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13. ABSTRACT <i>(Maximum 200 words)</i>  Overexpression of the c-erbB2 gene is correlated with poor prognosis and the number of lymph node metastases in breast cancer patients. Our previous work has demonstrated that erbB2 enhances the intrinsic metastatic potential of human breast cancer cells. We hypothesize that the erbB2-encoded receptor tyrosine kinase (RTK) may enhance metastatic potential through the RTK-signaling molecules. The purpose of this study is to test whether the increased c-erbB2 tyrosine kinase activity and tyrosine autophosphorylation on the carboxyl-terminal tail may be required for the downstream signaling involved in breast cancer metastasis. We have completed the Tasks 1, 2, 3 as stated in the Statement of Work of our proposal. Tasks 4, 5, 6, of the proposal are ongoing. We have published a paper on <i>Cancer Research</i> , which is resulted from this grant support. Currently, we are studying the metastatic potential of FACS sorted wild-type and erbB2 mutant transfectants in vitro and in vivo. Also, we are studying the erbB2 downstream signaling molecules which may contribute to erbB2 enhanced cancer metastasis.			
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## Progress Report for 1998-1999

### A. Introduction

Breast cancer is one of the most common malignancies among women in the United States, and metastasis from this cancer is the major cause of death for these patients. Therefore, it is extremely important to uncover the basis of breast cancer metastasis. Overexpression of the c-erbB2 (also known as HER-2, *neu*) gene has been shown to be correlated with poor prognosis and the number of lymph node metastases in breast cancer patients. Our recent work has demonstrated that stable transfection of the human c-erbB2 gene into the low c-erbB2-expressing MDA-MB-435 human breast cancer cells (named 435.eB transfectants) indeed enhanced the intrinsic metastatic potential of these cells (1). Because overexpression of the c-erbB2 gene has been found in ~ 30% of breast tumors, it is very important to examine the molecular mechanisms underlying the enhanced metastatic potential induced by c-erbB2 overexpression and then to design new strategies to treat this type of breast cancer metastasis. We hypothesize that the c-erbB2-encoded receptor tyrosine kinase (RTK) may enhance metastatic potential through the RTK-signaling molecules. The purpose of this proposed study is to test whether the increased c-erbB2 tyrosine kinase activity and tyrosine autophosphorylation on the carboxyl-terminal tail may be required for the downstream signaling involved in breast cancer metastasis. To address this question we will study: 1) The requirement of the tyrosine kinase domain and tyrosine autophosphorylation sites in the c-erbB2 receptor for mediating signals leading to metastasis. 2) The downstream signals of c-erbB2 that may contribute to increased metastatic potential.

### B. Study Results and Significance

We have completed the Tasks 1, 2, and 3. The tasks 4, 5, 6, 7 are currently undergoing. To determine whether the tyrosine kinase activity and other structural motifs in the cytoplasmic

domain of erbB2 receptor are required for enhancing metastatic potential, we have subcloned and transfected the kinase-deficient dominant-interfering mutant (K753M), autophosphorylation-site mutant (Y1248F), c-terminal-deletion mutant (C1025) and constitutively activated mutant (V659E) of the c-erbB2 receptor into human breast cancer MDA-MB-435 cells. We have established a panel of erbB2 gene transfected stable transfectants (see our first year report). In vivo tyrosine kinase activities of wild-type and mutant erbB2 proteins by western blot analysis has been performed. As described in the first year report, very low level of tyrosine phosphorylation of erbB2 protein has been found in transfectants that expressing the kinase-defective K753M mutant, reduced tyrosine phosphorylation of erbB2 proteins has been found in Y1248F and C1025 mutants, compared to those expressing the wild-type erbB2 protein. Further more, we detected a higher tyrosine phosphorylation level of erbB2 proteins in the constitutive activated V659E mutant compared to those expressing the wild-type erbB2 protein (see our first year report).

One of our aims is to compare wild-type with erbB2 mutant transfectants that express mutated erbB2 proteins for their cell signaling. Since Ras-Raf-ERK pathway and PI3-kinase pathway are two important signaling pathways, we tested whether expression of these mutants altered protein expression of Shc, ERK, p85 subunit of PI3-kinase, and PI3-kinase downstream signaling molecule Akt. The results showed that the erbB2 mutant protein expression has no effect on the expression of these signaling molecules (Appendices, Fig 1). We also tested the activation of Ras-Raf-ERK and PI3-kinase pathways in erbB2 wild-type and mutant cell lines. We used phosphorylated ERK and phosphorylated Akt specific antibodies (from New England BioLab) to test the activation of these two pathways. However, we did not see significant difference among these cells for ERK and Akt phosphorylation levels (Appendices, Fig 2). To compare erbB2 wild-type with mutant transfected cells for their metastatic potentials, we also performed in vitro motility assay and invasion assay. Our preliminary results show that there are no significant difference in motility and invasion among these cell lines (data not shown).

