UNCLASSIFIED

AD NUMBER

ADB236753

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Infor.; Mar 98. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Fort Detrick, MD 21702-5012.

AUTHORITY

USAMRMC ltr dtd 26 Jan 2000

THIS PAGE IS UNCLASSIFIED

AD_____

AWARD NUMBER DAMD17-94-J-4169

TITLE: Regulation of Breast Cancer Cell Repression and
Differentiation by ErbB2 Ligand

PRINCIPAL INVESTIGATOR: C. Richter   King, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
                          Washington, DC 20057

REPORT DATE: March 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
                U.S. Army Medical Research and Materiel Command
                Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government
agencies only (proprietary information, Mar 98). Other requests
for this document shall be referred to U.S. Army Medical Research
and Materiel Command, 504 Scott Street, Fort Detrick, Maryland
21702-5012.

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

19980721 155

NOTICE

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE March 1998	3. REPORT TYPE AND DATES COVERED Final (1 Sep 94 - 28 Feb 98)

4. TITLE AND SUBTITLE
Regulation of Breast Cancer Cell Repression and Differentiation by ErbB2 Ligand

5. FUNDING NUMBERS
DAMD17-94-J-4169

6. AUTHOR(S)
King, C. Richter   , Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains best available photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only
(proprietary information, Mar 98). Other requests for this document
shall be referred to U.S. Army Medical Research and Materiel Command,
504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

This proposal has addressed the action of Heregulin family members on breast cancer cells. Three projects have been undertaken. Project one studied the activities of Heregulin 1 in altering the phenotype of human cancer cells growing in culture. Project two identified a new member of the heregulin family NRG-3 and showed that it is expressed in human cancer and can alter the growth of human cancer cells. Project three was designed to address the importance of cdc42, rac and rho in growth signaling generated by erbB family signalling.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
60

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

__✓__ Where copyrighted material is quoted, permission has been obtained to use such material.

__✓__ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

__✓__ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__✓__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__✓__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

__✓__ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

_C. Richler K_____ _April 1, 1998_
PI - Signature ⟶   Date

Table of Contents

PROJECT 1

Report for Period 1/94 - 6/95

Biological Activities of Heregulin on Breast Cancer Cells

INTRODUCTION:

The heregulins (HRGs) are a novel family of EGF-like growth factors which activate members of the subclass 1 tyrosine kinase receptors. HRGα1 was first isolated as a putative ligand for the *erbB2* receptor (p185) (1,2,3) but has since been shown to bind *erbB3* (4) and *erbB4* (5) and activate *erbB2* by *erbB2/erbB3* or *erbB2/erbB4* heterodimerization. HRG is a 45 kDa glycoprotein homologous to gp30 (6), neural differentiation factor (NDF) in the rat (7), the glial growth factors (GGFs) (8) and acetylcholine receptor inducing activity (ARIA) (9). Several isoforms of HRG, all of which arise from splice variants of a single gene (10), have been cloned and classified into the alpha and beta subgroups based structural differences in their EGF binding domains. Most isoforms are generated as transmembrane molecules and cleaved to generate soluble ligand (11). The HRGs have been identified as critical ligands in development of the heart and nervous system during embryogenesis (12,13), are involved in the regulation of ductal morphogenesis in the mammary gland (14,15) and induce both growth and differentiation of breast cancer cells (3,16,17,18). They have been classified with other members of the EGF growth factor family, including epidermal growth factor (EGF) (19), transforming growth factor alpha (TGF-alpha) (20), heparin-binding EGF (HB-EGF) (21), amphiregulin (22) and betacellulin (23). They are biologically distinct since they do not bind to the EGF receptor, and structural analysis suggests that the N-terminal of the EGF domain of HRG is critical for binding to its receptors (24).

Transregulation events mediated by HRG ligand activation of the *erbB3* and *erbB4* receptors which lead to signalling from the *erbB2* receptor may be key players in tumor progression to the invasive state. The results of several recent studies support this concept, suggesting that the dynamics of *erbB* receptor heterodimerization with other *erbB* family members contribute significantly to the regulation of signal transduction events within the cell. Activated *erbB2/erbB4* heterodimers demonstrate higher levels of tyrosine kinase activity when compared to *erbB4* homodimers (25). Also, although *erbB3* can only weakly bind HRG on its own, *erbB3* heterodimerization with *erbB2* constitutes both a high affinity dimer for HRG (26) and enhances development of the transformed phenotype (27). Ligand binding of HRG to its receptors activates the ras/mitogen activated protein kinase (MAPK) signalling pathway (ERK1 and ERK2) and p70/p85 S6 kinase (17). HRG-induced tyrosine kinase activity also leads to phosphorylation of the p85 subunit of PI-3 kinase and to its association with *erbB3* (28) and *erbB4* (29). PI 3-kinase has been shown to be a substrate for erbB2 and is regulated by its oncogenic activation (30).

At this stage several questions remain in regards to the biological mechanisms through which the *erbB* receptors and their ligands contribute to breast cancer onset and progression. Overexpression of *erbB2* is known to correlate with poor prognosis in breast, ovarian, stomach and lung cancers (31,32,33). However, the clinical significance of the *erbB3* and *erbB4* receptors in malignancy has not yet been fully established. Although *erbB2* has been shown to be a poor prognostic indicator for invasive breast cancer (34,35), 70% of breast cancers overexpressing *erbB2* are classified as noninvasive intraductal carcinomas (32). This suggests that *erbB2* overexpression may be a positive indicator of proliferation and transformation but may not be sufficient for the development of the metastatic phenotype. Since the process of metastasis requires changes in cell adhesion properties, release of basement membrane degrading enzymes, cell motility and eventual recolonization at selective target organs, additional co-factors interacting with *erbB2* may therefore be required for induction of the invasive phenotype. Data in this study would suggest that HRGs fall into this category.

In terms of biological activity, HRG has previously been shown to exert a biphasic effect in breast cancer cells. Relatively low concentrations promoted cell growth, whereas higher

concentrations induced differentiation by upregulating expression of cell adhesion molecules (36,37) and stimulating production of alpha-lactalbumin (16). Transfection studies in the estrogen receptor positive MCF-7 breast cancer cell line showed that HRG enhanced cell proliferation, induced estrogen independent growth by estrogen receptor downregulation and stimulated metastasis (18, 31, 38). Loss of estrogen receptor has been linked to more aggressive forms of breast disease (39,40) and to higher invasive and metastatic indices in cell lines (41).

The research objectives of this postdoctoral fellowship were to further investigate the biological effects of heregulin. The work presented here examines a specific role for HRG in the induction of the invasive phenotype in breast cancer cells. Addition of exogenous HRG ligand to the non-invasive SKBR-3 breast cancer cell line, which expresses high levels of *erbB2* and intermediate levels of *erbB3*, resulted in formation of lamellopodia and pseudopodia, phagocytic activity and chemomigration. A specific function for HRG in the induction of the invasive phenotype was also demonstrated by use of a neutralizing polyclonal antibody targeting the N-terminal of the EGF binding domain of HRG. The anti-HRG antibody was capable of significantly reducing both the growth and invasive properties of the HRG-expressing MDA-MB-231 cells and the chemomigration response to HRG seen with SKBR-3 cells. The results obtained provide additional insights into the biological mechanisms through which HRG and the *erbB* receptors may contribute to the invasive phenotype and promote progression to the metastatic state in breast cancer. The methods, results and discussion of the work completed for this fellowship from 1/94 through 6/95 are presented in the following pages.

MATERIALS AND METHODS:

Cell Lines

MDA-MB-231 and SKBR3 human breast adenocarcinoma cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD). Cell lines were cultured in improved minimum essential media (IMEM) (Gibco), supplemented with 10% fetal bovine serum/ 1% glutamine and maintained in 5% CO_2/ 95% air atmosphere at 37°C.

Generation of recombinant heregulinß2 and purification of HRG from MDA-MB 231 breast cancer cell conditioned media.

The heregulinß2 (HRGß2) cDNA (aa.1- aa.426) was inserted into the baculovirus transfer vector pVL 1393 (Invitrogen, San Diego, CA) at the Bam HI and XbaI sites. Potential positive clones were verified by the dideoxy chain termination method using sequenase version 2.0 (USB, Cleveland, OH) and were then amplified in bacteria (strain DH5α). The pVL1393/HRGß2 cDNA was then co-transfected along with linearized wild type AcMNPV DNA by calcium phosphate mediated infection into *spodoptera frugiperda* (Sf9) cells. The recombinant virus was identified by plaque screening and then propagated. The high-titer recombinant virus stock was produced by 4-5 rounds of re-infection to give a titer of 1 X 10^8 pfu/ml. Conditioned medium from infected Sf9 cells was collected and recombinant HRGß2 was purified by heparin affinity chromatography. Purification of gp30/heregulin (gp30/HRG) from MDA-MB-231 conditioned media has been described previously (42), and such material will be referred to as gp30/HRG throughout the manuscript to distinguish it form the recombinant from of HRGß2.

Generation of the heregulin neutralizing antibody

Generation of the HRG-neutralizing antibody has been described previously (43). Briefly, amino acid sequences corresponding to different regions of the EGF-like domain of heregulin were identified as potential sites from which to develop selective neutralizing antibodies. Synthetic peptides corresponding to the selected sequences were generated and termed α1, α2 and α3. These were conjugated to a lysine rich core matrix and injected into rabbits (Biosynthesis, Inc., Lewisville, TX) and the resulting polyclonal antibodies (α1, α2 and α3) were affinity purified and tested for HRG binding specificity by enzyme immunoabsorbant assay (ELISA). The polyclonal anti-HRG Ab (α3) directed to the N-terminal region of the EGF-like domain was found to have the greatest neutralizing activity in the presence of HRG and was therefore used to investigate the biological activities of HRG in the experiments described below.

Boyden Chamber Motility Assays

a) Chemomigration and chemoinvasion assays

Methodology for the Boyden chamber assay has been described extensively in previous publications (41). Chemomigration and chemoinvasion assays were conducted with SKBR-3

4

breast cancer cells in the Boyden chamber assay. Cells were plated at 50,000 cells/well (in quadruplicate) onto polycarbonate filters (12 um pore, PVP free, Nucleopore, Pleasanton, CA) coated with either gelatin (0.1 mg/ml) for the chemomigration or with Matrigel (Collaborative Biomedical Products, Bedford, MA, 1:20 dilution of stock) for the chemoinvasion assays respectively. gp30/HRG (0, 0.02, 0.2, 4.0 and 8.0 ng/ml) was added to the upper chamber with the cells and to the lower chambers which contained NIH3T3 fibroblast conditioned media (NIH3T3 CM) as a chemoattractant. NIH3T3 CM was prepared according to previously established protocols (41). Cells were incubated at 37°C, 5%CO_2/ 95% air for 16 h. Filters were then fixed, stained with hematoxylin and cells on the top surface of the filters were removed. Filters were mounted onto glass slides and the number of cells which migrated through the pores were assessed for each treatment group by image analysis systems or by microscopy.

b)   Measurement of chemotactic activity of HRGβ2 and inhibition by the anti-HRG (α3) neutralizing antibody.

SKBR-3 cells breast cancer cells were plated at 25,000 cells per well (in quadruplicate) in 0.1% BSA/IMEM onto gelatin-coated 12 uM pore filters in the Boyden chamber. Recombinant HRGß2 (0, 0.02, 0.2, 2.0 or 6.0 ng/ml) was added to the upper chamber to test for induction of cell migration or to the lower chamber to test for chemotactic activity. Specificity of the chemotactic response was determined by preincubating HRGß2 (0.2 or 2.0 ng/ml) with the anti-HRG Ab (α3) (0, 10, 20 , 40 and 80 ug/ml) or rabbit IgG (Sigma, St. Louis, MO)for 30 min at 37°C before addition to the lower wells of the Boyden chamber. Cells were incubated at 37°C, 5% CO_2/ 95% air for 16 h, filters were then fixed and analyzed as described above.

c) Inhibition of motility of MDA-MB 231 breast cancer cells by the anti-Hrg Ab.

MDA-MB-231 breast cancer cells were preincubated for 30 min at 37°C, 5% CO_2/ 95% air in the presence of the anti-HRG Ab (α3) (0, 10, 20, 40 and 80 ug/ml) or rabbit IgG and were then plated at 25,000 cells/ well (in quadruplicate) in 0.1% BSA/IMEM onto gelatin-coated 12 uM pore filters and incubated for 6 h at 37°C, 5%CO_2/ 95% air. NIH3T3 CM was used in the lower chambers as a chemotactic agent. Cell migration results were analyzed as described above.

Matrigel Outgrowth Experiments

MB-MDA-231 cells were plated (Day 0) at 5000 cells/well in IMEM/10% FBS on a preset Matrigel layer (0.2 ml) in 46-well plates and grown for 7 days at 37 °C, 5% CO_2/ 95% air in the absence or presence of the anti-HRG Ab (α3) or rabbit IgG. Anti-Hrg Ab (α3) (20, 40 or 80 ug/ml) or IgG (40 or 80 ug/ml) were added on Days 1, 3, 5 and growth patterns were photographed on Days 3, 5 and 7 using Hofman optics on the Leitz Labovert photomicroscope.

Cytoskeletal Staining of F-Actin

F-actin staining was assessed in SKBR-3 and MDA-MB-231 cell lines. SKBR3 cells were plated at a sub-confluent density of 35,000 cells/well in IMEM/10% FBS onto 13 mm sterile glass coverslips in 24-well tissue culture plates and incubated at 37 °C, 5%CO_2/ 95% air for 24 h. Cells were then treated in 10% FBS/IMEM with gp30/ HRG (0, 0.2, 2.0 and 6.0 ng/ml) or gp30/HRG (0.2 ng/ml) in combination with Wortmanin (0.01 uM; Sigma, ST. Louis, MO) for 24 h after which they were fixed in 3.7% formaldehyde/PBS for 30 min, rinsed in PBS, permeabilized in PBS/0.3% Triton-X 100 and stained for F-actin with Texas Red immunofluorescent-labeled phalloidin (0.25 ug/ml; Sigma, St. Louis, MO.) for 30 min. MDA-MB-231 cells were plated (Day 0) at 10,000 cells/well in IMEM/10% FBS onto 13 mm sterile glass coverslips in 24-well

tissue culture plates and incubated at 37°C, 5% CO_2/ 95% air and incubated for 6 days in the absence or presence of the anti-HRG Ab ($\alpha3$) (0, 20, 40 and 80 ug/ml) or rabbit IgG (40 and 80 ug/ml) added on days 1, 3 and 5. Coverslips were fixed for examination on days 1, 3 and 6 and stained for F-actin as described above. Coverslips were mounted onto glass slides and actin staining in SKBR3 and MDA-MB 231 cells was analyzed with a Zeiss fluorescent microscope.
FACS Phagocytosis Assay

Precooled 24-well plates were coated with a thin film of fluorescein isothioncyanate (FITC)-labeled gelatin/sucrose solution and cross-linked with glutaraldehyde. Preparation of fluorescenlty labeled gelatin has been described previously (44). SKBR-3 cells were seeded at 300,000 cells/ well in 0.5 ml serum-containing medium and immediately treated with gp30/HRG (0, 0.02, 0.2 and 2.0 ng/ml). Cells were allowed to attach and spread overnight at 37°C, 7.5% CO2/ 95% air. After approximately 16 h cells were detached with trypsin/ EDTA, washed in serum-containing medium and PBS and fixed in 3% paraformaldehyde/ PBS. The amount of phagocytosed FITC-gelatin was determined by Fluorescent Activated Cell Sorting (FACS) (Star Plus, Becton and Dickinson) and expressed as fold background (cells incubated on unlabeled gelatin films).

