Del Mar, CA) as described in (29). Briefly, MDA-MB453 cells were incubated with 1.5 mCi/ml of [^{32}P]-orthophosphate for 3.5 hours followed by 30 min of stimulation with insulin in the presence or absence of LY294002. Endogenous p21$^{Cip1/WAF1}$ was immunoprecipitated, blotted, and visualized by autoradiography. The ^{32}P-labelled p21$^{Cip1/WAF1}$ protein on nitrocellulose were excised and digested with TPCK-trypsin (Sigma Co). The completed digested phosphopeptides were spotted on 20 x 20 cm thin-layer cellulose plates and separated in the first dimension by electrophoresis at 1000 V for 35 min in pH 1.9 buffer (2.2% v/v formic acid, 7.8% v/v acetic acid). The cellulose plates were then placed in a chromatography tank containing phosphochromatography buffer (38% v/v n-butanol,

25% v/v pyridine, 7.5% v/v acetic acid) for 6-8 h to separate the phosphopeptides in the second dimension. The dried cellulose plate was finally exposed to Kodak X-AR film.

Edman Degradation

Modified manual Edman degradation was performed as described in (30). Briefly, phosphopeptides were covalently coupled to Sequelon-AA discs (Perseptive Biosystem Inc.) and subjected to consecutive cycles of the Edman degradation. After each cycle, the disc was treated with trifluoroacetic acid to cleave and release the N-terminal amino acid, and the activity of radioisotope released by the ^{32}P-labelled amino acid was determined by Cerenkov counting.

In vitro **Kinase Assay**

293T cells (0.2×10^6) were transfected with 20 µg of HA-tagged CA-Akt or DN-Akt. After 48 hr of transfection, Akt was immunoprecipitated from cell extracts and incubated with 5 µg of purified GST-p21$^{Cip1/WAF1}$ (wild-type or mutant) in the presence of 5 µCi of [γ-^{32}P]ATP and 50 mM cold ATP in a kinase buffer for 30 min at 30°C. The reaction products were resolved by SDS-PAGE, and the ^{32}P-labeled proteins were visualized by autoradiography.

^{32}**P-orthophosphate Labeling**

293T cells (0.2×10^6)

were cotransfected with 18 µg of CA-Akt or DN-Akt and 2 µg of wild-type or mutant p21$^{Cip1/WAF1}$. After 36 hr of transfection, the cells were starved for 12 hr and then incubated with phosphate-free medium for 1 hr. The cells were then labeled with 1 mCi/ml ^{32}P-orthophosphate for 3 hr. The cells were then lysed, and p21$^{Cip1/WAF1}$ was immunoprecipitated from the cell extracts and separated by 12% SDS-PAGE. The incorporation of ^{32}p-phosphate was measured by autoradiography.

Immunoprecipitation and Immunoblotting

Cells were washed twice with PBS, scraped into 500 μl of lysis buffer. After a brief sonication, the lysate was centrifuged at 14,000 xg for 10 min at 4°C to remove the insoluble cell debris. Immunoprecipitation and immunoblotting were performed as described previously (4).

In situ Immunofluorescent Staining

Approximately 0.2 X 10^6 p21$^{-/-}$ MEF cells were plated in 100-mm plates and cotransfected with 9 μg of CA-Akt or DN-Akt and 1 μg of wild-type or mutant p21$^{Cip1/WAF1}$ by using liposome. After 36 hr of incubation, the cells were trypsinized and plated into chamber slides for another 12 hr. After fixation of the samples in cold acetone for 10 minutes at 4°C, the cellular localization of p21$^{Cip1/WAF1}$ was determined by using a monoclonal antibody against human p21$^{Cip1/WAF1}$ (Santa Cruz Biotech.) diluted 1: 100. After extensive washing in phosphate-buffered saline, the samples were further incubated with Texas Red-conjugated goat anti-mouse IgG (diluted 1:400) plus dapi (0.1 μg/ml) for 1 hr. After extensive washing, the samples were examined under a fluorescent microscope (Zeiss). The nonspecific reaction of secondary antibody was ruled out by the absence of fluorescence under the microscope.

Cellular Fractionation

Approximate 2 X10^7 cells were pelleted and resuspended in 800 μl of buffer A (10 mM HEPES, pH7.4, 1 mM EDTA, and 1 mM DTT) containing the protease inhibitors PMSF, leupeptin, aprotinin and pepstain. After incubation on ice for 10 min, the cells were homogenized with 10 strokes in a Dounce homogenizer. Then, the cells were examined under the microscope to confirm that more than 98% of the cells were lysed. After a brief centrifugation at 4°C, the supernatant (celluar fraction) was collected and the pellet

was washed twice with 400 µl of buffer B, and then resuspended in 150 µl of buffer C with gentle rocketing for 30 min at 4°C (23). After centrifugation, the supernatant (nuclear fraction) was collected. The amount of protein in the cytoplasmic and nuclear fractions were determined with a protein assay kit (Bio-rad) and the protein was subjected to immunoblotting.