Above described results are not compliant with our original expectation. These results indicate that under our experimental conditions, the erbB2 mutant transfected cells did not show

significant difference in ERK, Akt activation, cell motility, and invasion abilities. The causes for these results could be, 1) After passed in the tissue culture dishes for about 10 passages, the erbB2 mutant protein expression levels became lower, which may cause erbB2 expression level not high enough to reach the threshold to exert their effects. We used these cells to perform in vitro metastasis assays; therefore we can not see difference. 2) Under quiescent condition, the signals transduced by erbB receptors may be too weak to exert above mentioned functions. To solve these problems, first, we used heregulin to stimulate the cells before our assays. Second, we selected higher erbB2 expression cell populations from each of these mutant transfectants by flow cytometry analysis and fluorescence activated cell sorting (FACS) (2).

Heregulin (HRG) is a family of polypeptide growth factors derived from alternatively spliced genes. HRG can bind to receptor tyrosine kinases erbB3 and erbB4, thereby inducing erbB3 and erbB4 heterodimerization with erbB2, leading to receptor tyrosine phosphorylation, and activating downstream signal transduction (3). Since we did not see significant difference in ERK, Akt activation, we used HRG to stimulate these cells before our assays. We used phosphorylated ERK and phosphorylated Akt specific antibodies to test the activation of ERK and Akt by western analysis (Appendices, Fig 3). After HRG treatment, the phosphorylation levels of ERK in wild-type, V659E, Y1248F cell lines increased dramatically. However, in the C1025, very weak phosphorylation of ERK can be observed and in K753M mutant only moderately phosphorylation of ERK can be seen. The phosphorylation levels of Akt were increased dramatically in Wild-type, V659E, Y1248F cells. Slight phosphorylation can be observed in C1025 cells, but not in K753M cells. These results indicate that C-terminal of erbB2 protein is required for HRG induced Ras-ERK pathway signaling and erbB2 kinase activity is required for HRG induced PI3-kinase pathway activation. These result also indicated that erbB2 receptor is required for HRG induced Ras-Raf-ERK pathway and PI3-kinase pathway signaling. Further studies on wild-type and mutants for their signaling molecules which may contribute to erbB2 enhanced breast cancer cell metastatic potential are undergoing.

Our ultimate goal is to study erbB2 receptor signaling and metastasis. Therefore we used Flow cytometry analysis and fluorescence activated cell sorting (FACS) to select erbB2 high expressing cell population from erbB2 transfectants. After FACS sorting, the erbB2 expression levels of wild-type and mutants were increased significantly (Appendices, Fig 4). The assays on cell signaling and metastatic potential are undergoing with these FACS sorted cells. To compare the metastatic potentials of these cells *in vivo*, we have injected the sorted wild-type and V659E, Y1248F, K753M and C1025 cells into SCID mice as we described in previous study (1). We expected to get the animal experiment results next month.

During previous finding periods, while investigating the erbB receptor signaling and metastasis-associated properties when stimulated by their ligand, we found that HRG can enhance aggregation of MCF-7 and SKBR3 human breast cancer cells (Appendices, Fig 5). Since cell aggregation is one of the important metastasis-associated properties. We further investigated the downstream signals involved in HRG-enhanced cell aggregation. We found that HRG increased the kinase activities of extracellular signal-regulated protein kinase (ERK) and PI3-kinase in these cells (Appendices, Fig 6). By using the MAPK/ERK kinase 1 (MEK1) inhibitor PD98059 and PI3-kinase inhibitors wortmannin and LY294002, we found that blocking the MEK1-ERK pathway had no effect on HRG-enhanced cell aggregation; however, blocking the PI3-kinase pathway greatly inhibited HRG-mediated cell aggregation (Appendices, Fig 7). Our study indicated that the HRG-activated MEK1-ERK pathway has no demonstrable role in the induction of cell aggregation, whereas HRG-activated PI3-kinase is required for enhancing breast cancer cell aggregation. Since aggregation can contribute to invasion/metastasis phenotype of cancer cells, our results have provided one mechanism by which HRG-activated signaling of erbB receptors may affect invasive/metastatic properties of MCF-7 and SKBR3 breast cancer cells. This part of work has been published on peer reviewed journal, in which we acknowledged U.S. Army Breast Cancer Research Program for support.

## **C. Conclusions**

During this finding period, we are performing task 4, 5, 6, 7. We compared erbB2 wild-type and mutant transfectants for their metastasis associated properties and downstream signaling molecules. We found that Ras-ERK pathway, PI3-kinase pathway activation, cell motility and invasion abilities among our erbB2 transfectants have no significant difference. We analyzed the possible causes for these results and used two alternative methods to solve the problem. We used FACS to select erbB2 higher expressing cells from wild-type and mutant erbB2 transfectants. We used HRG to stimulate the cells before assay. In our preliminary studies on these cells, we found that ERK activation in C1025 mutant is impaired and PI3-kinase downstream molecule Akt activation is impaired when cells were stimulated by HRG. These are important findings, since they provided us a very nice system to analyze which signaling pathway is responsible for which metastasis-associated propertie(s). These results provided direct evidence that erbB2 is required for HRG induced cell signaling. To evaluate the metastatic potential of wild-type and mutant erbB2 transfected cells in vivo, we have injected FACS sorted cells into SCID mice tail vein. The forthcoming results will tell us which structure motifs of erbB2 receptor are required for breast cancer cell metastasis. We investigated metastasis-associated properties when human breast cancer cells were stimulated by erbB receptor's ligand HRG. We found that HRG can enhance aggregation of MCF-7 and SKBR3 human breast cancer cells and we investigated the downstream signals involved in HRG-enhanced cell aggregation. We published a paper on *Cancer Research*, which is resulted from this grant support.