RESULTS:

Gp30/HRG induces chemomigration and chemoinvasion of SKBR-3 cells.

gp30/HRG was tested for induction of cell motility in the non-HRG expressing SKBR-3 breast cancer cell line as an exogenously added reagent. This cell line was selected because it expresses high levels of *erbB2* in addition to *erbB3* and *erbB4*. Cells were tested in Boyden chamber chemomigration and chemoinvasion assays in the presence of gp30/HRG (0, 0.02, 0.2, 4.0 and 8.0 ng/ml) added to both upper and lower chambers (*Figure 1*). Chemomigration and chemoinvasion were measured according to the number of cells traversing gelatin or matrigel-coated filters respectively. The response to gp30/HRG was dose-dependent and showed migration and invasion to be maximal at 0.2 ng/ml ligand. This decreased significantly at the higher concentrations of 4 and 8 ng/ml. Although fewer cells were able to passage through Matrigel when compared to the gelatin-coated filters, motility profiles in both assays followed the same dose response pattern to gp30/HRG. MCF-7 cells, which express low levels of erbB2, 3 and 4, when tested in the Boyden chamber assay showed minimal chemomigration responses to gp30/HRG.

HRGβ2 acts a chemotactic agent and can be inhibited by the anti-HRG Ab.

Purified recombinant HRGβ2 protein was tested in the Boyden chamber assay to confirm the results obtained with gp30/HRG. In addition, the experiments were designed to examine the mechanism of action in the induction of cell motility. Results presented in *Figure 2* show that when HRGβ2 was added to the bottom wells, a biphasic chemotactic response was induced in the SKBR-3 cells. Chemotaxis was maximal at 0.2 ng/ml ligand and was still present at 2.0 ng/ml although at lower levels. A minimal increase in motility was observed at 0.02 ng/ml ligand and 0.1% BSA/PBS alone did not elicit a migratory response. In contrast, HRGβ2 incubated in the top chamber with the cells did not induce a statistically significant increase in motility of SKBR-3 cells over the control group at 0.2 and 2.0 ng/ml, although migration was reduced at 6.0 ng/ml. (Data not shown).
In order to inhibit the chemotactic response induced by HRGβ2, the anti-HRG Ab ($\alpha3$) (0, 20, 40 and 80 ug/ml) or rabbit IgG (40 and 80 ug/ml) were added to the bottom wells of the

chamber in the presence of HRGß2 at 0.2 ng/ml, the dose which induced the greatest chemotactic response. Incubation of HRGß2 in the presence of the anti-HRG Ab reduced the chemomigration response by 40% at 40 ug/ml and by 60% at 80 ug/ml antibody when compared to the 0.2 ng/ml control group (*Figure 2*). Rabbit IgG at the same concentrations did not inhibit the chemotactic response in the presence of 0.2 ng/ml HRG.

HRG induces formation of pseudopodia and stress fibers in SKBR-3 cells.

Based on the migration and invasion results obtained in the Boyden chamber assay, SKBR-3 cells were then treated with HRG and tested for cytoskeletal responses to ligand stimulation. To allow for observation of membrane ruffling and pseudopodia formation, cells were plated at sub-confluent densities, incubated in the presence of gp30/HRG (0, 0.2, 2.0 and 6.0 ng/ml) for 24 h and stained for F-actin with Texas Red-labeled phalloidin. Untreated SKBR3 cells (*Figure 3a*) show bands of stress fibers close to the cell periphery and punctate actin staining within the cell. Cells treated with gp30/HRG (0.2 - 6.0 ng/ml) however exhibit dramatic cytoskeletal changes characterized by a 2-3 fold increase in size and specific rearrangement of the actin cytoskeletal structure based on the dose of ligand. At 0.2 ng/ml gp30/HRG extensive formation of membrane ruffles and/or pseudopodia are clearly apparent. Cell morphology is characterized by F-actin distribution into the pseudopodia and ruffles at the cell borders, often accompanied by complete clearing of the actin cytoskeleton in the cell center (*Figure 3b*). Stress fibers and radiating actin structures close to cell periphery start to appear at 2.0 ng/ml (*Figure 3c*). At 6.0 ng/ml the cells are still enlarged and spread, and extensive actin stress fibers traverse the cell body forming a complex network rather than even parallel bands (*Figure 3d*). The observed morphological changes induced by gp30/HRG correlate with the previously shown migration data in the Boyden chamber assay. At 0.2 ng/ml ligand SKBR3 cells form large pseudopodia and lamellopodia and demonstrate maximal chemomigration and invasion activity. At higher doses of ligand stress fiber formation is induced and cell motility is decreased.

Since cell membrane ruffling is believed to be mediated by activation of PI-3 kinase, we tested whether HRG-induced formation of pseudopodia and lamellopodia could be inhibited by the PI-3 kinase inhibitor Wortmanin (45). SKBR-3 cells were co-incubated in the presence of Wortmanin (0.01 uM) and 0.2 ng/ml gp30/HRG for 24 h and stained for F-actin. Wortmanin (0.01 uM) alone did not show any significant effect on the SKBR-3 baseline cytoskeletal actin network (*Figure 3e*) but was capable of inhibiting the HRG-induced ruffling and formation of pseudopodia (*Figure 3f*).

HRG stimulates phagocytosis in SKBR-3 cells.

The FACS phagocytosis assay has been shown to correlate with existing in vitro invasion assays and depends on both the gelatinolytic activity and the phagocytotic capacity of the cells (44). Compared to invasive breast cancer cell lines such as MDA-MB-231 and HS578T, the invasive capacity of SKBR-3 cells in the FACS phagocytosis assay is low, but can be significantly stimulated by increasing doses of gp30/HRG. The results of FACS analysis of FITC gelatin phagocytosis by SKBR-3 cells stimulated with gp30/HRG (0.02, 0.2 and 2.0 ng/ml) are shown in *Figure 4*. Data (mean values and SEM of duplicates) quantitatively express the overnight uptake of FITC-gelatin by SKBR-3 cells. The stimulation by gp30/HRG is dose-dependent, reaching a plateau at 0.2 ng/ml where it shows a 2-fold increase and is statistically significant (Student T-test, $p < 0.05$). Fibronectin degradation and phagocytosis most likely involve proteases.

Inhibition of invasive properties of MDA-MB 231 by the anti-HRG Ab.

In order to test for a specific effect of HRG in promoting a more aggressive and invasive phenotype in breast cancer cell lines, the HRG neutralizing antibody was tested in proliferation and invasion assays. MDA-MB-231 cells which express HRG were tested in the Boyden chamber assay in the presence of the anti-HRG Ab (α3) (0, 10, 20, 40 and 80 ug/ml) or rabbit IgG. NIH3T3 CM was added as a chemoattractant to the lower chambers and cells were allowed to migrate over a 6 h incubation period. Results presented in *Figure 5* show a dose-dependent reduction in cell motility in the presence of antibody. Motility of MDA-MB-231 cells was inhibited by 29, 43, 59 and 73% at 10, 20, 40 and 80 ug/ml neutralizing antibody respectively. Rabbit IgG had no effect on cell motility up to 80 ug/ml (data not shown). In the presence of the anti-HRG Ab (α3) (40 and 80 ug/ml), the reduction in cell migration was accompanied by a morphological change from the characteristic spindle shape to a flattened phenotype with an enlarged cytoplasm.

HRG neutralizing antibody alters outgrowth patterns of MDA-MB-231 cells in Matrigel.

Highly invasive breast cancer cell lines such as MDA-MB-231, HS578T and MCF-7/ADR, have been shown to have a characteristic invasion and stellate outgrowth pattern when cultured in Matrigel, while non-invasive cell lines grow clonally and do not migrate into the surrounding matrix (41). The effect of the anti-HRG Ab (α3) on the Matrigel outgrowth pattern of MDA-MB-231 cells was therefore assessed. Cells were plated (day 0) in IMEM/10% FBS on a preset Matrigel layer and grown for 7 days in the presence of the HRG neutralizing antibody or rabbit IgG. MDA-MB-231 cells were plated sparsely (5000 cells/well) to observe outgrowth patterns in response to antibody treatment. Anti-HRG Ab (α3) (20, 40 or 80 ug/ml) or IgG (40 or 80 ug/ml) were added on days 1, 3 and 5. Control untreated and IgG treated cells moved outward across the surface of the Matrigel as they replicated, forming pathways of cells multiple layers in width by day 5 (*Figure 6c*). Matrigel outgrowth of MDA-MB-231 cells was significantly reduced in the presence of antibody (40 and 80 ug/ml) over the 7 day incubation period. When Ab was present the cells tended to form foci and by day 5 pathways only a single cell in width were observed leaving these foci (*Figure 6d*).

Anti-HRG Ab induces a more differentiated phenotype in MDA-MB-231 Cells.

In order to understand the mechanism by which the anti-HRG Ab (α3) decreased motility and inhibited growth of MDA-MB 231 cells, we tested whether treatment with antibody could alter the actin cytoskeleton. MDA-MB 231 cells were plated (Day 0) in IMEM/10% FBS at 10,000 cells/well onto sterile glass coverslips in 24-well tissue culture plates and were incubated for 7 days in the presence of the anti- HRG Ab (α3) (0, 20, 40 and 80 ug/ml). On day 5, untreated MDA-MB-231 cells generally stained diffusely for F-actin in the cytoplasm and showed ruffling activity at the cell membrane (*Figure 6a*). In striking contrast, incubation in the presence of the HRG neutralizing antibody induced cell flattening, an increase in cell size and formation of stress fibers (*Figure 6b*). Reorganization of the cell cytoarchitecture initially become apparent by Day 1 with 80 ug/ml antibody (day 3 at 40 ug/ml) and continues to progress up to day 7. Actin staining in the IgG group (40 and 80 ug/ml) was similar to the control.

DISCUSSION:

The process of metastasis consists of several steps involving changes in cell adhesion properties, release of basement membrane degrading enzymes, cell migration and eventual recolonization at selective target organs (46). The *erbB2* protooncogene is a major prognostic

parameter for invasive breast cancer but is expressed more often in non-invasive disease, where it lacks prognostic significance. It's role in promoting tumor progression to metastatic disease is therefore not clear and suggests that erbB2 overexpression alone may be a good indicator of proliferation and transformation, but that additional interacting co-factors may potentiate the metastatic phenotype. Signaling from members of the subclass 1 tyrosine kinase receptors (EGFR, erbB2, 3 and 4) and their ligands are known to be involved in growth regulation of breast carcinoma cells (47,48). Although HRG does not directly bind erbB2, several recent studies indicate that HRG ligand interactions with erbB3 and erbB4 are directly influenced by the presence of the erbB2 receptor in the development of the transformed phenotype (49,50,51,52,53). The results presented in this paper suggest that HRG expression and its biological functions may be very important in the maintenance and activation of a more aggressive and invasive phenotype in breast carcinoma cells.

Specific involvement of HRG in promoting a more aggressive and invasive phenotype was demonstrated with the HRG-neutralizing antibody. The anti-HRG Ab tested on the highly aggressive HRG-producing MDA-MB-231 breast cancer cell line was capable of significantly inhibiting both proliferative and invasive behavior and also induced morphological changes indicative of more a differentiated phenotype. The anti-HRG Ab was however not capable of completely inhibiting the growth and invasive properties of the MDA-MB-231 cells. This may be due to presence of the mutated ras gene in these cells and therefore a constituitively activated MAPK signalling pathway and/ or the production of additional growth factors (54). Since the antibody is polyclonal it is difficult to assess what portion comprises the active fraction.

Actions of HRG as a paracrine agent in the induction of a more invasive phenotype was tested by addition of exogenous ligand. The erbB2/erbB3 overexpressing SKBR-3 breast cancer cell line which lacks HRG production and exhibits minimal invasive behavior, responded to low concentrations of HRG in the Boyden chamber assay by showing increased chemoinvasion and chemotaxis. Specificity of the chemotactic response to HRG was confirmed by co-incubation of the ligand in the presence of the anti-HRG neutralizing antibody. In addition, treatment of SKBR-3 cells with low concentrations of gp30/HRG resulted in extensive membrane ruffling and formation of pseudopodia, and also enhanced phagocytic activity. The secretion of proteases as a result of ligand treatment is implied by both the fibronectin phagocytosis assay which simulates basement membrane degradation and the Boyden chamber invasion assays. Protease secretion and basement membrane remodelling in response to HRG have not been characterized at present.

The actions of low concentrations of HRG on cell motility, formation of large pseudopodia and lamellopodia and increased phagocytic activity reflect behavior which is functionally consistent with the induction of a more invasive phenotype. Treatment of SKBR-3 cells with higher concentrations of ligand however decreased chemomigration and invasion and induced formation of a more differentiated phenotype. The latter was characterized by an increased cytoplasmic to nuclear ratio and a complex actin cytoskeletal framework of stress fibers. Biphasic effects of HRG have been described previously in the AU-565 and SKBR-3 erbB2 overexpressing cell lines (16), where low ligand levels enhanced proliferation and higher concentrations induced differentiation. These results support the effects observed here with HRG in the induction of a more invasive or alternatively more differentiated phenotype. These biological endpoints suggest that HRG ligand and erbB receptor levels in normal or transformed breast epithelial cells may define a balance between promotion of proliferation and invasion or differentiation (14,15).

The signalling pathway activated by HRG in SKBR-3 cells leading to the formation of pseudopodia and membrane ruffles appears to involve PI-3 kinase activation since this response was prevented by Wortmanin. PI-3 kinase is known to associate with the erbB2, erbB3 and erbB4 receptors upon ligand binding of HRG and tyrosine kinase activation (27, 28, 29). Cell membrane ruffling signals through the small GTPase protein rac (55) which also directly interacts with PI-3 kinase (56), implicating this pathway in signaling by HRG. The association between the erbB2 receptor and motility in the SKBR-3 cell line has also been demonstrated in previous studies. A 50 kd protein isolated from COLO-16 cells, which has not been characterized but has been suggested to be a member of the heregulin family, stimulated motility of SKBR-3 cells (57)

and aggregation of phosphorylated *erbB2* receptor in pseudopodia and membrane ruffles (58). EGF has also been shown to induce formation of lamellopodia and act as a chemotactic agent for a metastatic mammary adenocarcinoma cell line derived from the rat (59). Biological characteristics such as cell motility, phagocytosis and formation of membrane ruffles in cancer cells have been positively correlated with the invasive phenotype and increased metastatic index (60).