Colony-Formation Assay

The colony-formation assay was used to measure the inhibition activity of p21$^{Cip1/WAF1}$ and its mutant. Wild-type or mutant p21$^{Cip1/WAF1}$ (T145A or T145D) or the vector pcDNA3 (2 µg of each) was transfected into NIH3T3, *HER-2/neu* 3T3, and DN-Akt 3T3 cells in six-well plates by using liposome. After 48 hr of transfection, the cells were trypsinized and evenly distributed into four 100-mm culture plates. The cells were selected with 700 µg/ml G418 (or 100 µg/ml hygromycin for DN-Akt 3T3 cells, as they contains the neomycin-resistance gene) for 3 weeks.

Bromodeoxyuridine (BrdU) Incorporation

The Brdu incorporation assay was also used to measure the inhibition activity of p21$^{Cip1/WAF1}$ and its mutant. Vector containing membrane-bound green fluorescence protein (GFP) (1 µg) and wild-type or mutant p21$^{Cip1/WAF1}$ (T145A or T145D) or the vector pcDNA3 (9 µg of each) were cotransfected into NIH3T3, *HER-2/neu* 3T3, and DN-Akt 3T3 cells by using liposome. After 48 hr of incubation, the cells were labeled with Brdu for 1 hr and then fixed with 70% ethanol. The cells were then stained with anti-Brdu antibody and incubated with fluorescent-conjugated secondary antibody. After extensive washing, the cells were sorted for GFP, and the incorporation of Brdu was measured by using FACS analysis.

Acknowledgements

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FIGURE LEGENDS

Figure 1 The Akt pathway was required for *HER-2/neu*-mediated cell proliferation.

(A) Blocking the Akt pathway reduces the growth of *HER-2/neu* 3T3 cells. The cells (3×10^3) were seeded in 96-well plates and grown in Dulbecco's modified Eagle's medium/F12 medium plus 1% fetal bovine serum. The growth rate was monitored by the MTT assay. The results are presented as the average $\pm$ SE of three independent experiments performed in quadruplicate. **(B)** Cells (3×10^3) were grown as described in (A) with 1 μCi of [^{3}H]thymidine for 12 hr. The cell replication rate was determined by measuring [^{3}H]thymidine incorporation. The results are presented as the average $\pm$ SE of three independent experiments performed in quadruplicate. **(C)** Blocking the Akt pathway did not significantly induce apoptosis in *HER-2/neu* 3T3 cells. The cells were grown in 1% serum, and the percentage of cells in apoptosis was measured by fluorescence-activated cell sorting (FACS) as described previously (Zhou et al., 2000). The results are presented as the average $\pm$ SE of three independent experiments. **(D)** Blocking the Akt pathway also reduced cell growth in MDA-MB453 cells. The cells were grown in six-well plates as described in (A), and their growth rates were measured by cell counting with a Coulter Counter. The results are presented as the average $\pm$ SE of three independent experiments. **(E)** The DNA synthesis rate of each of the cell lines in (D) was determined as described in (B). **(F)** Overexpression of *HER-2/neu* induced the expression of p21$^{\text{Cip1/WAF1}}$. Lysates (50 μg) from each cell lines were analyzed by 12% SDS-PAGE. After the protein was transferred to a nitrocellulose membrane, the expression of p21$^{\text{Cip1/WAF1}}$ was measured with a monoclonal antibody against p21$^{\text{Cip1/WAF1}}$ (Phamingen).

Figure 2 Threonine 145 of p21$^{Cip1/WAF1}$ was phosphorylated in vivo. (A) Endogenous p21$^{Cip1/WAF1}$ was phosphorylated in vivo. Breast cancer MDA-MB453 cells were incubated with 1.5 mCi/ml of [^{32}P]-orthophosphate for 3.5 hours followed by 30 min of insulin stimulation in the presence or absence of PI-3K inhibitor, LY294002. Cells were lysed and the endogenous p21$^{Cip1/WAF1}$ was immunoprecipitated with specific antibody and subjected to two-dimensional phosphopeptide analysis as described in Methods. (B) Tyrosine residue in p21$^{Cip1/WAF1}$ was not phosphorylated. Breast cancer MDA-MB453 cells were treated as described above, one third of the lysate was analyzed in western blot for the activation of Akt and the tyrosine phosphorylation of HER-2/neu (2 second exposure with anti phospho-tyrosine antibody, clone 4G10 from Upstate Biotech.). The remained lysate was subjected to immunoprecipitation with p21$^{Cip1/WAF1}$ specific antibody. The immunoprecipitated complexes were analyzed in western blotting with specific antibodies against p21$^{Cip1/WAF1}$ and phospho-tyrosine (20 min exposure with same antibody as described above). (C) Threonine 145 of p21$^{Cip1/WAF1}$ was phosphorylated. Breast cancer MDA-MB453 cells were treated as described in (A), and the p21$^{Cip1/WAF1}$ was immuoprecipitated and subjected to trypsin digestion followed by amino acid sequencing using Edman's degradation. The activity of radioisotope for ^{32}P-labelled amino-acid released from each cycle of Edman's degradation was detected in Cerenkov counter. The activity of radioisotope from each cycle has been subtracted from the background count, which usually ranges from 50 to 60 cpm.