**Key accomplishments:**

- We compared erbB2 wild-type and mutant transfectants for their metastasis associated properties and downstream signaling molecules in vitro.
- We used FACS to select erbB2 higher expressing cells from wild-type and mutant transfectants. We injected FACS sorted cells into SCID mice to measure the metastatic potential in vivo.
- We used heregulin to stimulate the cells before assay. We found that ERK activation in C1025 mutant is impaired and PI3-kinase downstream molecule Akt activation is impaired in K753M mutant. This provides us a very nice system to analyze signal transduction pathways and metastasis-associated properties. These results also provided direct evidence that erbB2 is required for HRG induced cell signaling.
- We found that HRG can enhance aggregation of MCF-7 and SKBR3 human breast cancer cells through erbB receptors and we investigated the downstream signaling. We published a paper on *Cancer Research*, which is resulted from this grant support.

#### **D. Reference**

1. Tan M., Yao J., and Yu D. C-erbB-2 overexpression enhanced intrinsic metastatic potential in human breast cancer cells. *Cancer Res.* 57: 1199-1205, 1997.
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# Appendices

## Figures and Legends

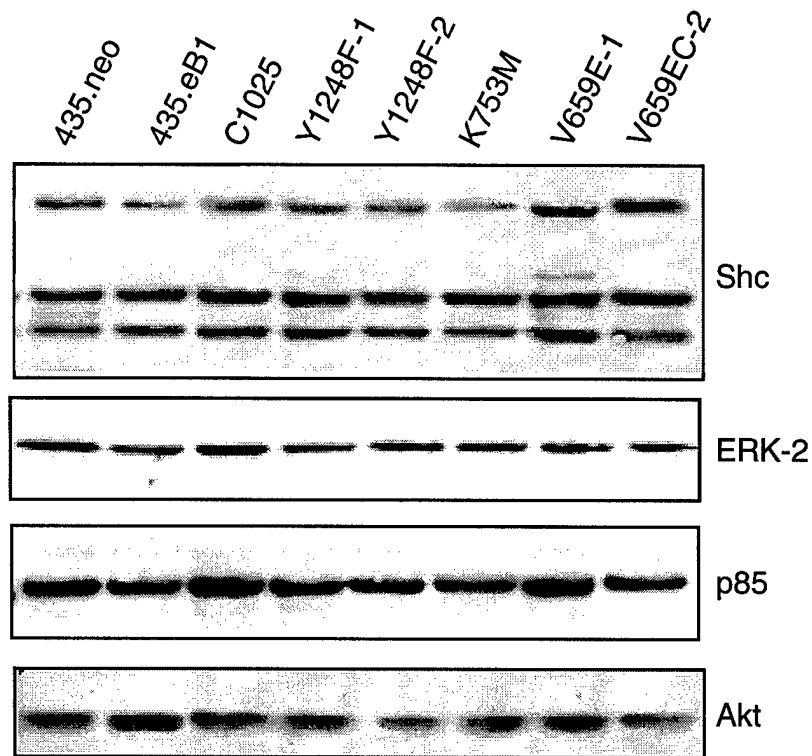


Fig.1 erbB2 gene trasfection has no effect on Shc, ERK, p85, and Akt expression levels. Immunoblot analysis for the Shc, ERK, p85 subunit of PI3-kinase, and Akt in the wild-type and mutant erbB2 transfected MDA-MB-435 cells. 100  $\mu$ g of protein from each sample was electrophoresed on SDS-PAGE and transferred to nitrocellulose. The filters were immunoblotted with the indicated antibodies. Positions of the proteins are indicated on the right.

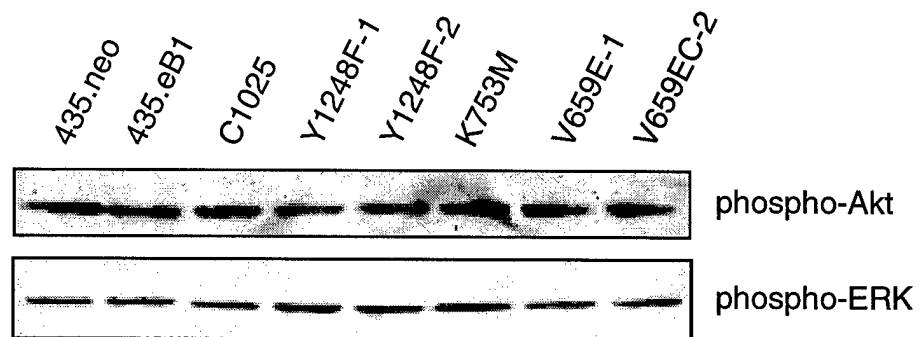


Fig.2 ERK and Akt activation in erbB2 transfectants. Cell lysates containing equal amounts of proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies against phospho-Akt (top) and phospho-ERK (lower). The membrane were visualized by ECL kit.