CONCLUSIONS:

Signaling events initiated by the *erbB-2, 3* and *4* receptors and the HRG family of growth factors can provide us with additional insights into the biological mechanisms which may contribute to the progression of breast cancer. The results of this work describe specific functions for HRG in the induction and maintenance of the invasive phenotype in breast cancer cells as demonstrated by use of an HRG neutralizing antibody. HRG appears to be acting by an autocrine mechanism in the MDA-MB-231 cell line, but can also function as a paracrine agent to promote motile and invasive behavior when added as exogenous ligand to the non-invasive SKBR-3 cell line. The overall results presented here therefore imply that growth factor ligands such as HRG rather than the receptors may participate in enhancing the metastatic process. Expression of HRG in breast tumors has not been characterized so far. HRG may be present in the tumor in a subset of malignant cells which could initiate metastatic events. Alternatively, it may be synthesized by surrounding stromal fibroblasts and promote invasiveness of cancer cells by juxtacrine and paracrine mechanisms. Further studies on tumor expression of HRG and its biological functions may lead to the development of new therapies which target its actions on cancer cell proliferation and invasion.

REFERENCES:

1) Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis, G.D., Shepard, H.M., Kuang, W.-J., Wood, W.I., Goeddel, D.V. and Vandlen, R.L. Identification of Heregulin, a specific activator of p185erbB2. Science, *256*: 1206 - 1210, 1992.

2) Lupu, R., Colomer, R., Kannan, B. and Lippman, M.E. Characterization of a growth factor that binds exclusively to the erbB-2 receptor and induces cellular response. Proc Natl Acad Sci USA., *89*: 2287 - 2291, 1992.

3) Peles, E., Bacus, S.S, Koski, R.A., Lu, H.S., Wen, D., Ogden, S.G., Ben Levy, R. and Yarden, Y. Isolation of the Neu/HER-2 stimulatory ligand: A 44 kd glycoprotein that induces differentiation of mammary tumor cells. Cell, *69*: 205 - 216, 1992.

4) Carraway, K.L., III, Sliwkowski, M.X., Akita, R., Platko, J.V., Guy, P.M., Nuijenes, A., Diamonti, A.J., Vandlen, R.L., Cantley, L.C. and Cerione, R.A. The erbB-3 gene product is a receptor for Heregulin J. Biol. Chem., *269*, 14303-14306, 1994.

5) Plowman, G.D., Green, J.M.,Colouscou, J.-M., Carlton, G.W., Rothwell, V. and Buckley, S. Heregulin induces tyrosine phosphorylation of HER4/p180erbB-4. Nature, *366*: 473 - 475, 1993.

6) Lupu, R., Colomer, R., Zugmaier, G., Sarup, J., SHepard M., Salmon D. and Lippman, M.E. Direct interaction of a ligand for the erbB-2 oncogene product with the EGF receptor and

p185erbB-2. Science, *249*: 1552 - 1555, 1990.

7) Wen, D., Peles, E., Cupples, R.L., Suggs, S.V., Bacus, S.S, Luo, Y., Trail, G., Hu, S., Silbiger, S.M., Levy, R.B., Koski, R.A., Lu, H.S. and Yarden, Y. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell, *69*: 559-572, 1992.

8) Marchionni, M.A., Goodearl, A.D.J., Chen, M.S., Berminghan-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J.B., Hsuan, J.J., Totty, N.F., Otsu, M., Mcburney, R.N., Waterfield, M.D., Stroobant, P. and Gwynne, D. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous sysyem. Nature, *362*: 312 - 318, 1993.

9) Falls, D.L., Rosen, K.M., Corfas, G., Lane, W.S., and Fischbach, G.D. ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell,*72*: 801 - 815, 1993.

10) Wen, D., Suggs, S.V., Karunagaran, D., Liu, N., Cupples, R.L., Luo, Y., Janssen, A.M., Ben-Baruch, N., Trollinger, D.B., Jacobsen, V.L., . Structural and functional aspects of the multiplicity of neu differentiationm factors. Mol. Cell. Biol., *14*: 1909-1919, 1994.

11) Orr Utregger, A.L., Trakhtenbrot, L., Ben Levy, R., Wen, D., Rechavi, G., Lonai, P. and Yarden, Y. Neural expression and chromosomal mapping of NEu differentiation factor to 8p12-p21. Proc. Natl. Acad. Sci. USA, *90*: 1867-1871, 1993.

12) Meyer, M., and Birchmeier, C. Multiple essential functions of neuregulin in development. Nature, *378*: 386-390, 1995.

13) Gassman, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G. Aberrant neural and cardiac development in mice lacking the erbB4 neuregulin receptor. Nature, *387*: 390-394, 1996.

14) Yanmin, Y., Spitzer, E., Meyer, D., Sachs, M., Nieman, C., Hartman, G., Weidner, K.M., Birchmeier, C. and Brichmeier, W. Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J. Cell Biol., *119*: 215-226, 1995.

15) Krane, I. and Leder P. NDF/heregulin induces persistence of terminal end budsand adenocarcinomas in the mammary glands of transgenic mice. Oncogene, *12*, 1781-1788, 1996.

16) Bacus, S.S, Huberman, E., Chin, D., Kiguchi, K., Simpson, S., Lippman, M.E., and Lupu, R. A ligand for the erbB-2 oncogene product (gp30) induces differentiation of human breast cancer cells. Cell, Growth and Diff., *3*: 401-411, 1992.

17) Marte, B.M., Graus-Porta, D., Jeschke, M., Fabbro, D., Hynes, N.E. and Taverna, D. NDF/heregulin activates MAP kinase and p70/p85 S6 kinase during proliferation or differentiation of mammary epthelial cells. Oncogene, *10*: 167-175, 1995.

18) Tang, C.K., Perez, C., Grunt, T., Waibel, C., Cho, C. and Lupu, R. INvolvement of heregulinβ2 in the aquisition of the hormone-independent phenotype of breast cancer cells. Cancer Res., *56*: 3350-3358, 1996.

19) Cohen, S., Ushiro, H., Stoscheck, C. and Chinkers, M. A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. J. Biol. Chem., *257*: 1523-1531, 1982.

20) Derynck, R. Transforming growth factor α. Cell, *54*: 593-595, 1988.

21) Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C. and Klagsburn, M. A heparin-binding growth factor secreted by macrophage-like cells thaat is related to EGF. Science, *251*: 936-939, 1991.

22) Shoyab, M., Plowman, G.D., McDonald, V.C., Bradley, J.G. and Todaro, G.J. Structure and function of human amphiregulin: a member of the epidermal growth factor family. Science, *243*: 1074-1076, 1989.

23) Shing, Y., Cristofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K. and Folkman, J. Betacellulin: a mitogen from pancreatic b cell tumors. Science, *259*: 1604-1607, 1993.

24) Burgess, T.L., Ross, S.L., Qian, Y., Brankow, D. and Hu, S. Biosynthetic processing of neu differentiation factor. J. Biol. Chem., *270*: 19188-19196, 1995.

25) Plowman, G.D.,Colouscou, J-M, Whitney, G.S., Green, J.M., Carlton, G.W., Foy, L., Neubauer, M.G. and Shoyab M. Ligand-specific activation of HER4/ p180erbB4, a fourth member of the epidermal growth factor receptor family. Proc Natl Acad Sci USA, *90*: 1746 - 1750, 1993.

26) Sliwkowski, M.X., Schaefer, G., Akita, R.W., Lofgren, J.A., Fitzpatrick, V.D., Nuijens, A., Fendly, B.M., Cerione, R.A., Vandlen, R.L. and Carraway, K.L. 3rd. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J. Bio.l Chem., *269*: 14661-5, 1994.
27) Kraus, M.H., Fedi, P., Starks, V., Muraro, R. and Aaronson, S.A. Demonstration of ligand-dependent signaling by the erbB-3 tyrosine kinase and its constitutive activation in human breast tumor cells. Proc. Natl. Acad. Sci. USA, *90*:2900-4, 1993.

28) Carraway, K.L. 3rd, Soltoff, S.P., Diamonti, A.J. and Cantley, L.C. Heregulin stimulates mitogenesis and phosphatidylinositol 3-kinase in mouse fibroblasts transfected with erbB2/neu and erbB3. J. Biol. Chem., *270*: 7111-6, 1995.

29) Sepp-Lorenzino, L., Eberhard, I., Ma, Z., Cho, C., Serve, H., Liu, F., Rosen, N.and Lupu, R. Signal transduction pathways induced by heregulin in MDA-MB-453 breast cancer cells. Oncogene, *12*: 1679-87, 1996.

30) Peles, E., Lamprecht, R., Ben-Levy , R., Tazhar, E. and Yarden, Y. Regulated coupling of the neu receptor to phosphoinositol 3'-kinase and its release by oncogenic activation. J. Biol. Chem. *267*: 12266 - 12274, 1992.

31) Slamon, D.J., Godolphin, W., Jones, L.A., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Udove, J., Ullrich, A. and Press, M.F. Studies of the HER-2/neu proto-oncogene in Human breast and ovarian cancer. Science, *244*: 707-712, 1989.

32) Paik S, Hazan R, Fisher ER, Saas, R.E., Fisher, B., Redmond, C., Schlessinger, J., Lippman, M.E. and King, C.R. Pathologic findings from the national surgical adjuvant breast and bowel project: prognostic significance of erbB-2 protein overexpression in primary breast cancer.

J. Clin. Oncol., *8*: 103-112, 1990.

33)   Gusterson, B.A., Gelber, R.D., Goldhirsch, K.N., Price, K.N., Save-Soderbiugh, J., Anbazaghan, R., Styles, J., Rudenstam, C.M., Golouh, R., and Reed, R. Prognostic importance of c-erbB2 expression in breast cancer. J. Clin. Oncol., *10*: 1049-1056, 1992.

34)   Gusterson, B.A., Machin, L.G, Gullick, W.J., Gibbs, N.M., Powels, T.J., Price, P., McKinna, A. and Harrison A. Immunohistochemical distribution of c-erbB-2 in infiltrating and in situ breast cancer. Int.J.Cancer, *42*: 842-845,1988.

35)   Allred, D.C., Clark, G.M., Molina, R., . Overexpression of HER-2/neu and its relationship with other prognostic factors change during the progression of insitu to invasive breast cancer. Human Pathol, *23*: 974-979, 1992.

36)   Bacus, S.S., Gudkov, A.V., Zelnick, C. R. *et al* . Neu differentiation factor (heregulin) induces expression of intrrercelular adhesion molecule 1; implications for mammmary tumors. Cancer Res, *53*: 5251-5261, 1993.

37)   Staebler, A., Sommers, C., Mueller, S.C., Byers, S., Thompson. E.W. and Lupu. R. Modulation of breast cancer progression and differentiation by the gp30/heregulin. Br Cancer Res & Treatm, *31*: 175-182, 1994.

38)   Grunt, T., Saceda, M., Martin, M.B., Lupu,R., Dittrich, E., Krupitza, G., Harant, H., Huber, H., and Dittrich, C.   bidirectional interactions between the estrogen receptor and the c-erbB2 signalling pathways: HRG inhibits estrogenic effects in breast cancer cells. Int. J. Cancer, *63*: 560-567, 1995.

39)   Godolphin, W., Elwood, J.M., and Spinelli, J.J. Estrogen receptor quantitation and staging as complimentary prognostic indicators in breast cancer. Int. J. Cancer, *28*: 677-683, 1981.

40)   McGuire, W.L., Clarke, G.M., Dressler, L. G., and Owens, M. A. Role of steroid hormone receptors as prognostic factors in primary breast cancer. J. Natl. Cancer. Inst. Monogr., *1*: 19-24, 1986.

41)   Thompson EW, Paik SM, Brunner N, Sommers CL, Zugmaier G, Clarke R, Shima TB, Torri J, Donahue S, Lippman ME, Martin GR, Dickson RB (1992). Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol 150: 534-544.

42)   Lupu, R., Wellstein, A., Sheridan, J., Ennis, B.W., Zugmaier, G., Katz, D., Lippman, M.E. and Dickson, R. B. Purification and characterization of a novel growth factor from human breast cells. Biochemistry, *31*: 7330-7340, 1992.

43)   Cho, C. and Lupu, R. Keystone Symposia, Breast and Prostate Cancer, Abstract , 1994.

44)   Coopman, P.J., Thomas, D.M., Gehlsen, K.R. and Mueller, S.C.   Integrin $\alpha 3\beta 1$ participates in the phagocytosis of extracellular matrix molecules by human breast cancer cells. Mol Biol Cell, *7*: 1789-1804. 1996.

45)   Powis, G., Bonjouklian, R., Berggren, M.M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W.F., Dodge, J., Grindey, G. and Vlahos, C.J. Wortmanin, a potent an

selective inhibitor of phosphatidylinositol-3-kinase. Cancer Res, *54*: 2419-2423, 1994.

46) Liotta, L. Tumor invasion and metastases: Role of the basement membrane. Am. J. Pathol., *117*: 339-348, 1984.

47) Lemoine, S. A., and Lemoine, N. R. The type 1 (EGFR-related) family of growth factor receptors and their ligands. Prog. Growth Factor Res., *4*: 1-24, 1992.

48) Dickson, R. B., and Lippman, M. E. Growth factors in breast cancer. Endocrine Reviews, *16*: 559-589, 1995.

49) Ram, T.G., Kokeny, K.E., Dilts, C.A. and Ethier, S.P. Mitogenic activity of neu differentiation factor /heregulin mimics that of epidermal growth factor and insulin-like growth factor-I in human mammary epithelial cells. J. Cell. Physiol., *163*: 589-596, 1995.

50) Zhang, K., Sun, J., Liu, N., Wen, D., Chang, D., Thomason, A., and Yoshinaga, S. K. Transformation of NIH3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. J. Biol. Chem., *271*: 3884-3890, 1996.

51) Beerli, R.R., Graus-Porta, D., Woods-Cook, K., Chen, X., Yarden, Y. and Hynes, N.E. Neu differentiation factor activation of ErbB-3 and ErbB-4 is cell specific and displays a differential requirement for ErbB-2. Mol. Cell. Biol., *156*: 496-505, 1995.

52) Karunagaran, D., Tzahar, E., Beerli, R.R., Chen, X., Graus-Porta, D., Ratzkin, B.J., Seger, R., Hynes, N.E. and Yarden, Y. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. EMBO J, *15*: 254-64, 1996.

53) Alimandi, M., Romano, A., Curia, M.C., Muraro, R., Fedi, P., Aaronson, S.A., Di Fiore, P.P. and Kraus, M.H. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. Oncogene, *10*: 1813-21, 1995.

54) Normanno, N., Qi, C.F., Gullick, W.J., Persico, G., Yareden, Y., Wen, D., Plowman, G., Kenney, N., Johnson, G., Kim, N., Brandt, R., Marinez-Lacaci, I., Dickson, R.B and Salomon, D.S. Expression of amphiregulin, cripto-1, and heregulin alpha in human breast cancer cell lines. Int. J. Oncol., *2*: 903-911, 1993.