Figure 3 Akt interacted with p21$^{Cip1/WAF1}$ and phosphorylated it at threonine 145. (A) the consensus Akt phosphorylation motif is highlighted. Sequence comparison of

p21$^{Cip1/WAF1}$ and other known Akt substrates is shown. **(B)** Immunoprecipitation of p21$^{Cip1/WAF1}$ and detection of Akt. HA-tagged DN-Akt or CA-Akt (10 μg) and flag-tagged wild-type or mutant p21$^{Cip1/WAF1}$ (10 μg) were cotransfected into 293T cells by the calcium phosphate methods. The cells were lysed in RIPA buffer after 48 hr and p21$^{Cip1/WAF1}$ was immunoprecipitated with anti-flag antibody. After transfer to a nitrocellular membrane, Akt was detected with HA antibody. **(C)** Immunoprecipitation of Akt and western blotting of p21$^{Cip1/WAF1}$. Akt was immunoprecipitated with an HA antibody and p21$^{Cip1/WAF1}$ was detected with a flag antibody. **(D)** Akt phosphorylated p21$^{Cip1/WAF1}$ at threonine 145. HA-tagged CA-Akt or DN-Akt (20 μg) was transiently transfected into 293T cells as described above. After 48 hr of incubation, CA-Akt or DN-Akt was immunopricipitated with an HA antibody and incubated with 5 μg of either GST-wild-type p21$^{Cip1/WAF1}$ or GST-mutant p21$^{Cip1/WAF1}$ (T145A) in a kinase buffer containing 5 μCi of [γ^{32}P]ATP for 30 min at 30°C. The kinase reaction was terminated with SDS-PAGE buffer, and the samples were assayed by autoradiography. The bottom two panels show western blots of Akt and the GST fusion protein used in the phosphorylation reaction, detected with antibodies against Akt and p21$^{Cip1/WAF1}$. **(E)** CA-Akt or DN-Akt (18 μg) and flag-tagged wild-type or mutant p21$^{Cip1/WAF1}$ (T145A) (2 μg) were cotransfected into 293T cells. After 48 hr, the cells were labeled with 1 mCi/ml [^{32}P]-orthophosphate for 3 hr. The p21$^{Cip1/WAF1}$ was immunoprecipitaed from the lysate and analyzed by either autoradiography to detect phosphorylation of p21$^{Cip1/WAF1}$ *in vivo* (upper panel) or western blotting as a control to measure p21$^{Cip1/WAF1}$ protein level (bottom panel). The histogram shows the amount of labeled p21$^{Cip1/WAF1}$ relative to the amount of immunoprecipitated p21$^{Cip1/WAF1}$ on the western blots.

Figure 4 Akt affected the cellular localization of p21$^{Cip1/WAF1}$. A 9:1 ratio of CA-Akt or DN-Akt (9 μg) and wild-type or mutant p21$^{Cip1/WAF1}$ (1 μg) were cotransfected into p21$^{-/-}$ MEF cells. After 36 hr of incubation, the cells were trypsinized and plated into chamber slides for another 12 hr. After fixation, the cellular localization of p21$^{Cip1/WAF1}$ was detected by using a monoclonal antibody against human p21$^{Cip1/WAF1}$. After extensive washing in phosphate-buffered saline, the samples were further incubated with Texas Red-conjugated goat anti-mouse IgG plus dapi and examined under a fluorescent microscope (Zeiss).

Figure 5 Cellular localization of endogenous p21$^{Cip1/WAF1}$. **(A)** Cellular fractionation was performed to determined the cellular localization of p21$^{Cip1/WAF1}$ in *HER-2/neu*3T3 cells, MDA-MB453 cells, and their DN-Akt transfectants as described in Methods. Equal amounts (40 ug) of cellular fraction (C) and nuclear fraction (N) from each samples were analyzed by 12% SDS-PAGE. Actin and PCNA were used as markers of the cellular and nuclear fractions, respectively. In each cellular fraction, the protein content in cytoplasm and nucleus was about 10 to 1 ratio. **(B)** Cellular localization of endogenous p21$^{Cip1/WAF1}$ can be regulated by the activation of Akt. Breast cancer MDA-MB453 cells were stimulated with insulin in the presence or absence of PI-3K inhibitor, LY294002. Cellular fraction was performed as described in (A). Equal amount of cellular or nuclear fraction from each samples were analyzed by western blotting.