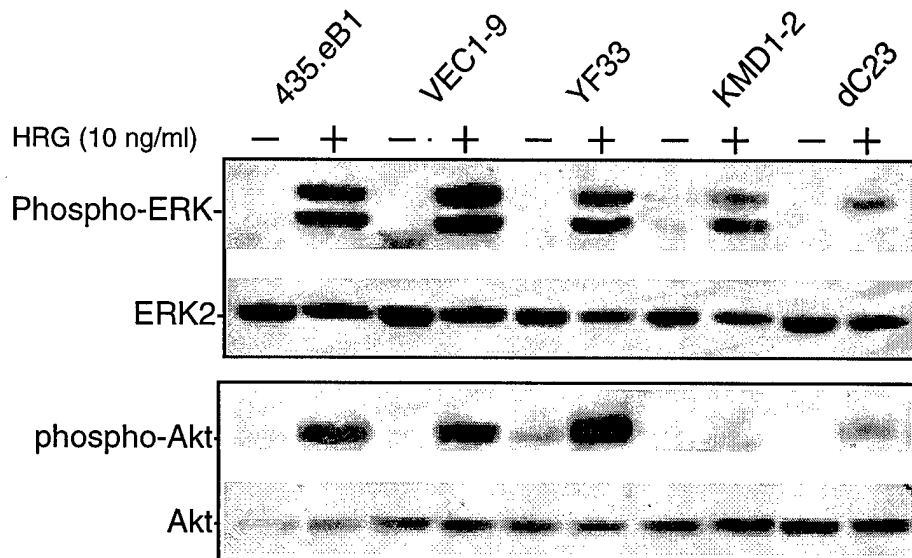


Fig.3 ERK and Akt activation in erbB2 transfectants by HRG. Serum-starved cells were treated with HRG- $\beta$ 1 at the indicated concentrations. Cell lysates containing equal amounts of proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies against phospho-ERK (top) and phospho-Akt (lower), the membranes were stripped, and rehybridized with antibodies against ERK or Akt.

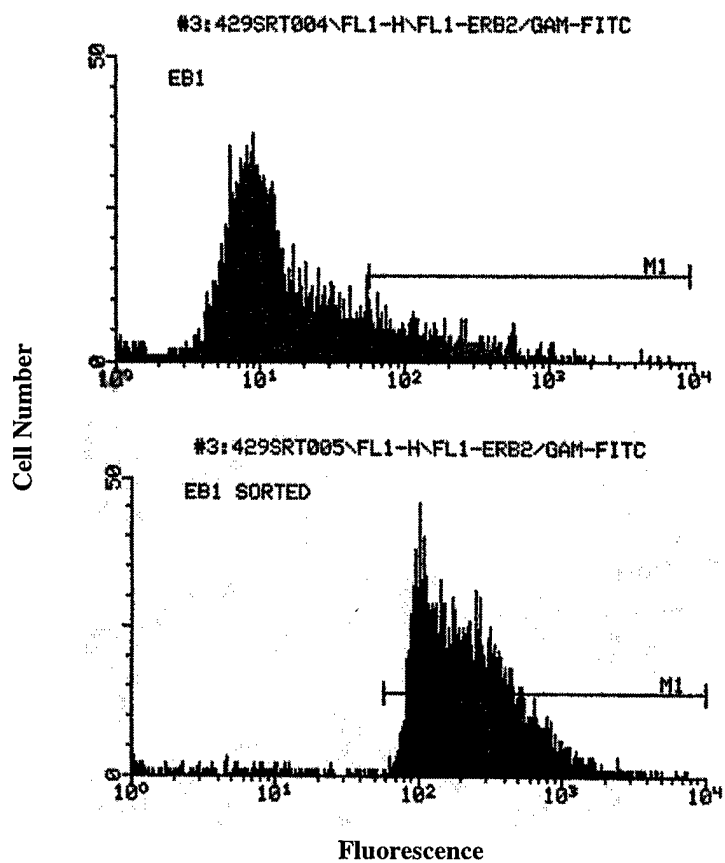


Fig. 4 Fluorescence Activated Cell Sorting (FACS) of 435.eB1 cells. Cells were incubated with monoclonal erbB2 specific antibody for 45 min, washed and labeled with anti-mouse antibodies conjugated fluorescence (FITC). Fluorescence labeled cells were subjected to flow cytometry sorting.