55) Hall A. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu. Rev. Cell Biol., *10*:31-54, 1994.

56) Nobes, C.D., Hawkins, P., Stephens, L. and Hall, A. Activation of the small GTP-binding proteins rho and rac by growth factor receptors. J.Cell Sci., *108*: 225-33, 1995.

57) De Corte, V., De Potter, C., Vandenberghe, D., Van Laereebeke, Azam, M., Roels, H., Mareel, M. and Vandekerckhove, J. A 50 kd protein present in conditioned medium of COLO-16 cells stimulates cell spreading and motility, and activates tyrosine phosphorylation of Neu/Her-2, in human SKBR3 mammary cancer cells. J. Cell. Sci., *107*: 405-416, 1994.

58) De Potter, C. and Quatacker, J. The p185erbB2 protein is localized on cell organelles invoved in cell motility. Clin. Exp. Met., *11*: 453-461, 1993.

59) Segall, J.E., Tyerech, S., Boselli, L., Masseling, S., Helft, J., Chan, A., Jones, J., and Condeelis, J. EGF stimulates lamellopod extension in metastatic adenocarcinoma cells by an actin-

dependent mechanism. Clin. Exp. Metastasis, *14*: 61-72, 1992.

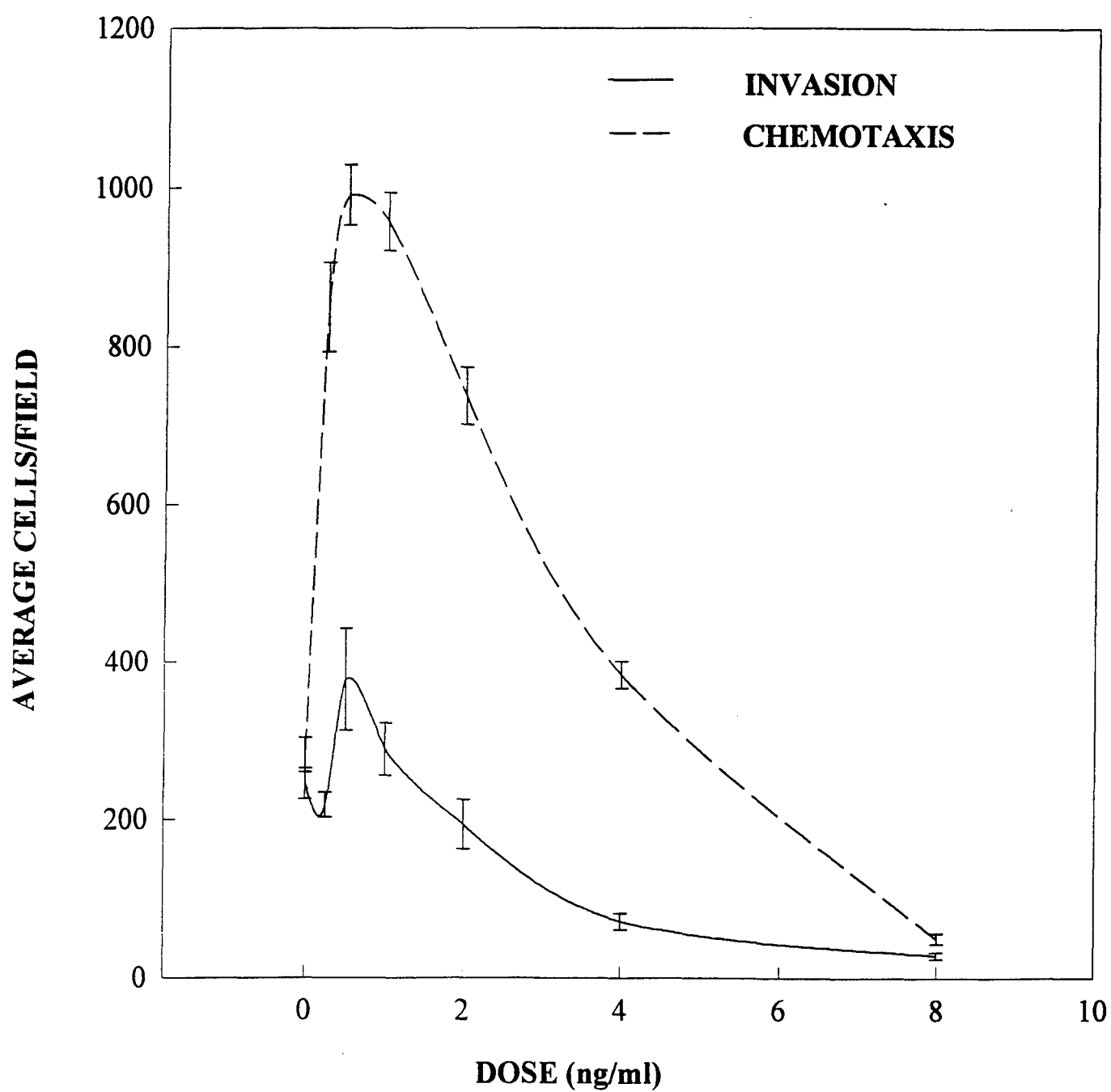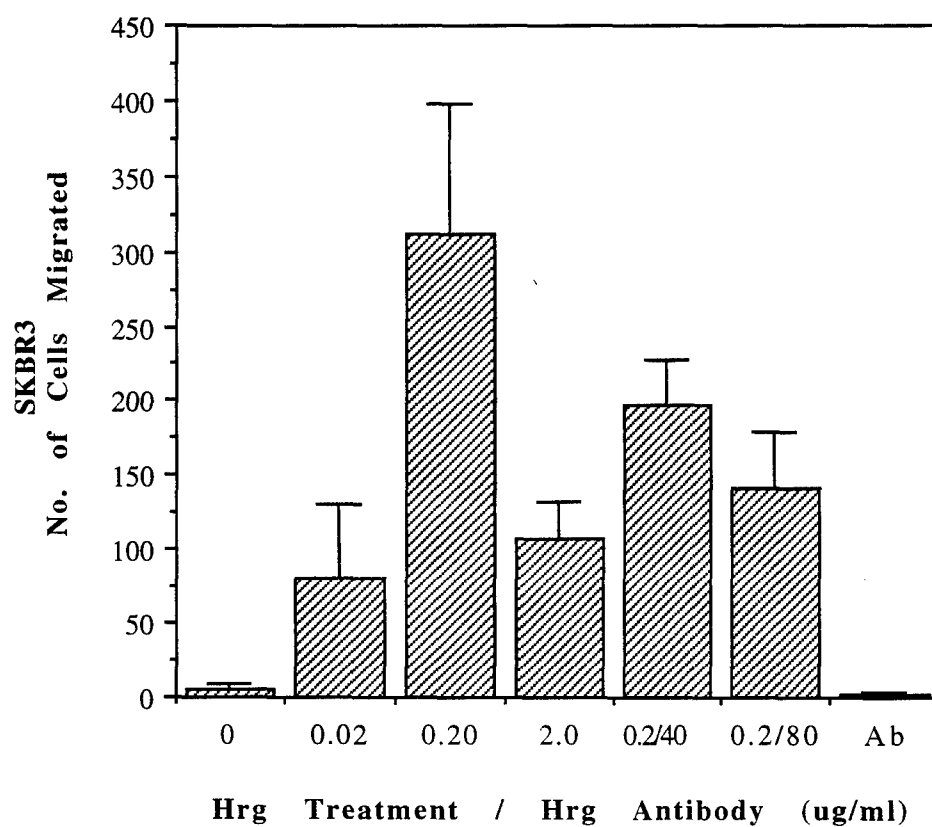60) Mohler, J.L. Cellular motility and prostatic carcinoma metastases. Cancer Metastasis Rev., *12*: 53-67, 1993.
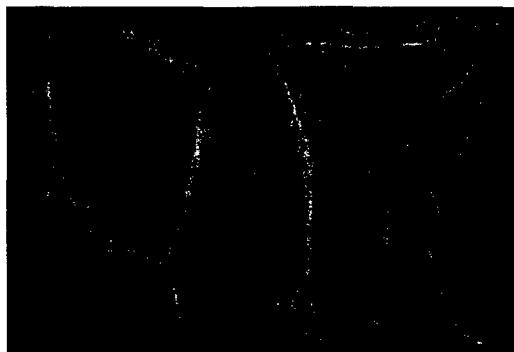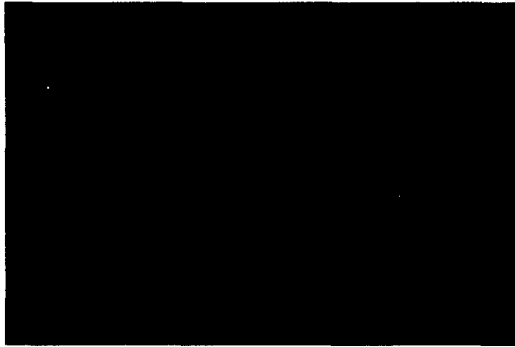
Figure 1:

Figure 2:

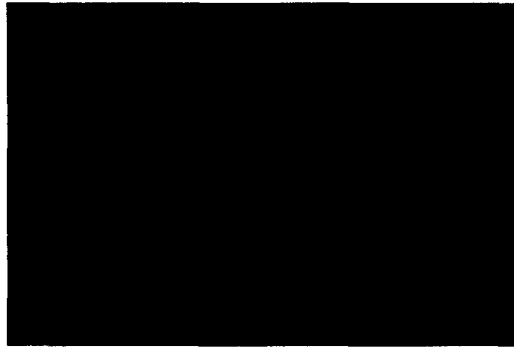Figure 3 a-f:
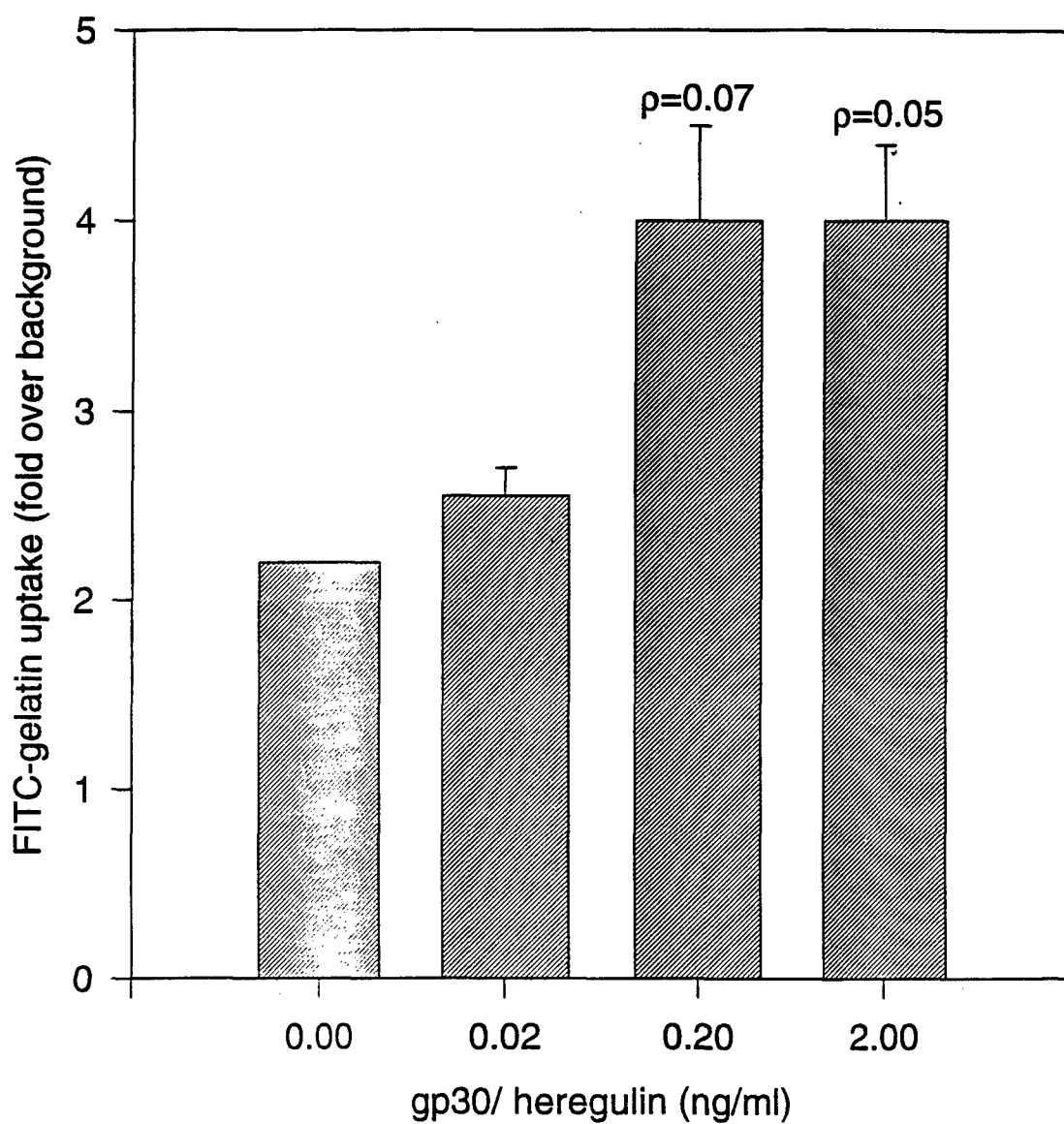
a)

b)

c)

d)

e)

f)

SKBR3

Phagocytosis of proteolyzed FITC-gelatin

error bars=std.dev.
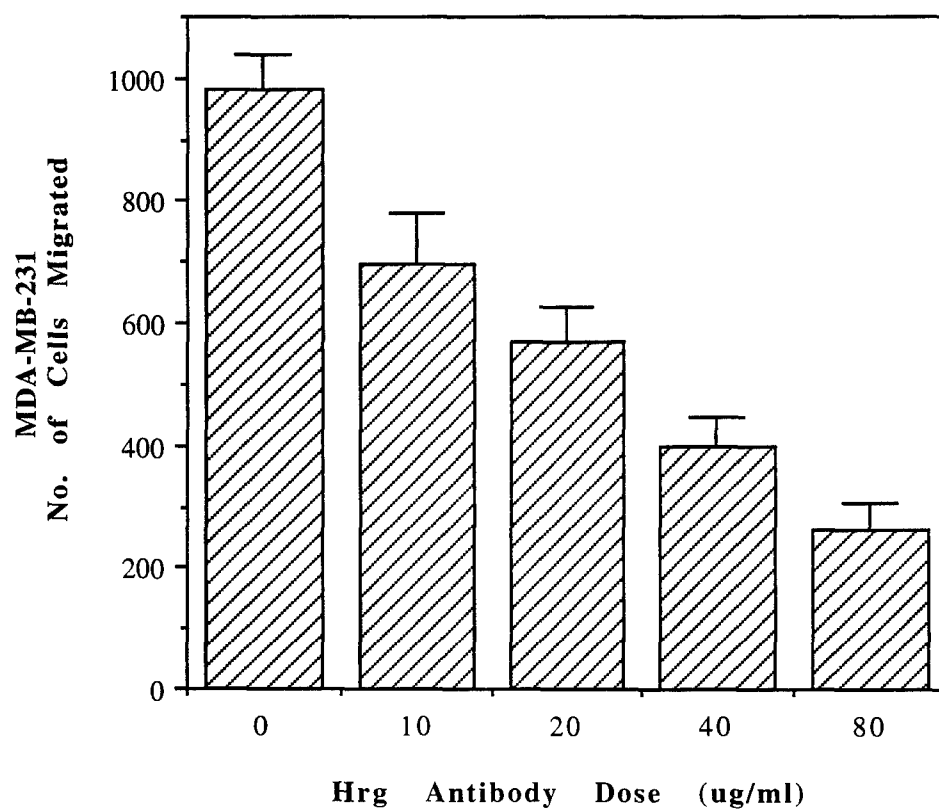HIJAZI1b.spw

Figure 5:
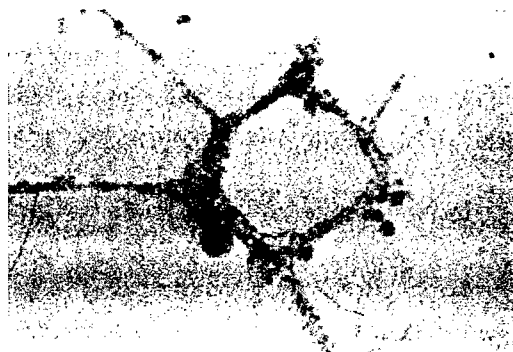
Figure 6 a-d:

a)

b)

c)

d)

Figure Legends:

Figure 1: Chemomigration and chemoinvasion of SKBR-3 cells. Cells were tested in the Boyden chamber assay in the presence of gp30/HRG (0, 0.02, 0.2, 4.0 and 8.0 ng/ml) and chemotaxis and chemoinvasion were measured according to the number of cells traversing gelatin or matrigel-coated filters respectively.