Figure 6 HER-2/neu activates Akt and induces the cytoplasmic localization of p21$^{Cip1/WAF1}$ in breast tumor tissues. Five tissue sections from the HER-2/neu positive

adenocarcinoma (a-c) and five sections from HER-2/neu negative adenocarcinoma (d-f) were stained with antibodies specific to HER-2/neu (a, d), phosphorylated Akt (b, e), p21$^{Cip1/WAF1}$ (c, f), or normal rabbit serum (data not shown). The immunostaining was visualized with secondary antibody conjugated with peroxidase. One set of the representive staining was shown in here.
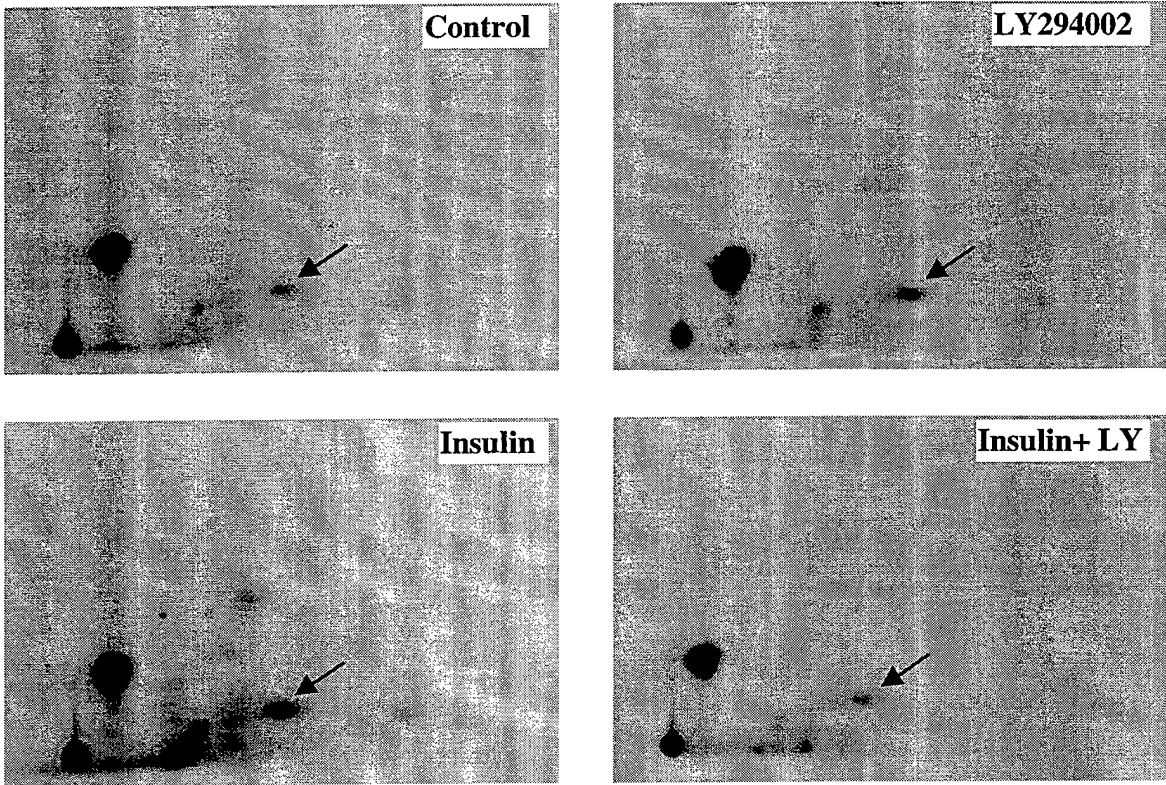
Figure 7 p21$^{Cip1/WAF1}$ (T145D) lost its inhibition activity whereas p21$^{Cip1/WAF1}$ (T145A) retained its inhibition activity independent of Akt. (A) The colony-formation assay was used to measure the inhibition activity of p21$^{Cip1/WAF1}$ and its mutant. Wild-type or mutant p21$^{Cip1/WAF1}$ (T145A or T145D) or the vector pcDNA3 (2 ug of each) was transfected into NIH3T3, *HER-2/neu* 3T3, and DN-Akt 3T3 cells. The number of colonies from each transfectant was determined by using crystal violet staining. The percentages of colonies from wild-type and mutant p21$^{Cip1/WAF1}$ were calculated by defining the number obtained from vector transfection alone as 100%. The results are presented as the average ± SE of four independent experiments. **(B)** The Brdu incorporation assay was also used to measure the inhibition activity of p21$^{Cip1/WAF1}$ and its mutant. Vector containing membrane-bound GFP (1 μg) and wild-type or mutant p21$^{Cip1/WAF1}$ (T145A or T145D) or vector pcDNA3 (9 μg of each) were cotransfected into NIH3T3, *HER-2/neu* 3T3, and DN-Akt 3T3 cells by using liposome. After 48 hours of incubation, the cells were labeled with Brdu for 1 hour and then fixed with 70% ethanol. The cells were then stained with anti-Brdu antibody and incubated with fluorescent-conjugated secondary antibody. After extensive washing, the cells were sorted for GFP, and the incorporation of Brdu was measured by using FACS analysis. The percentage of