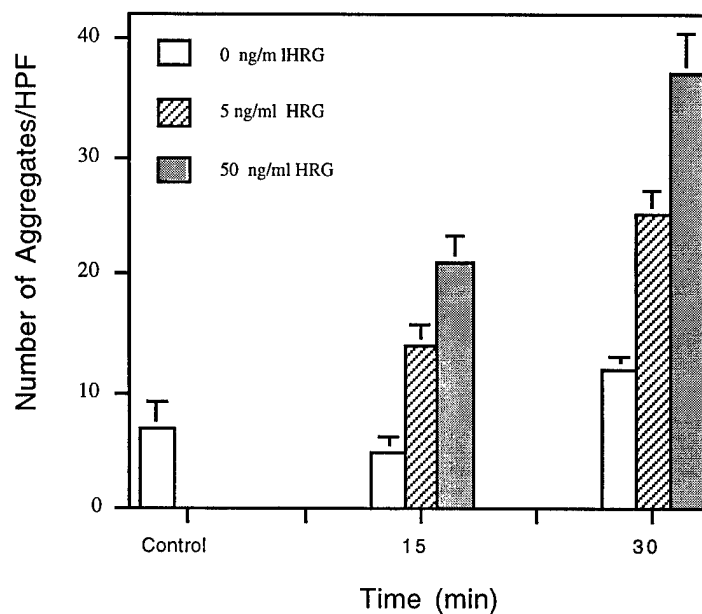


Fig. 5 Homophilic aggregation of MCF-7 breast cancer cells was enhanced by heregulin- $\beta$ 1. 500  $\mu$ l of single-cell suspension ( $5 \times 10^4$  cells/ml in DMEM/F12 medium containing 0.5% BSA) in the presence or absence of HRG- $\beta$ 1 were plated into each well of a 24-well low-binding plate. The plate was incubated at 37°C on a rotating platform for 30 min. Cells were then fixed and quantitated. The number of cell aggregates represents the average number determined from four random high power fields (HPFs).

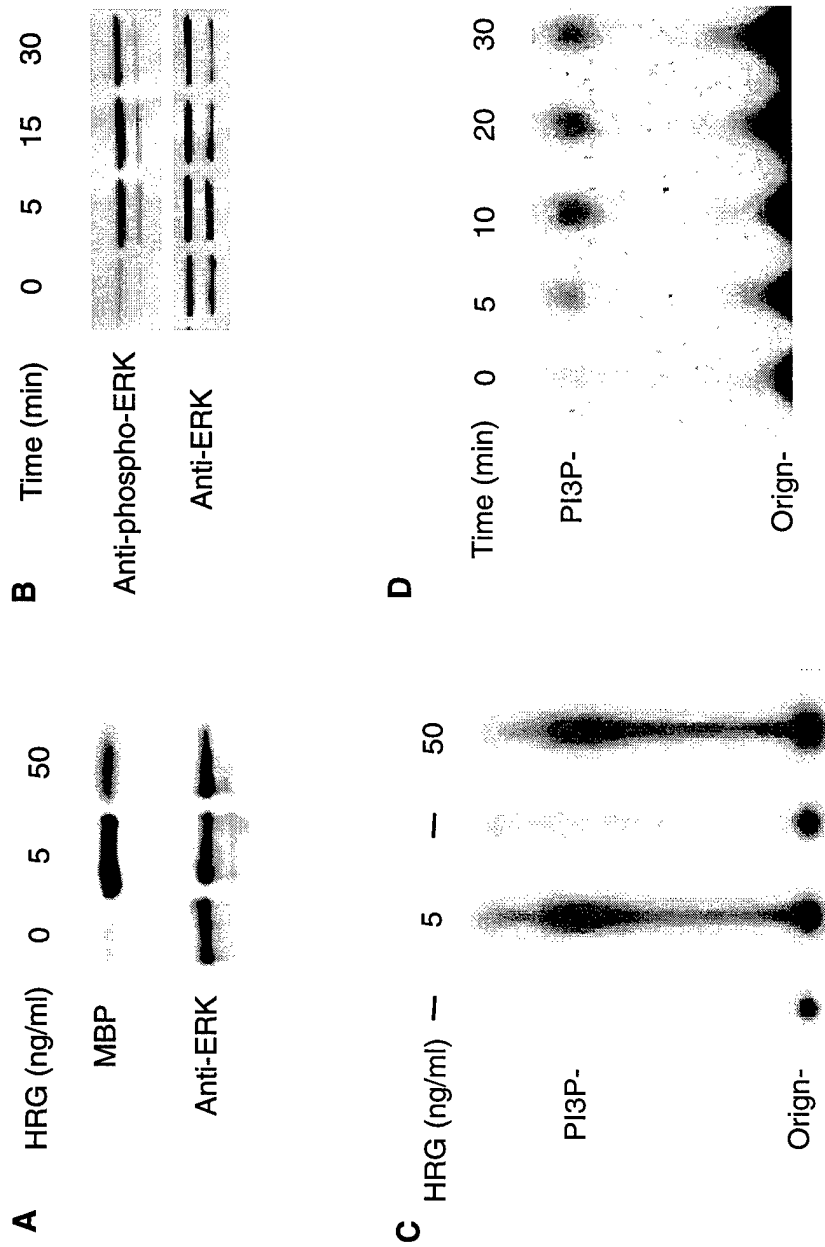
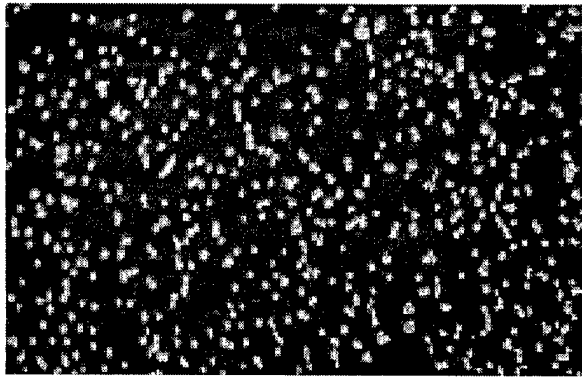
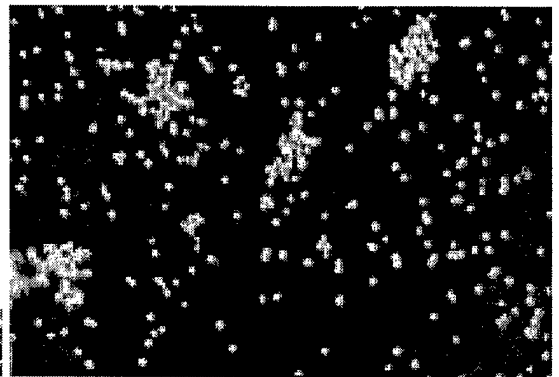