Figure 2: Chemotactic activity of HRGß2 and inhibition by the anti-HRG Ab. SKBR-3 cells were allowed to migrate in the Boyden chamber assay over a 16 h incubation period in response to HRGß2 (0, 0.02, 0.2, 2.0 or 6.0 ng/ml in 0.1% BSA/PBS) added to the bottom wells as a chemoattractant. To test for a specific response to HRG, the anti-HRG Ab (α3) (40 and 80 ug/ml) was added to the bottom wells of the chamber in the presence of HRGß2 at 0.2 ng/ml. Antibody alone had no effect on cell migration.

Figure 3 (a-f): Regulation of the actin cytoskeleton by gp30/HRG. SKBR-3 cells were incubated in the presence or absence of gp30/HRG (0, 0.2, 2.0 and 6.0 ng/ml) for 24 h and stained for F-actin with TRITC phalloidin. Untreated cells (a) show bands of stress fibers at the cell periphery and punctate actin staining within the cell. 0.2 ng/ml gp30/HRG induces extensive membrane ruffling and formation of large pseudopodia accompanied by complete clearing of polymerized actin from the cell center. At 2.0 ng/ml gp30/HRG (c) stress fibers and radiating actin structures start to appear, which becomes more evident at 6.0 ng/ml (d) where actin stress fibers form complex networks rather than even parallel bands. Wortmanin (0.01 uM) alone (e) does not affect actin distribution but inhibits HRG-induced (0.2 ng/ml) ruffling and formation of pseudopodia (f). (Magnification 630x).

Figure 4: FACS analysis of FITC gelatin phagocytosis by SKBR-3 cells stimulated with gp30/HRG (0, 0.02, 0.2 and 2.0 ng/ml). Data (mean values and SEM of duplicates) quantitatively express the overnight uptake of FITC-gelatin by SKBR3 cells.

Figure 5: Inhibition of motility of MDA-MB-231 cells by the anti-HRG Ab (α3). MDA-MB-231 cells were treated in the Boyden chamber assay with the anti-HRG Ab (α3) (0, 20, 40 and 80 ug/ml) or rabbit IgG (80 ug/ml). After a 6 h incubation period the number of migrated cells were counted. Chemomigration was inhibited by 36% at 20 ug/ml, 57% at 40 ug/ml and 84% at 80 ug/ml antibody. Rabbit IgG (80 ug/ml) had no effect on migration (data not shown).

Figure 6 (a-d): Results of HRG neutralizing Ab treatment on MDA-MB-231 morphology and outgrowth. Actin staining of MDA-MB-231 cells show dramatic morphological changes in response to treatment with anti-HRG Ab (α3) (80 ug/ml) by day 5 reflecting an increase in cell size, stress fibers formation and a more fibroblastic appearance (b). Untreated cells (a) are not well adherent and show ruffling at the cell borders. (Magnification 630x). Inhibition of proliferation and outgrowth of MDA-MB-231 cells was also observed in the matrigel growth assay with anti-HRG (80 ug/ml) (d) treatment and compared to cells grown in the presence of rabbit IgG (80 ug/ml) (c).

Project 2

NRG-3 in human breast cancers; activation of multiple erbB family proteins

Mai Hijazi[1]*, Paul E. Young[2]* Michele K. Dougherty1, Dana S. Bressette[2], Tin T. Cao[1], Jacalyn Pierce[3], Ling Mei Wong[3], and Maurizio Alimandi[3], and C. Richter King[1]~

[1] Lombardi Cancer Center, Georgetown University Medical Center, Washington DC

[2] Human Genome Sciences, Inc. Department of Molecular Biology, Rockville MD

[3] Laboratory of Cellular and Molecular Biology, NCI, Bethesda, MD.

* These authors contributed equivalently

~ To whom correspondence should be addressed:

C. Richter King

Room 508, The Research Building, Georgetown University Medical Center, 3970 Reservoir Road, Washington, DC 20007.

ABSTRACT

Ligands of the EGF/Heregulin family control the growth of epithelial cells by binding to receptors of the erbB family. By searching a large database of cDNA sequences at Human Genome Sciences Inc we have identified a new encoded protein sequence containing all the conserved elements of the EGF/Heregulin family. The same sequence has recently been independently identified as NRG-3. The EGF like domain of NRG-3 was generated as a recombinant protein in E. coli and used to test the specificity of receptor binding. In human breast cancer cells and in 32D cells transfected by erbB family members NRG-3 activated multiple erbB family members. These include EGF receptor (erbB1) and erbB4 when expressed individually. ErbB2 and erbB3 were activated by NRG-3 expressed in the same cells. Recombinant NRG-3 EGF-like domain was shown to alter the growth of human breast cancer cells growing *in vitro*. NRG-3 was found to be expressed in cell lines derived from breast cancer. These results indicate that NRG-3 is a potential *in vivo* regulator of normal and malignant breast epithelial cells.

INTRODUCTION

Increased activity of members of the *erb*B family has been implicated in the development of cancer. Different molecular mechanisms of activation have been identified. The ligands of the EGF/Heregulin family are inappropriately expressed in breast cancers (1). EGF, αTGF, Amphiregulin and Heregulin have all been reported to be expressed in breast cancers containing appropriate receptors thus leading to autocrine growth stimulation (2,3). Autocrine growth stimulation is clear in the transformation of NIH/3T3 cells with high levels of EGF receptor. In these cells, full morphological transformation requires the co-expression of αTGF (4).

The causative role of autocrine growth stimulation by αTGF in breast cancer is demonstrated in experiments using transgenic animals where the expression of αTGF acts synergistically to produce frequent breast cancers (5). These findings indicate that the co-incident expression of *erb*B receptor proteins with their ligands can result in aberrant cell growth. Moreover, in 20% of breast cancers amplification of the *erb*B2 gene results in overexpression of p185*erb*B-2 (6,7). Overexpression of p185*erb*B-2 is an oncogenic event in experimental systems (8,9). In these cancers activation of signalling has been thought to be independent of ligand activation. To date no ligand has been isolated that binds only to *erb*B2. However, recent studies show that *erb*B2 can form part of a receptor complex for Heregulin (10-12). Overexpression of the *erb*B1 (EGFR) protein is common,

although gene amplification is infrequent in breast cancer (13).

The *erb*B receptors bind their ligands as dimers formed from two identical *erb*B proteins (homodimers) or from two different proteins (heterodimers) (14,15). EGF can bind homodimers of *erb*B1 (EGFR) or heterodimers of *erb*B1 and *erb*B2 (16,17). Similarly, Heregulin1ß can bind homodimers of *erb*B4 or heterodimers of *erb*B2 and *erb*B3. Other ligands of the EGF/Heregulin family have receptors formed by homo and heterodimers of *erb*B proteins(16,18-20). Ligand binding and dimer formation leads to increased autophosphorylation of the receptor proteins and substrates activating intracellular signalling pathways.

The cellular consequences of receptor stimulation by members of the EGF/Heregulin family vary with the ligand and cellular context. EGF and αTGF can stimulate the growth of many cells in culture, but in cases of breast cancers that overexpress the EGF Receptor, EGF can be growth inhibitory at concentrations above ~10 nM (21,22). In a similar way, Heregulin can both stimulate growth of some human cancer cells as well as inhibit those that overexpress *erb*B2 (23,24). Heregulin clearly has effects on cell morphology as evidenced by changes in the actin cytoskeleton (25). The display of *erb*B proteins on breast cancer cells is not uniform. Many cells lack one or more of the family and others greatly overexpress *erb*B1 or *erb*B2. Heregulin seems to play a number of specialized roles in appropriate regulation of the neuro-muscular junction (26), in neuronal and glial cells (27,28) and in schwann cell development (28). In prenatal development Heregulin and *erb*B2 and *erb*B4 control in morphogenesis of brain and heart (29,30). These findings indicate that members of the EGF/Heregulin family can have

differing effects on cell phenotype.

During the preparation of this manuscript other investigators reported NRG-3, a product of the same gene (31). Our results extend the published information to indicate that NRG-3 binds and activates multiple members of the erbB family of receptors. We demonstrate that NRG-3 is expressed in human breast cancer samples and can alter the growth of human breast cancer cell lines. These results indicate that NRG-3 may have *in vivo* effects on the growth of normal and malignant breast epithelial cells.

RESULTS

NRG-3 contains an EGF like domain.

The ligands of the EGF/Heregulin family have a well-defined sequence similarity which we used to identify NRG-3. Figure 1 shows a compilation of known ligands of the EGF/Heregulin family; all contain 6 cysteines. Between the fourth and sixth cysteine is the common EGF-like folding motif containing a conserved hydrophobic amino acid (Y37) and a conserved glycine (G39). This region apparently forms a very stable core structure that is used in many extracellular proteins. Sequence similarity among the ligands is not limited to this folding motif. There is an exactly conserved arginine (R41) and hydrophobic amino acids at positions 14 and 16. A hydrophobic amino acid that is required for binding activity is found at position 46 or 47. The number of amino acids between cysteines is similar among the ligands with the notable exception of loop B and loop C. Heregulins have a loop C which is three amino acids longer than the EGF-like ligands. The overall sequence similarity among the EGF/Heregulin family members is 19-42% (except between Hrg1α and Hrg1ß which are derived from the same gene). Recently, NRG2 has been identified in rat brain (32,33). NRG2 is most closely related to Hrg1ß with sequence identity of 42% in the EGF like domain.

Using a consensus sequences derived from EGF and Heregulin sequences we screened the HGS database of over 800,000 expressed cDNA sequences. As shown in Figure 1, one cDNA encoded the NRG-3 sequence which has 34-38% similarity to EGF and Heregulin family within the EGF-like domain. Importantly, in the NRG-3

sequence all of the conserved cysteine residues, the R41, and the G39 are exactly conserved. There is additional sequence conservation in NRG-3, notably hydrophobic amino acids at positions 13, 15, 37 and 46-47. The length of the B and C loops are more similar to heregulin than EGF. Within the coding frame defined by our current cDNA clone there is a sequence of hydrophobic amino acids that is consistent with a transmembrane domain C-terminal to the EGF-like domain. This structure is similar to the transmembrane domains found in αTGF EGF, Heregulin and other ligand precursor proteins (not shown in figure 1). The sequence attributes of the NRG-3 cDNA make it a strong candidate as encoding a novel growth factor binding one or several of the *erb*B family of receptors. NGR2 is 36% identical to NRG-3 in the highly conserved EGF-like domain it is extremely likely that they are products of distinct genes. Don-1 has also been recently and independently identified by sequence similarity to EGF/Heregulin (34) and is apparently the product of the same gene as NRG-2.

An independent group of investigators identified NRG-3 using sequence similarity to Heregulin-1 (31) during preparation of this manuscript. That study reports a sequence that is identical in the EGF-like domain but varies near the C-terminus of the intact protein probably due to alternative splicing of the transcript.

Demonstration that NRG-3 activates *erb*B family proteins.

In order to obtain an initial estimation for the action of NRG-3 as a ligand for *erb*B family of receptors we generated recombinant protein in E.coli using a GST fusion system. The EGF-like domain of NRG-3 was released and purified from the

GST by thrombin cleavage. The resulting protein contained a single polypeptide when analyzed by SDS-PAGE.

To test for the ability of recombinant NRG-3 to activate receptors of the *erb*B family we used a tyrosine kinase activation assay. Tyrosine phosphate containing proteins were then identified by immunoblotting using anti-phosphotyrosine antibodies. As shown in figure 2, there is a clear increase in the tyrosine phosphorylation at ~p185 when recombinant NRG-3 is applied to MCF-7 cells. In MCF-7 cells recombinant Heregulin-1ß results in a large increase in tyrosine phosphorylated proteins at about this size. These results indicate that recombinant NRG-3 is able to activate phosphorylation of at least one of the members of the *erb*B family expressed in MCF-7 cells.

NRG-3 Activates multiple *erb*B proteins.

In order to begin the analysis of the NRG-3 receptor we used an experimental system where the display of *erb*B proteins can be controlled. The 32D cell is a murine myeloid cell line which is devoid of expression of genes of the *erb*B family (35). Growth of 32D cells is dependent on the IL-3 present in WEHI conditioned media (35). When expression constructs encoding an *erb*B protein are introduced into 32D cells the resulting cell can survive in the absence of IL-3 if an appropriate EGF/Heregulin family member is present. For example, the introduction of EGF Receptor expression leads to growth of 32D cells in the presence of EGF or αTGF and introduction of *erb*B4 allows growth with Heregulin (15). Similar experimental systems have been used to examine the receptor specificity of the newly discovered

NRG-2 (36). Figure 3 shows that growth of 32D cells in the presence of NRG-3 occurs only when EGF Receptor or *erb*B4 are present singly or when *erb*B2 and *erb*B3 are present in combination. The expression of *erb*B2 or *erb*B3 alone does not lead to NRG-3 induced growth. To confirm that this growth stimulation was the result of receptor activation we determined whether NRG-3 induces the tyrosine phosphorylation of EGF Receptor, or *erb*B2 or *erb*B3 when expressed together. Figure 4 shows the appearance of an appropriate sized band when cell lysates of these 32D cells are probed by antiphosphotyrosine antibodies. These results are strong evidence that NRG-3 can activate *erb*B1 homodimers (the EGF receptor), and *erb*B2 + *erb*B3 heterodimers. Our results using 32D cells confirm the previously reported finding (31) that NRG-3 can activate erbB4 homodimers.