28

Brdu incorporation for wild-type and mutant p21$^{Cip1/WAF1}$ was calculated by defining the number obtained from vector (pcDNA3) alone as 100%. The results are presented as the average ± SE of two separate experiments.
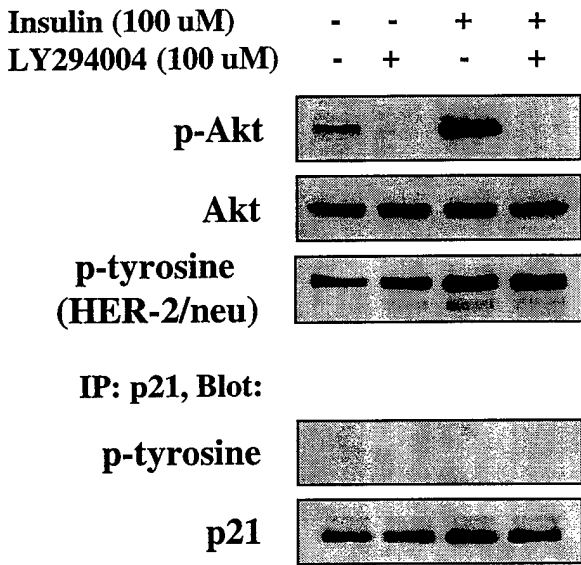
Figure 8   A model proposed to illustrate that *HER-2/neu* induces the cytoplasmic localization of p21$^{Cip1/WAF1}$ via Akt. The dashed line was derived from the studies of Deiehl *et al.* (1998) and Asada *et al.* (1999). The straight lines are derived from the study reported here.
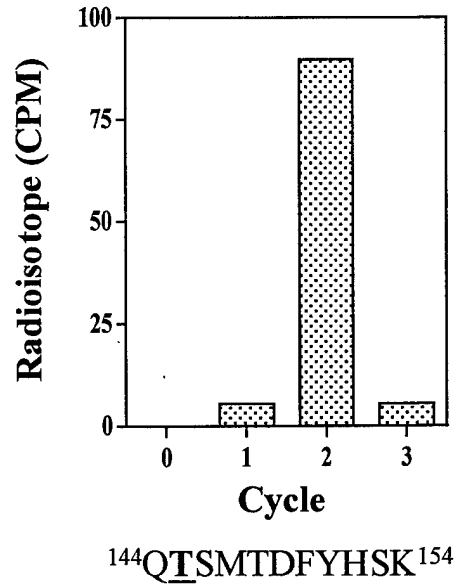
A

Legend:
- □ HER-2/neu3T3
- ◇ NIH3T3
- ● DN-Akt3T3
- ▲ HER-2/Wortmannin

Y-axis: Percentage of cell growth
X-axis: Time (hours)

D

Legend:
- □ MDA-MB453
- ◆ DN-Akt1
- ● DN-Akt2
- ▲ DN-Akt3
- ▽ Revertant

Y-axis: Percentage of cell growth
X-axis: Time (hours)

B

Y-axis: Thymidine incorporation (%)
X-axis categories: HER-2/neu3T3, NIH3T3, DN-Akt, HER-2/neu3T3 + Wort

E

Y-axis: Thymidine incorporation (%)
X-axis categories: MDA453, DN-Akt-C1, DN-Akt-C2, Revertant

C

Y-axis: Percentage of apoptosis
X-axis categories: HER-2/neu3T3, NIH3T3, DN-Akt, HER-2/neu3T3 + Wort

F

HER-2/3T3   DN-Akt   NIH3T3

Wortmannin   −   +   −   −
(100 nM)

p21

Actin

A

Control

LY294002

Insulin

Insulin+ LY

B

| Insulin (100 uM) | - | - | + | + |
| LY294004 (100 uM) | - | + | - | + |

p-Akt

Akt

p-tyrosine
(HER-2/neu)

IP: p21, Blot:

p-tyrosine

p21

C

^{144}Q**T**SMTDFYHSK^{154}

A

Akt phosphorylation motif: RxRxxS/T

GSK3β	**R**ARTS**S**	(9)
PFK2	**R**MRRN**S**	(466)
PFK2	**R**PRNY**S**	(483)
BAD	**R**GRSR**S**	(136)
Caspase-9	**R**RRFS**S**	(196)
FKHRL1	**R**PRSC**T**	(32)
	RRRAV**S**	(253)
	RSRTN**S**	(315)
IKKα	**R**ERLG**T**	(23)
p21 (human)	**R**KRRQ**T**	(145)
p21 (mouse)	**R**KRRQ**T**	(140)