Fig. 6 Activation of ERK and PI3K by HRG- $\beta$ 1. Serum-starved MCF-7 cells were treated with HRG- $\beta$ 1 at the indicated concentrations and time intervals. A, Cell lysates were immunoprecipitated with anti-ERK2 antibodies and the immunocomplexes subjected to an ERK assay. Phosphorylation of myelin basic protein (MBP) by ERK was visualized by autoradiography (top). ERK protein level was determined by western blotting using anti-ERK antibody (bottom); B, Cell lysates containing equal amounts of proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies against phospho-ERK (top), stripped, and rehybridized with antibodies against ERK (bottom). C and D, cell lysates were immunoprecipitated with the anti-erbB3 antibodies and the immunocomplexes subjected to PI3K assay. The products of the reaction were analyzed by thin-layer chromatography and visualized by autoradiography. The product of PI3K, PI3P, is indicated on the left.

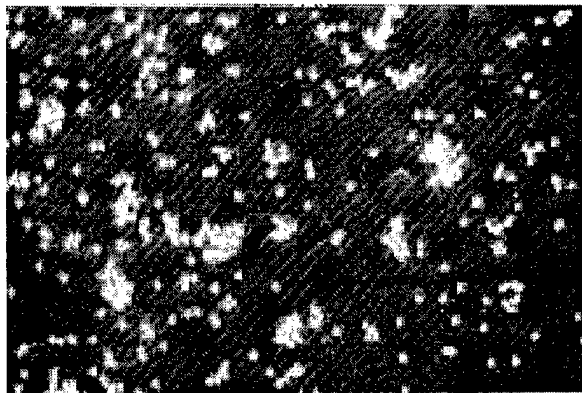
A. HRG 0 ng/ml



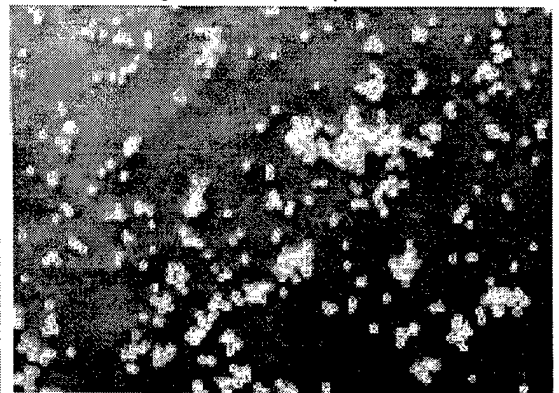
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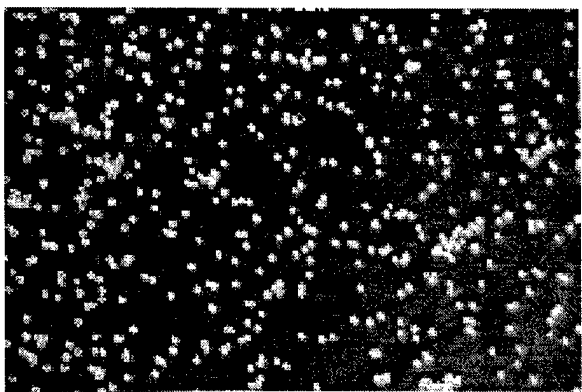
C. HRG 5 ng/ml + PD 50  $\mu$ M