The results of 32D experiments indicate that the receptor binding pattern of NRG-3 is complex. In order to test if NRG-3 activates proteins other than erbB4 in MCF-7 cells we determined the phosphorylation of erbB3. Following stimulation by NRG-3, *erb*B3 was immunoprecipitated the level of tyrosine phosphorylation on *erb*B3 determined by immunoblot using antiphosphotyrosine antibodies. As shown in figure 5, phosphorylation on tyrosine is clearly a consequence of NRG-3 stimulation.

Biological Activity of NRG-3.

The effects of the EGF/Heregulin family vary significantly. Differing cellular phenotypes can be induced by different ligands in the same cell system and the same ligand can cause differing effects among different cells. Mitogenic activity of

NRG-3 has been detected in 32D cell experiments. Growth stimulation of MCF-7 cells is observed in the breast cancer cell line MCF-7. The MCF-7 cell is dependent on estrogens in the media either in the form of phenol red or present in the fetal bovine serum. Little proliferation is seen in phenol red free media containing serum treated with charcoal to remove steroids. Heregulin is able to promote growth in the absence of estrogen. As shown in figure 6, when NRG-3 is added there is also a clear growth stimulation. Growth inhibitory effects have also been observed. NRG-3 inhibits the growth of the breast cancer cell line MDA-MB-468. These cells overexpress the EGF receptor and can be stimulated by EGF at low concentrations (1 ng/ml) and growth inhibited at higher concentrations (>10 ng/ml). As shown in figure 7, NRG-3 can inhibit growth of these cells at concentrations that are active in producing growth stimulation of 32D cells containing EGF Receptor. No growth suppression or stimulation are seen when NRG-3 is applied to MCF-7 cells when they are grown in media containing agonists for the estrogen receptor.

NRG-3 mRNA expression in breast cancer.

Detection of NRG-3 mRNA by northern blotting methods showed a weak signal for NRG-3 mRNA in adult brain with a size of approximately 2 KD (data not shown). Similar northern blot results are reported in the recent NRG-3 study (31). We have extended this analysis using RT-PCR to detect NRG-3 mRNA. RT-PCR experiments employed two primer sets. The two primer sets generated concordant results. In addition all assays included control reactions lacking reverse

transcriptase in order to detect the presence of contaminating DNA. The results of a representative RT-PCR assay is shown in figure 8. The observed band at 340 bp corresponds to the predicted size based on the NRG-3 cDNA. It was cloned sequenced and shown to contain NRG-3 coding information. The bands shown at 500 bp and 120 bp. were also subjected to sequence analysis. These do not contain NRG-3 coding information and thus likely represent miss priming by the RT-PCR oligonucleotides on unrelated mRNAs. These results are strong evidence that NRG-3 is expressed in at least some human breast cancer cell lines. We also confirm (31) expression of NRG-3 mRNA in human brain tissue(figure 8).

DISCUSSION

The results reported here confirm and extend the results of the recent neuregulin-3 study (31) concerning the receptor specificity of NRG-3. In that study a NRG-3 EGF-like domain fused to an immunoglobulin Fc domain. The resulting recombinant protein was shown to bind and activate tyrosine kinase phosphorylation of *erb*B4. No binding was observed for *erb*B1, *erb*B2, or *erb*B3 when each was expressed alone. Studies on the activation of tyrosine phosphorylation of these proteins was not reported nor were mitogenic assays conducted. Activity of recombinant NRG-3 on heterodimer combinations of *erb*Bs was also not investigated. Our results suggest that NRG-3 can bind and activate *erb*B1 and heterodimers of *erb*B2 + *erb*B3 in addition to activating *erb*B4. Taken together the available data suggests that the precise receptor binding and activation profile of NRG-3 is complex. This conclusion is also suggested in the of the NRG-3 publication (31). Our results demonstrate that *erb*B3 can be phosphorylated as a consequence of NRG-3 binding (figure 5). The NRG-3 induced increases in tyrosine phosphorylation on *erb*B3 suggests that the *erb*B3 protein can be part of an NRG-3 receptor. Our studies of 32D cells supports this conclusion where *erb*B2 is the other member of the heterodimeric receptor with *erb*B3.   Still to be determined is whether NRG-3 can bind to *erb*B1 + *erb*B3 heterodimers or *erb*B3 + *erb*B4 heterodimers or *erb*B2 + *erb*B4 heterodimers. Our preliminary data does conclusively demonstrate that NRG-3 is a new ligand for the *erb*B family of receptors. These results suggest that NRG-3 may have a receptor specificity

somewhat analogous to ß-Cellulin (20,37).

In adult tissue, expression levels of NRG-3 are low but detectable using sensitive methods such as RT-PCR. NRG-3 is expressed at the highest levels in the brain where it is likely to play a critical role in the morphogenesis (29-31). Interestingly, we identify NRG-3 expression a breast cancer cell line, MCF-7, that clearly have receptors that can be activated by HLGF. Our results also show that NRG-3 can cause alteration of growth of MCF-7 cancer cells *in vitro*. The ability to cause growth of MCF-7 cells in the absence of estrogen is similar to that previously reported for Heregulin (31). Our results suggest that effects on cell phenotype by HLGF may depend on the cell line. MDA-MB-468 which has high levels of EGFR are growth inhibited by NRG-3 *in vitro.*

The results in this paper together with those recently reported earlier (31) identify NRG-3 as a new ligand for the erbB family of growth factor receptors and suggest a role for NRG-3 in the growth regulation of normal and malignant breast epithelial cells.

MATERIALS AND METHODS

Preparation of Recombinant NRG-3 The coding segment containing the EGF like domain of NRG-3 (nucleotide 79 to 279 of HGS38) were amplified by PCR and inserted into the pGEX3 plasmid for expression as a fusion protein with bacterial glutathione S transferase. Protein was prepared using standard methods. Bacterial were cultured to an OD_{600} of ~0.4 and induce to express recombinant protein by addition of 0.1 mM IPTG. Bacteria were collected by centrifugation resuspended in 1X PBS and lysed by sonication. Recombinant protein was collected by incubation with glutathione beads. After washing, the recombinant NRG-3 protein was cleaved from the GST bound to the beads by thrombin cleavage for 18 hours. Thrombin was removed by incubation with p-Aminobenzamidine agarose beads. Refolding followed the methods used for the refolding of recombinant antibody fragments (38). Briefly, recombinant NRG-3 was denatured in 6M guanidine HCL containing 65mM DTE. This was rapidly diluted 100 fold to a final protein concentration of 100 ug/ml into 0.4 M Arginine 0.1M Tris pH 8.0, 0.9mM oxidized glutathione 2.0 mM EDTA. Refolding was allowed to proceed for 24 hours at 4 degrees C. Refolded protein was extensively dialyzed against PBS using 3000 kDa cutoff membranes. Protein preparations were stored at -20 C.

Detection of receptor activation by phosphotyrosine immunoblot. Cells were starved (24 hours for MCF-7, 4 hours for 32D derived cell lines) before addition of the indicated amounts of growth factors. Total cell lysates were prepared by addition of SDS PAGE sample buffer (1% SDS, 0.15M Tris pH 8.6, 5% BME and 1mM Sodium OrthoVanadate) directly to cells. Cell lysates and were run on 8-16% Tris-Glycine gradient gels (Novex). Proteins were transferred onto Hybond ECL nitrocellulose membranes (Amersham) and were immunoblotted with anti-phosphotyrosine MAb (Upstate Biochemicals Inc.)

32D cell experiments. 32D cells containing expression constructs for erbB1, erbB2, erbB3, erbB4 and erbB2 and erbB3 together were grown in IL3 containing (WEHI conditioned media) or Hrg-1β prior to the experiment. Expression of the erbB proteins was verified by FACS analysis using erbB specific antisera. Cells (10^4 per well)were plated in 24 well dishes in the absence of IL3 containing media (DMEM, 10% FCS) or in the presence of the indicated growth factors, Heregulin 1β 100 ng/ml, EGF 100ng/ml, and NRG-3 10ug/ml. Cells were allowed to grow for 3 days and viable cells counted using a hemocytometer.

Immunoprecipitation and Immunoblot of erbB3. Cells were plated in 80 cm^2 dishes (DMEM + 10% FCS) for until 80 % confluent. Cells were then allowed to become quiescent in serum free media (DMEM) for 24 hours. Cells were then stimulated with the indicated growth factors, Heregulin 1β 1 ug/ml, and NRG-3 10ug/ml for 15 minutes. Cells were lysed in 1% Triton X-100 in PBS containing 1 mM Sodium orthovanadate. Nuclei were removed by centrifugation. erbB3 proteins were immunoprecipitated (2 hours at 4^0 C) using monoclonal anti-erbB3 antibodies (Neomarkers) and collected on protein A sepharose. Proteins were released by incubation in 1% SDS containing PAGE sample buffer at 100^0C and electrophoresed on 8-16% gels (Novagen). Proteins were transferred to nitrocellulose. Proteins containing pTyr were detected using monoclonal anti-phosphotyrosine antibodies (Oncogene Science) and the ECL detection system (Amersham).

Growth Assays. MDA-MB-468 cells were plated in IMEM+ 10% FBS at 3000 cells per well in 96 well dishes. Cells were allowed to become quiescent in serum free IMEM for 24 hours and growth factors EGF 2 ng/ml and NRG-3 10 ug/ml were added to the media. Growth of cells at 1, 3, and 5 days was monitored using the XTT assay method. XTT was added at 10 ug/ml in IMEM and PMS (1.5 mg/ml in PBS) to 25% of volume of well for 4 hours at 37^0C. OD monitored at

540 nm. MCF-7 cells were plated in IMEM (containing no phenyl red) + 5% charcoal stripped calf serum (CCS) at 3000 cells per well in 96 well dishes. Cells were allowed to become quiescent in serum free IMEM for 24 hours and growth factors Hrg1 2 ng/ml and NRG-3 10 ug/ml were added to the media. Growth of cells at 1, 3, and 5 days was monitored using the XTT assay method. XTT was added at 10 ug/ml in IMEM and PMS (1.5 mg/ml in PBS) to 25% of volume of well for 4 hours at 37^{0}C. OD monitored at 540 nm.

Detection of NRG-3 mRNA. Total RNA was extracted from cultured cells using the RNazol B method (Tel-Test, CS-104). The final RNA pellet was resuspended in 135 ul DEPC treated H2O. DNase treatment was performed using the SNAP RNA isolation kit (Invitrogen, K1950-01). Briefly, 10X DNase buffer and RNase free DNaseI was added to each sample and incubated for 20' at 37o. RNA purification was performed as indicated in the kit. Concentration of each sample was determined, samples were dried and resuspended to give a final concentration of 2 ug/ul.

RT-PCR was performed using 2 ug of total RNA in the Gene Amp RNA PCR Core kit (Perkin Elmer, N808-0143). cDNA was synthesized using the downstream primer 5'-CCACGATGACAATTCCAAAG-3'. Samples were reverse transcribed 1h at 37o. RT was heat inactivated 5' at 99o, samples were cooled on ice. PCR was performed with the entire RT reaction using the upstream primer 5'-TACCACCACCACACCAGAAA-3'. The reaction was performed for 40 cycles, 1' at 94^{0}C, 1' 30s at 58^{0}C, 2' at 72^{0}C, followed by an extension for 8' at 72^{0}C. Samples were electrophoresed on an agarose gel and visualized with ethidium bromide staining.

Confirmation of the sequence of the bands was performed by purifying the bands from agarose gel slices (Wizard PCR preps DNA purification system, Promega, A7170) and cloning into a TA vector (Invitrogen, K2000-J10) for automated sequencing. Bands of unknown identity present in the reaction products were cloned and sequenced in a similar fashion.

FIGURE LEGENDS

Figure 1: The amino acid sequences of the EGF/ heregulin family of growth factors and of the NRG-3 novel sequences. Cysteines (**C**) defining the basic structure of the EGF domain and highly conserved amino acids are in bold. Listed are sequences for the EGF like domains of Transforming Growth Factor alpha, Amphigregulin, Epidermal Growth Factor, Heparin-Binding EGF, Beta Cellulin, human Heregulin 1-alpha and 1-beta, Heregulin Related Gene 2 and the novel gene described in this study and (39) NRG-3.

Figure 2   Demonstration of Biochemical Activity of a Recombinant NRG-3.
MCF-7 cells were treated with recombinant NRG-3 (10 ug/ml) or recombinant full length Hrg1ß (0.1 ug/ml) for 30 minutes. Cells were lysed and proteins separated by SDS-PAGE. Proteins phosphorylated on tyrosine detected using immuno-blotting and anti-phosphotyrosine antibodies.

Figure 3 NRG-3 is mitogenic for 32D cells with expressed erbB proteins.
The indicated cells were plated in media containing IL-3 (WEHI conditioned media) and then changed to media containing no IL-3 with the indicated erbB ligands. At day 5, cells were counted visually and the average of 4 determinations shown.

Figure 4 NRG-3 is stimulates tyrosine phosphorylation of erbB proteins in 32D cells. Cells (10^5 per well)were plated in 24 well dishes in the absence of IL3 containing media (DMEM) for 4 hours prior to incubation with the indicated growth factors, Heregulin 1β 100 ng/ml, EGF 100ng/ml, and NRG-3 10ug/ml for 15 minutes. Cells were collected by

centrifugation, lysed and separated by SDS-PAGE and proteins containing pTyr were detected using immunoblotting and monoclonal anti-phosphotyrosine antibodies. (UBI).

Figure 5 NRG-3 is stimulates tyrosine phosphorylation of erbB3 proteins in MCF-7 cells. MCF-7 cells were treated with NRG-3 (10ug/ml) for 15 minutes and lysed in PBS 1% Triton. erbB3 protein was immunoprecipitated and pTyr containing proteins identified by SDS-PAGE and anti-phosphotyrosine immunoblotting. The amount of erbB3 protein in each immunoprecipitation was determined by immunoblot anti-erbB3 antibodies (Neomarkers Inc.)

Figure 7 NRG-3 can inhibit growth of human breast cancer cells (MDA-MB-468). MCF-7 cells were grown in media containing charcoal stripped calf serum; phenol red free media and the indicated growth factors. The extent of cell growth was monitored at the indicated days using the XTT method.

Figure 7 NRG-3 can inhibit growth of human breast cancer cells (MDA-MB-468). MDA-MB-468 cells were grown in media containing FBS, Fetal Bovine Serum and the indicated growth factors. The extent of cell growth was monitored at the indicated days using the XTT method.