B

IP: p21; Blot: Akt

CA–Akt	–	+	–	+	+
DN–Akt	+	–	+	–	–
p21	+	+	–	–	+
p21(T145A)	–	–	+	+	–
Control Ab	–	–	–	–	+

Akt

p21
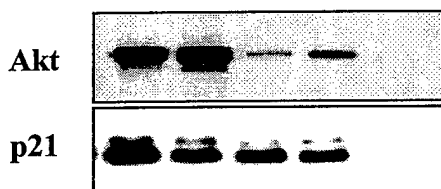
C

IP: Akt; Blot: p21

CA–Akt	–	+	–	+	+
DN–Akt	+	–	+	–	–
p21	+	+	–	–	+
p21(T145A)	–	–	+	+	–
Control Ab	–	–	–	–	+

p21

Akt
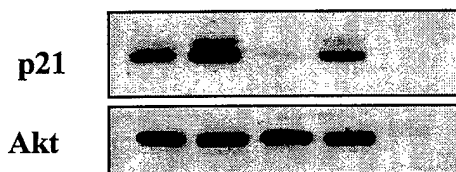
D

CA–Akt	+	–	+	–
DN–Akt	–	+	–	+
GST-p21	+	+	–	–
GST-p21(T145A)	–	–	+	+

200—
97—
68—
43—
29—

Akt

GST-p21

E

Densitometric ratio (^{32}P-p21/p21)

100
75
50
25
0

^{32}P-p21

p21
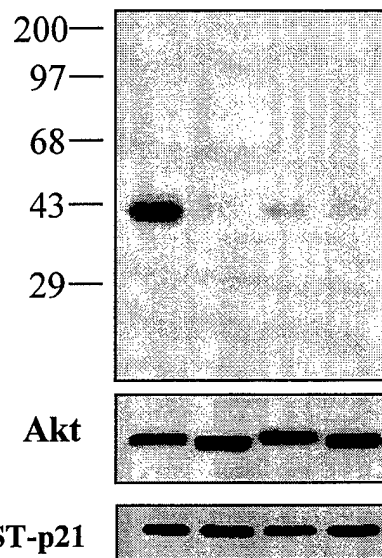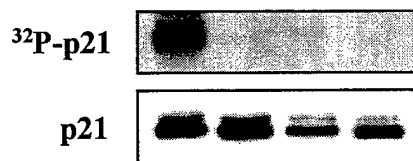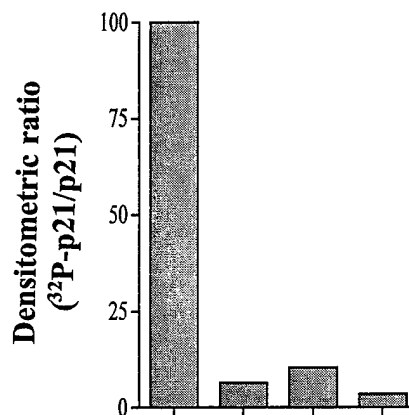
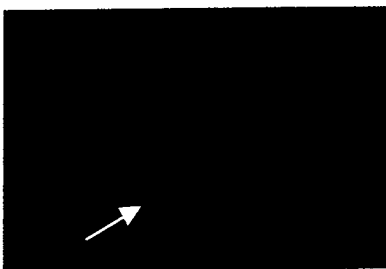|				
CA–Akt	+	–	+	–
DN–Akt	–	+	–	+
p21	+	+	–	–
p21(T145A)	–	–	+	+