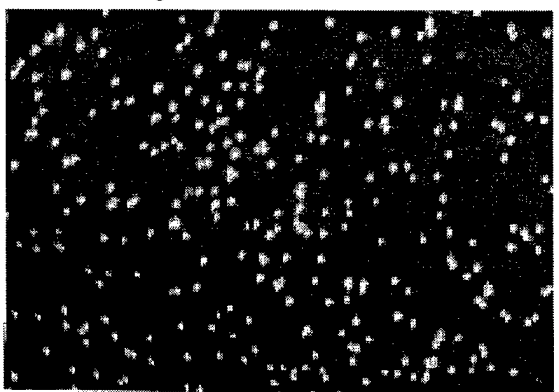
D. HRG 5 ng/ml + PD 100  $\mu$ M



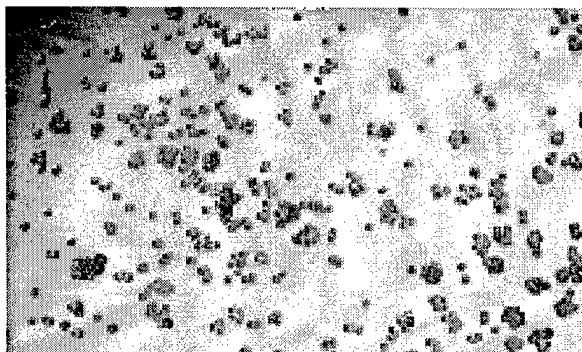
E. HRG 5 ng/ml + WN 50 nM



F. HRG 5 ng/ml + WN 100 nM



G. HRG 5 ng/ml + LY 30  $\mu$ M



H. HRG 5 ng/ml + LY60  $\mu$ M

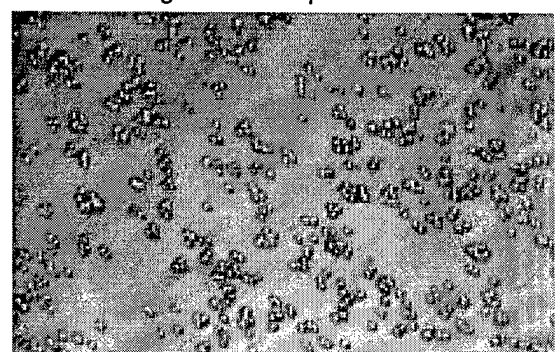


Fig. 7 HRG- $\beta$ 1-enhanced MCF-7 cell aggregation was inhibited by wortmannin but not by PD98059. Cells were pretreated with indicated concentrations of PD98059 for 2 h or wortmannin for 1 h, detached from tissue culture dishes using 10 mM EDTA and 0.1% BSA in PBS, and washed with DMEM/F12 serum-free medium. 500  $\mu$ l single-cell suspensions ( $5 \times 10^4$  cells/ml in DMEM/F12 medium containing 0.5% BSA) in the presence or absence of 5 ng/ml of HRG- $\beta$ 1 were plated into wells of the 24-well low-binding affinity plate. The plate was incubated at 4°C on a rotating platform for 30 min. PD, PD98059; WN, wortmannin. Photographs taken by Nikon N6006 camera. Magnification 200 X.

# Heregulin $\beta$ 1-activated Phosphatidylinositol 3-Kinase Enhances Aggregation of MCF-7 Breast Cancer Cells Independent of Extracellular Signal-regulated Kinase<sup>1</sup>

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## ABSTRACT

Heregulin (HRG) is a family of polypeptide growth factors derived from alternatively spliced genes. HRG can bind to receptor tyrosine kinases erbB3 and erbB4, thereby inducing erbB3 and erbB4 heterodimerization with erbB2, leading to receptor tyrosine phosphorylation and activating downstream signal transduction. Cell-cell homophilic adhesion (cell aggregation) is important in determining the structural organization and behavior of cells in tissues. In addition, tumor cell homophilic adhesion may affect invasive and metastatic potentials of cells. We report that HRG- $\beta$ 1 can enhance aggregation of MCF-7 and SKBR3 human breast cancer cells. While investigating the downstream signals involved in HRG- $\beta$ 1-enhanced cell aggregation, we observed that HRG- $\beta$ 1 induced tyrosine phosphorylation of erbB2 and erbB3 receptor heterodimers and increased the association of the dimerized receptors with the 85-kDa subunit of phosphatidylinositol 3-kinase (PI3K). HRG- $\beta$  also increased the kinase activities of extracellular signal-regulated protein kinase (ERK) and PI3K in these cells. By using the mitogen-activated protein kinase/ERK 1 (MEK1) inhibitor PD98059 and PI3K inhibitors wortmannin and LY294002, we found that blocking the MEK1-ERK pathway had no effect on HRG- $\beta$ 1-enhanced cell aggregation; however, blocking the PI3K pathway greatly inhibited HRG- $\beta$ 1-mediated cell aggregation. Our study indicated that the HRG- $\beta$ 1-activated MEK1-ERK pathway has no demonstrable role in the induction of cell aggregation, whereas HRG- $\beta$ 1-activated PI3K is required for enhancing breast cancer cell aggregation. Because aggregation can contribute to invasion/metastasis phenotype of cancer cells, our results have provided one mechanism by which HRG- $\beta$ 1-activated signaling of erbB receptors may affect invasive/metastatic properties of MCF-7 and SKBR3 breast cancer cells.

## INTRODUCTION

The erbB family of receptor tyrosine kinases has four known members: erbB1 (EGF<sup>3</sup> receptor), erbB2, erbB3, and erbB4 (1-4). The erbB receptors are widely expressed in epithelial, mesenchymal, and neuronal tissues and play fundamental roles during development. Their aberrant expression is frequently observed in human malignant diseases (5, 6). The precise mechanism by which erbB receptors are involved in human cancer progression remains poorly understood, but, presumably, it involves signal transduction pathways that are activated by ligand binding.