Figure 8 Detection of NRG-3 transcripts in brain and cell lines derived from breast cancer. RNA was prepared using RNAzol (Tel-Test Inc.). RT-PCR was conducted using standard procedures (RNA Gene AMP, Perkin-Elmer Inc.) The oligonucleotides were synthesized based on the HGS38 cDNA sequence and predict a 340 nucleotide amplification product. Lanes marked RT - were conducted identically to RT-PCR except that reverse

transcriptase was not added to the reaction. These lanes indicate the RNA dependence of the PCR reaction, suggesting that the bands observed are derived from NRG-3 mRNA and not from DNA contamination.

References

1. Narmanno, N. and Ciardiello, F. (1997) *J. Mammary Gland Biology and Neoplasia* **2**, 143-151

2. Bates, S. E., Davidson, N. E., Valverius, E. M., Freter, C. E., Dickson, R. B., Tam, J. P., Kudlow, J. E., Lippman, M. E., and Salomon, D. S. (1988) *Mol. Endocrinol.* **2**, 543-555

3. Normanno, N., Kim, N., Wen, D., Smith, K. ., Harris, A. L., Plowman, G. D., Colletta, G., Ciardiello, F., and Salomon, D. S. (1997) *Breast Cancer Research and Treatment* **35**, 293-297

4. Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. (1987) *Science* **237**, 178-182

5. Schroeder, J. A. and Lee, D. C. (1997) *J. Mammary Gland Biology and Neoplasia* **2**, 119-130

6. Slamon, D. J. (1990) *Cancer Invest.* **8**, 253

7. De Potter, C. R. and Schelfhout, A. M. (1995) *Virchows Arch.* **426**, 107-115

8. Di Fiore, P. P., Pierce, J. H., Fleming, T. P., Hazan, R., Ullrich, A., King, C. R., Schlessinger, J., and Aaronson, S. A. (1987) *Cell* **51**, 1063-1070

9. Hudziak, R. M., Schlessinger, J., and Ullrich, A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7159-7163

10. Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D., and Yarden, Y. (1993) *EMBO J.* **12**, 961-971

11. Marte, B. M., Jeschke, M., Graus-Porta, D., Taverna, D., Hofer, P., Groner, B., Yarden, Y., and Hynes, N. E. (1995) *Mol. Endocrinol.* **9**, 14-23

12. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L. (1994) *J. Biol. Chem.* **269**, 14661-14665

13. Fox, S. B. and Harris, A. L. (1997) *J. Mammary Gland Biology and Neoplasia* **2**, 131-142

14. Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996) *Mol. Cell Biol.* **16**, 5276-5287

15. Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M., and Yarden, Y. (1996) *EMBO J.* **15**, 2452-2467

16. King, C. R., Borrello, I., Bellot, F., Comoglio, P., and Schlessinger, J. (1988) *EMBO J.* **7**, 1647-1651

17. Stern, D. F. and Kamps, M. P. (1988) *EMBO J.* **7**, 995-1001

18. Riese, D. J., Kim, E. D., Elenius, K., Buckley, S., Klagsbrun, M., Plowman, G. D., and Stern, D. F. (1996) *J. Biol. Chem.* **271**, 20047-20052

19. Jones, F. E., Jerry, D. J., Guarino, B. C., Andrews, G. C., and Stern, D. F. (1996) *Cell Growth. Differ.* **7**, 1031-1038

20. Riese, D. J., Bermingham, Y., van Raaij, T. M., Buckley, S., Plowman, G. D., and Stern, D. F. (1996) *Oncogene.* **12**, 345-353

21. Filmus, J., Trent, J. M., Pollak, M. N., and Buick, R. N. (1987) *Mol. Cell Biol.* **7**, 251-257

22. Armstrong, D. K., Kaufmann, S. H., Ottaviano, Y. L., Furuya, Y., Buckley, J. A., Isaacs, J. T., and Davidson, N. E. (1994) *Cancer Res.* **54**, 5280-5283

23. Xu, F-J, Stack, S., Boyer, C., O'Briant, K., Whitaker, R., Mills, G. B., Yu, Y. H., and Bast, R. c. (1997) *Clin. Cancer. Res.* **3**, 1629-34

24. Staebler, A., Sommers, C., Mueller, S. C., Byers, S., Thompson, E. W., and Lupu, R. (1994) *Breast. Cancer Res. Treat.* **31**, 175-182

25. Hijazi, M. M., Thompson, E. W., Tang, C., Coopman, P., Torri, J. A., Yang, D., Mueller, S. C., and Lupu, R. (1997) *Submitted*

26. Sandrock, A. W.,Jr., Dryer, S. E., Rosen, K. M., Gozani, S. N., Kramer, R.,

Theill, L. E., and Fischbach, G. D. (1997) *Science* **276**, 599-603

27. Subramony, P. and Dryer, S. E. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 5934-5938

28. Syroid, D. E., Maycox, P. R., Burrola, P. G., Liu, N., Wen, D., Lee, K. F., Lemke, G., and Kilpatrick, T. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 9229-9234

29. Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R., and Lemke, G. (1995) *Nature* **378**, 390-394

30. Meyer, D. and Birchmeier, C. (1995) *Nature* **378**, 386-390

31. Zhang, D., Sliwkowski, M. X., Mark, M., Frantz, G., Akita, R., Sun, Y., Hillan, K., Crowley, C., Brush, J., and Godowski, F. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 9562-9567

32. Chang, H., Riese, D. J., Gilbert, W., Stern, D. F., and McMahan, U. J. (1997) *Nature* **387**, 509-511

33. Carraway, K. L., Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Gassmann, M., and Lai, C. (1997) *Nature* **387**, 512516

34. Busfield, S. J., Michnick, D. A., Chickering, T. W., Revett, T. L., Ma, J., Woolf, E. A., Comrack, C. A., Dussault, B. J., Woolf, J., Goodearl, A. D., and Gearing, D. P. (1997) *Mol. Cell Biol.* **17**, 4007-4014

35. Pierce, J. H., Ruggiero, M., Fleming, T. P., Di Fiore, P. P., Greenberger, J. S., Varticovski, L., Schlessinger, J., Rovera, G., and Aaronson, S. A. (1988) *Science* **239**, 628-631

36. Carraway, K. L., Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Gassmann, M., and Lai, C. (1997) *Nature* **387**, 512-516

37. Alimandi, M., Wang, L-M., Bottaro, D., Lee, C-C., Kuo, A., Frankel, M., Fedi, P., Tang, C., Lippman, M., and Pierce, J. H. (1997) *EMBO J.* **in press**,

38. Buchner, J., Pastan, I., and Brinkmann, U. (1992) *Anal. Biochem.* **205**, 263-270

39. Smyth, M. S., Stefanova, I., Hartmann, F., Horak, I. D., Osherov, N., Levitzki, A., and Burke, T. R.,Jr. (1993) *J. Med. Chem.* **36**, 3010-3014

Amino Acid Sequences of EGF Binding Domains

```
                      6        14  20                 31          42
>αTGF      SHFNDCPDSHTQFCFHG-TCRFLVQEDKP---ACVCHSGYVGARCEHADLLA
>EGF       RNSDSECPLSHDGYCLHDGVCMYIEALDKY---ACNCVVGYIGERCQYRDLKW
>HB-EGF    GKKRDPCLRKYKDFCIHG-ECKYVKELRÄP---SCICHPGYGGERCHGLSLP
>Amph      RKKKNPCNAEFQNFCIHG-ECKYIEHLEAV---TCKCQQEYFGERCGEKSMKT
>βCell     KGHFSRCPKQYKHYCIKG-RCRFVVAEQTP---SCVCDEGYIGARCERVDLFY


>Hrgα1     TSHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCQPGFTGARCTENVPMK

>Hrgβ1     TSHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCPNEFTGDRCQNYVMAS

>HRG-2     SGHARKCNETAKSYCVNGGVCYYIEGINQLS---CKCPVGYTGDRCQQFAMVN

>NRG-3     SEHFKPCRDKDLAYCLNDGECFVIETLTGSHK-HCRCKEGYQGVRCDQFLPKT
```

Figure 1

Figure 2

47

Expressed erbB proteins

F   i   g   u   r   e

Cells Treated with

p^{170} —

EGFR

NRG-3

EGF

—

NRG-3

Heregulin

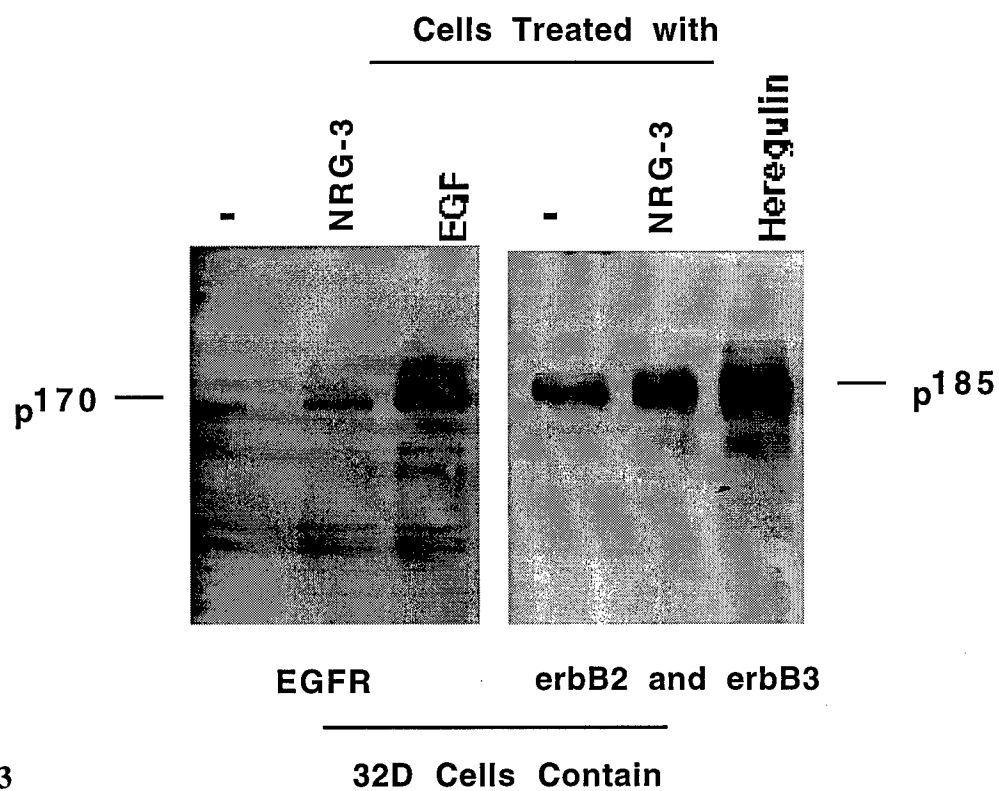— p^{185}

EGFR   erbB2 and erbB3

3   **32D Cells Contain**
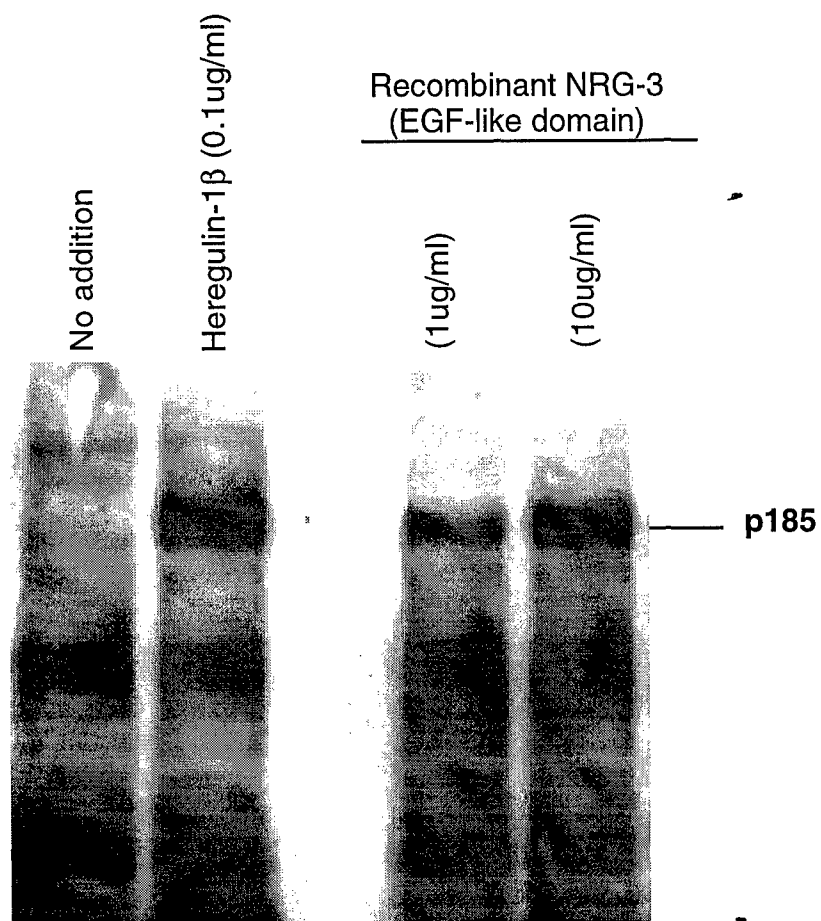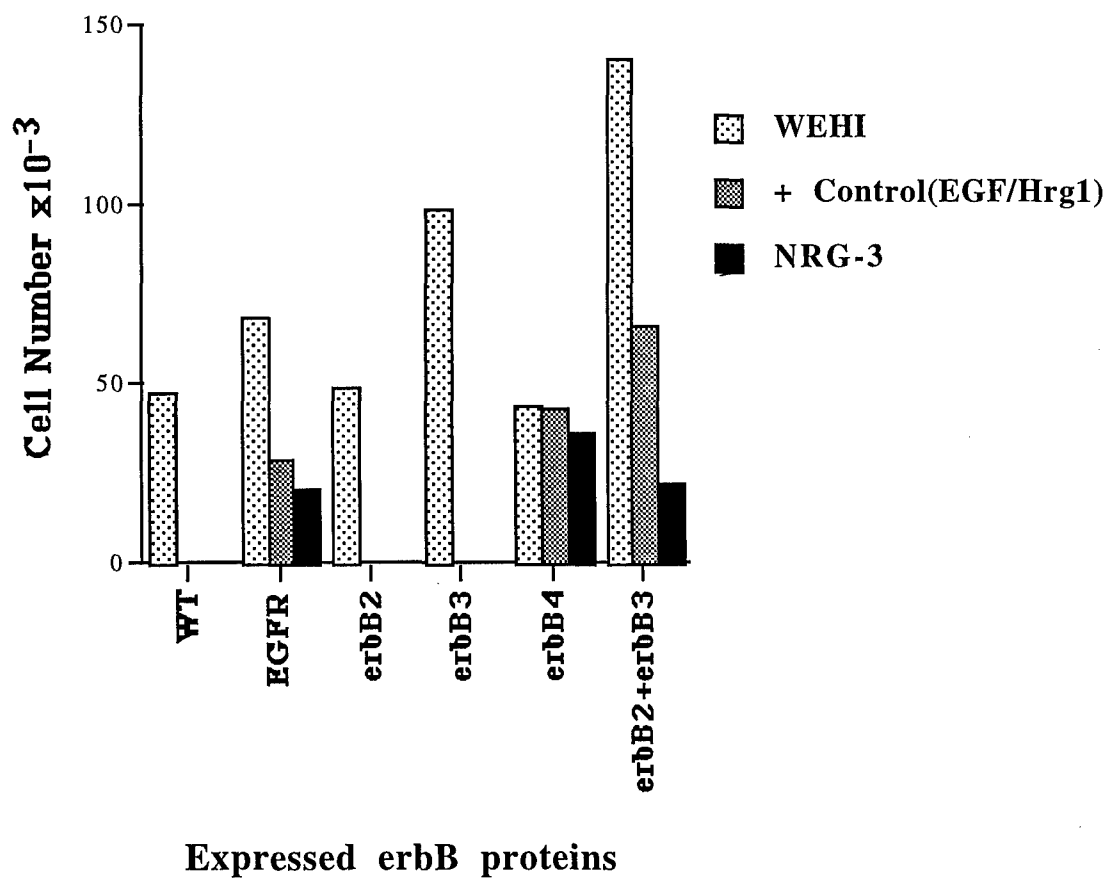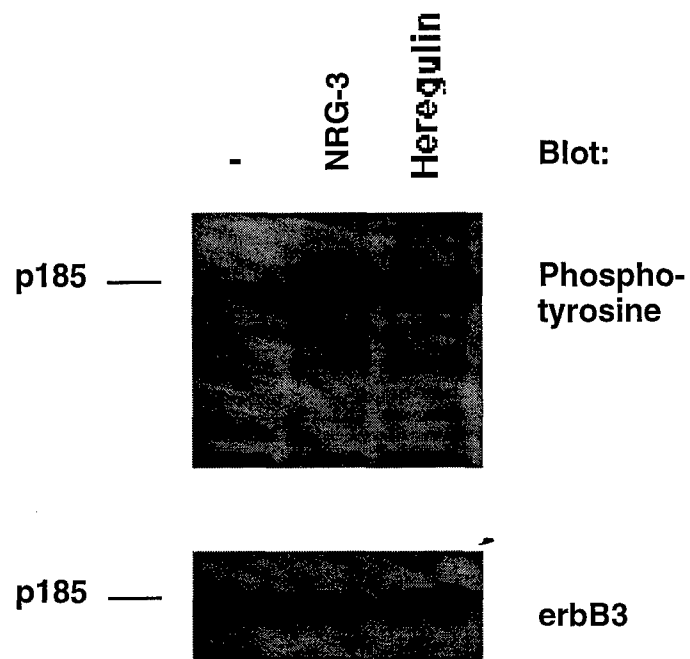
Figure 4