	Texas Red	**DAPI**	**Combination**

CA-Akt
p21 (WT)
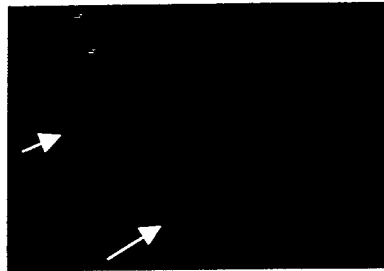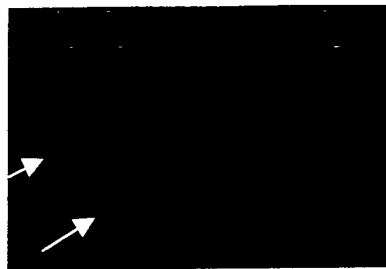
DN-Akt
p21 (WT)
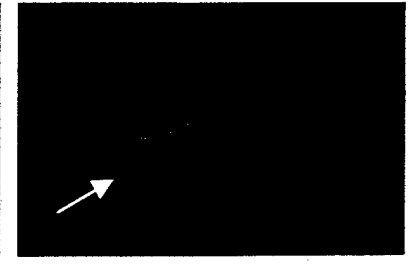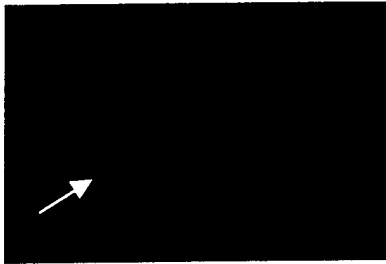
CA-Akt
p21(T145A)
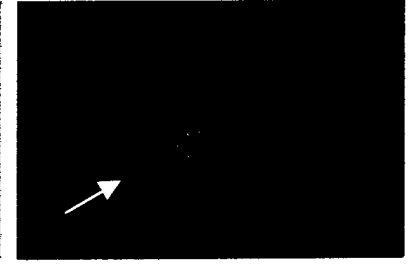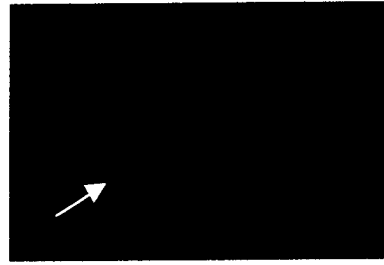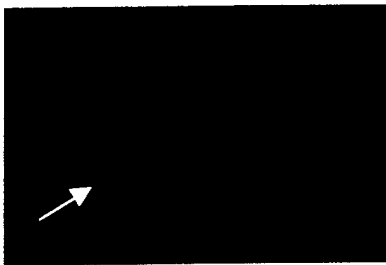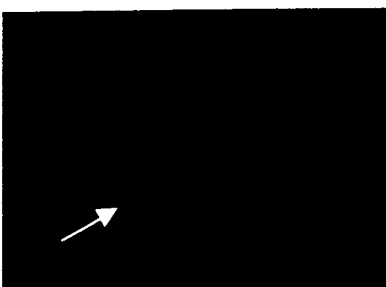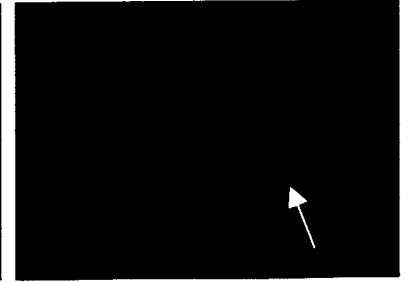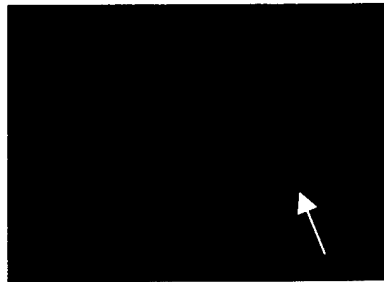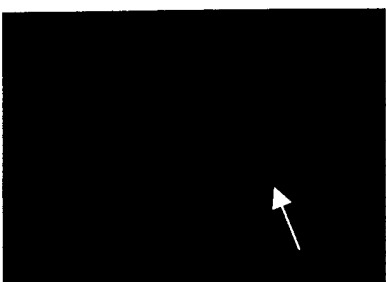
DN-Akt
p21(T145A)
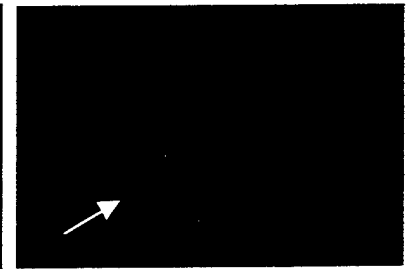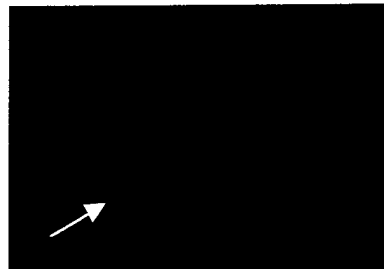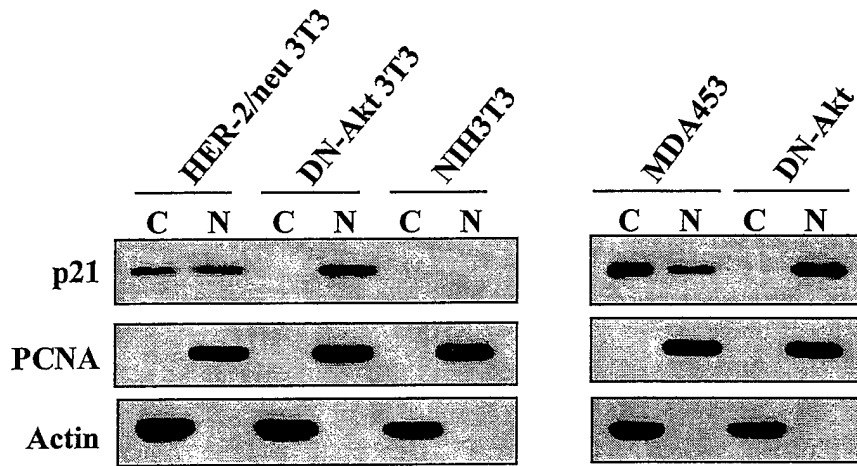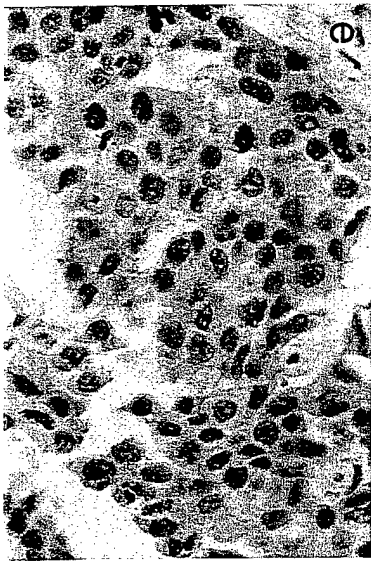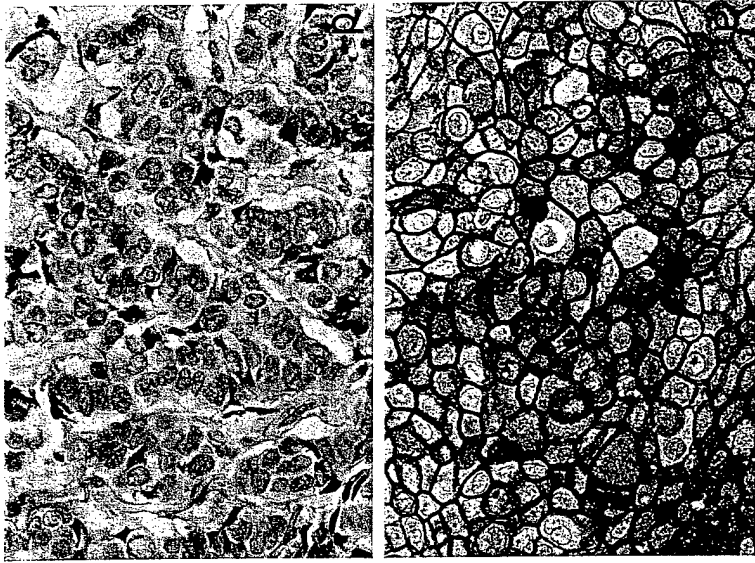
CA-Akt
p21(T145D)

DN-Akt
p21(T145D)

A

	HER-2/neu 3T3		DN-Akt 3T3		NIH3T3		MDA453		DN-Akt	
	C	N	C	N	C	N	C	N	C	N
p21										
PCNA										
Actin										

B

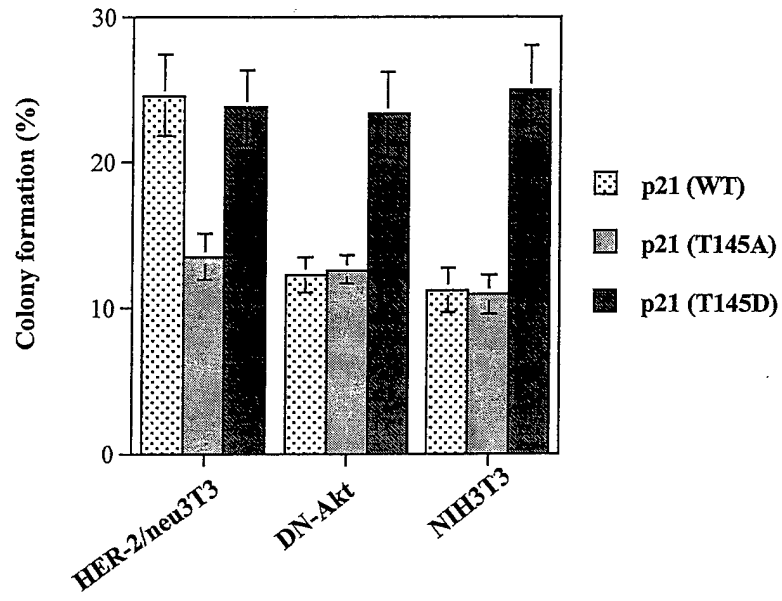|	Cytoplasm						Nucleus					
	MDA453			DN-Akt			MDA453			DN-Akt		
Insulin	-	+	+	-	+	+	-	+	+	-	+	+
LY294004	-	-	+	-	-	+	-	-	+	-	-	+
p21												
PCNA												
Actin												

HER-2/neu

Akt-Ⓟ

p21

A

B

HER-2/neu

PI3K

Akt

GSK-3β

Anti-apoptosis

Degradation ← Cyclin D   p21   p21 + Ask1

Proliferation