Figure 5

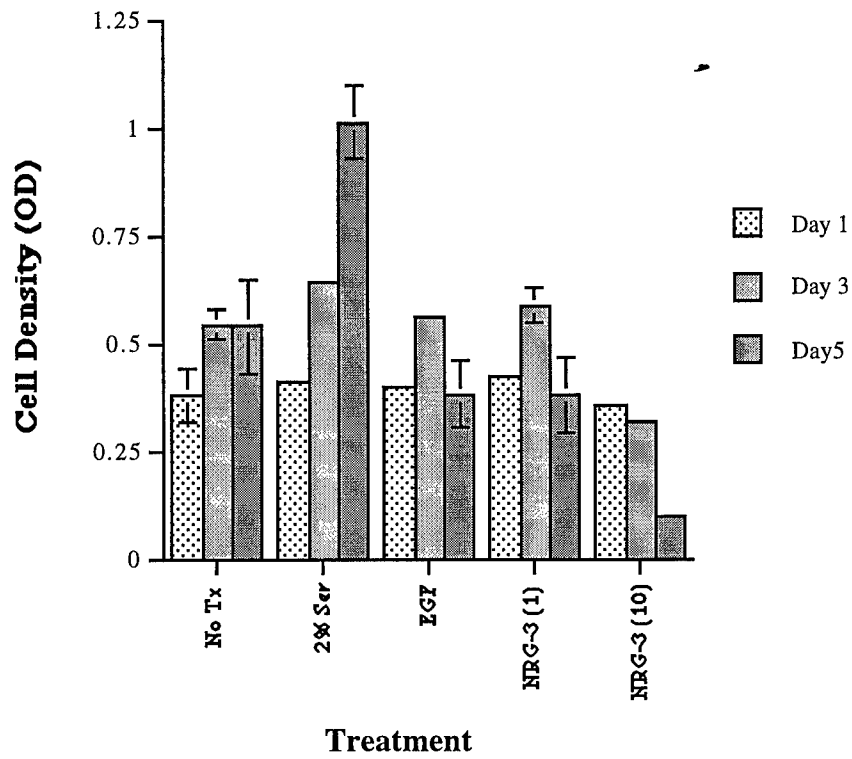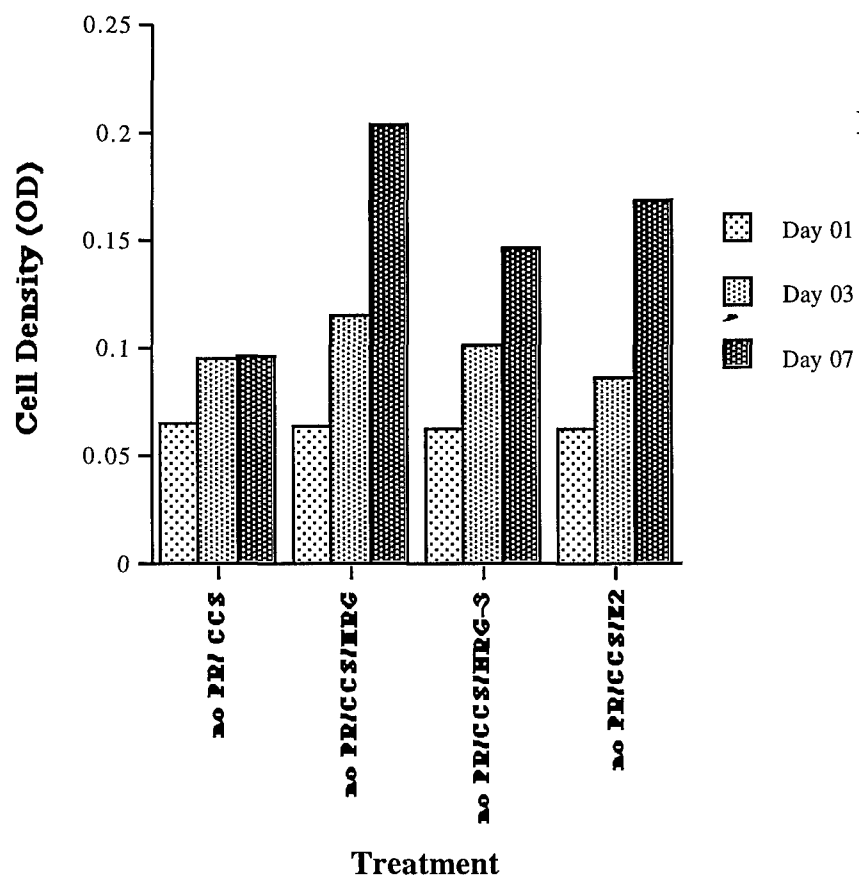## Growth of MDAMB-468 in NRG-3
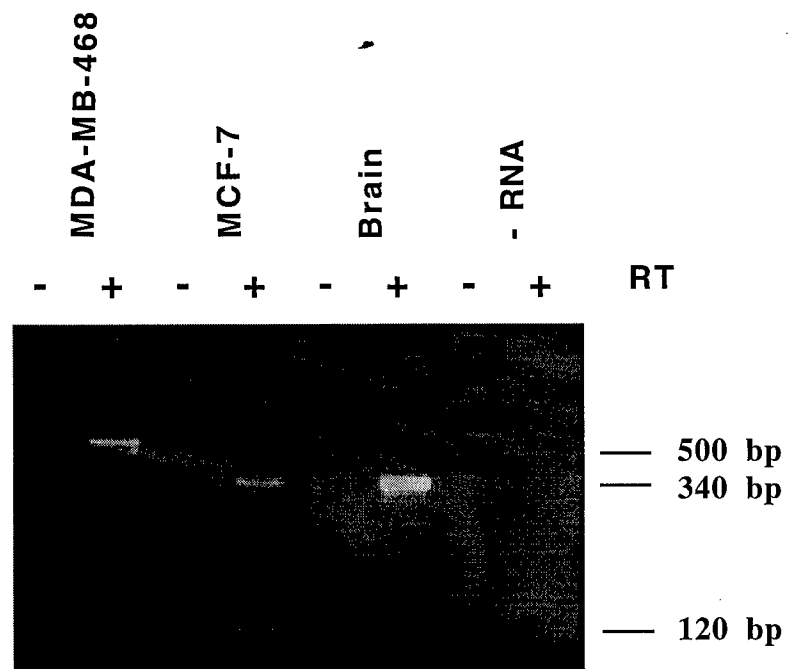

Figure 6

Figure 7

52

Figure 8

PROJECT 3: Functions of cdc42, rac and rho in growth factor siganlling in breast cancer.

Report for Period August 9, 1996-April 1997

Due to administrative issues this grant DAMD17-94-J-4169 was reassigned to our laboratory on August 9, 1996. The aims and work scope of the proposal were updated as described on the attached sheets. Progress on the updated specific aims has necessarily been limited due to the short period of activity in this setting. We have emphasized the establishment of the experimental systems that are proposed. Important elements of this progress are:

- Fibroblasts (NIH/3T3) that contain a chimeric human protein derived from the extracellular domain of EGFR fused to the intracellular tyrosine kinase of p185^{erbB-2} have been obtained and tested. The activation of the tyrosine kinase is comparable to a similar cell containing the intact human EGFR.

- Cytoskeletal changes in response to EGF and PDGF have been characterized for fibroblasts and breast cancer cells. This has involved careful optimization of the preparation and analysis of cells using phalloidin staining. In the fibroblasts, PDGF is a potent stimulator of membrane ruffling with EGFR while erbB-2 kinase activation is less potent.

- MCF-7 and SKBR-3 cells have been tested for the induction of the type of cytoskeletal changes associated with rho/rac/cdc42 activation. MCF-7 shows pronounced stimulation of pseudopodia in response to heregullin. SKBR-3 shows a strong response to both EGF and Heregullin.

- The NIH/3T3 fibroblasts containing the EGFR and the EGFR/p185^{erbB-2} chimera have been successfully micro-injected with rabbit IgG to label cells. BrdU staining assays have been tested with an increase in labelling observed in the presence of EGF.

- Micro-injection of normal rac into breast cancer cells has been shown to induce ruffling. This indicates that the cell responds to this stimulus and that the micro-injection of appropriate DNAs can be conducted in this laboratory.

- Dominant negative expression constructs of the small G proteins listed above have been obtained and prepared for micro-injection.

This project was inactivated as exciting findings were being obtained in project 2.

Publications:

Submitted

Mai Hijazi, Paul E. Young Michele K. Dougherty, Dana S. Bressette, Tin T. Cao, Jacalyn Pierce, Ling Mei Wong, and Maurizio Alimandi, and C. Richter King NRG-3 in human breast cancers; activation of multiple erbB family proteins

In preparation
Hijazi M.M., Mueller S.C., Coopman P., Torri J., Thompson E. and Lupu R. Heregulin regulates the actin cytoskeleton and promotes invasive properties in breast cancer cells. Manuscript to be submitted to Cancer Research.

Published Papers:
Lupu R., Cardillo M., Harris L.N., Hijazi M. M. and Rosenberg K. Interaction between erbB-receptors and heregulin in breast cancer tumor progression and drug resistance. Seminars in Cancer Biology, Vol 6, 135-145, 1995.

Lupu R., Cardillo M., Harris L., Hijazi M. M., Rosenberg K., Tang C. Thesignificance of heregulin in breast cancer tumor progression and drug resistance.Breast Cancer Research and Treatment, 38: 57-66, 1996.

Abstracts:
Hijazi M.M., King C.R. et al. Inhibition of Grb2 function and EGF induce mitogenesis by micro-injection of non-phosphorylated smallpeptides. AmericanAssociation for Cancer Research, Meeting on Basic and Clinical Aspects ofBreast Cancer, January 1997.

Hijazi M.M., Lupu R. Heregulin/gp30 polyclonal antibody inhibits invasive behavior in MB-MDA 231 breast cancer cells. American Association for Cancer Research, Vol 36, 445, 1995.

Hijazi M.M., Mueller S.C., Coopman P., Torri J., Lupu R., Thompson E.W.

Gp30/heregulin promotes invasive behavior and phenotype in SKBR3 cells and is blocked by gp30 polyclonal antibody. Clinical & Experimental Metastasis, Vol 12, 69, 1994.

C. Richter King*, Mai Hijazi*, Michele Dougherty*, Tin Cao*, Jackie Pierce+, Ling Mei Wong+, and Maurizio Alimandi+, and Paul Young# Identification of Heregulin Like Growth Factor (NRG-3): A new ligand for the erbB family of receptors expressed in human breast cancers. Era of Hope Conference 1997.

Personnel

King,   C. Richter PI   8/1/96-2/28/98

Hijazi,   Mai Magda   2/1/96   to   9/1/98
                              9/1/94   to   6/30/95

Lupu,   Ruth PI   9/1/94   to   7/31/96

Guerra de Vladusic, F.   9/1/95   to   1/31/96

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S   (70-1y)   26 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information
              Center, ATTN: DTIC-OCA, 8725 John J. Kingman
              Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has
reexamined the need for the limitation assigned to technical
reports written for the following Awards.

             DAMD17-86-C-6169   ADB116203
             DAMD17-94-J-4056   ADB218947
             DAMD17-94-J-4394   ADB220575
             DAMD17-94-J-4358   ADB236080
             DAMD17-94-J-4169   ADB236753
             DAMD17-94-J-4049   ADB234453
             DAMD17-94-J-4080   ADB218909
             DAMD17-94-J-4080   ADB233428
             DAMD17-94-J-4431   ADB220348
             DAMD17-94-J-4335   ADB234557
             DAMD17-94-J-4388   ADB218872
             DAMD17-94-C-4081   ADB246577
             DAMD17-94-J-4025   ADB238010
             DAMD17-94-J-4080   ADB241898
             MIPR 96MM6720   ADB240182
             MIPR 96MM6720   ADB226818

Request the limited distribution statement for Accession Document
Numbers be changed to "Approved for public release; distribution
unlimited." These reports should be released to the National
Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at
DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

FOR THE COMMANDER:

                              PHYLIS M. RINEHART
                              Deputy Chief of Staff for
                              Information Management