HRG, also called neu differentiation factor, is a family of polypeptide growth factors derived from alternatively spliced genes (7-10). HRG can bind to receptor tyrosine kinases erbB3 and erbB4, thereby

inducing erbB3 and erbB4 heterodimerization with erbB2, receptor tyrosine phosphorylation, and downstream signal transduction (2, 11, 12). Several signal transduction pathways activated by HRG have been reported recently. Activation of PI3K, ERK, and the stress-activated protein kinase/c-Jun N-terminal kinase have been observed in various systems (13-16). Studies of breast cancer cell lines have revealed that the physiological effects of HRG are diverse and cell type-dependent (9, 17). In addition to regulating cell growth and differentiation, HRG may be involved in regulation of other biological behaviors of cancer cells, such as apoptosis (18), cell adhesion, migration, and invasion (19, 20); but, the overall picture of its biological effects is still not clear. Moreover, little is known regarding the integration of HRG-activated signals leading to various biological effects.

Cell adhesion is crucial for maintaining the structural integrity of tissues. Cell-matrix adhesion is mediated by heterophilic interactions between cell-surface receptors and their matrix ligands, whereas cell-cell adhesion (cell aggregation) primarily involves direct homophilic interactions between cell-surface molecules such as the cadherins (21). Cell-adhesion molecules do not merely offer structural anchors for cells, but also transmit signals that are integrated with other cellular activities in the coordination of major aspects of cell behavior, including proliferation, differentiation, apoptosis, and cell movement (21, 22).

We report here that HRG- $\beta$ 1 can enhance aggregation of MCF-7 and SKBR3 human breast cancer cells. We demonstrated that PI3K is required for the induction of cell aggregation in response to HRG- $\beta$ , but that the mitogen-activated protein kinase (ERK) activated by HRG- $\beta$  has no demonstrable role in the induction of cell aggregation.

## MATERIALS AND METHODS

**Materials.** Recombinant human HRG- $\beta$  was purchased from NeoMarkers (Fremont, CA). Wortmannin, LY294002, and PD98059 were purchased from Calbiochem (La Jolla, CA). Antibodies against erbB2 were from Oncogene Science Products (Cambridge, MA); antibodies against erbB3 were from NeoMarkers; antibodies against PI3K 85-kDa subunit were from Upstate Biotechnology Inc. (Lake Saranac, NY); antibodies against phosphotyrosine and ERK2 were from Santa Cruz Biotechnology (Santa Cruz, CA); and antibodies against phospho-ERK were from New England Biolabs (Beverly, MA).

**Cell Culture.** The human breast carcinoma cell lines MCF-7 and SKBR3 were purchased from the American Type Culture Collection (Manassas, VA) and maintained in DMEM/F12 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies, Inc.).

**Cell Aggregation Assay.** Cells in subconfluent cultures were serum-starved for 24 h, treated with chemical kinase inhibitors or their solvent (DMSO), then detached from tissue culture dishes and washed with serum-free medium. Each well of a 24-well low-binding affinity tissue culture plate (Costar Corp., Cambridge, MA) contained 500  $\mu$ l of single-cell suspension at the concentration of  $5 \times 10^4$  cells/ml in DMEM/F12 containing 0.5% BSA. Cells were plated in the presence (5 or 50 ng/ml) or absence of HRG- $\beta$  or EGF. Plates were incubated at 4°C or 37°C on a rotating platform for 30 min. Aggregated cell mixtures were fixed with 2% glutaraldehyde. The aggregates were defined as cell clumps containing more than five cells. Aggregates in four randomly selected high-power fields were counted using light microscopy.

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<sup>3</sup> The abbreviations used are: EGF, epidermal growth factor; HRG, heregulin; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated kinase/ERK.

**Preparation of Cell Lysates and Immunoprecipitates.** Cells at 70–80% confluency were starved in serum-free medium for 24 h and treated with or without chemical kinase inhibitors, then stimulated without or with HRG- $\beta$  (5 or 50 ng/ml) at 37°C for 5 min. The cells were washed and lysed in lysis buffer (23), and the insoluble materials were removed by centrifugation. Equal amounts of protein were incubated with the indicated antibodies for 1 h at 4°C and precipitated with protein A-Agarose. The immunoprecipitates were washed four times with the lysis buffer and eluted by boiling for 5 min in sample buffer before separation by SDS-PAGE.

**Western Blot Analysis.** Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Western blotting was performed using the enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL). Horseradish peroxidase-conjugated antibodies against mouse IgG or rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies.

**PI3K Assay.** Cells at 70–80% confluency were stimulated with or without HRG- $\beta$ , lysed, and immunoprecipitated with anti-erbB3 antibody, as described

above. The PI3K assay was performed essentially as previously described (23), with minor modification.

**ERK Assay.** HRG-treated or -untreated cells (70–80% confluent) were lysed and immunoprecipitated with anti-ERK2 antibody, as described above. The ERK assay was performed as described previously (24).

## RESULTS

**Enhancement of Human Breast Cancer Cell Aggregation by HRG- $\beta$ .** HRG- $\beta$  was previously shown to enhance invasiveness of SKBR3 breast cancer cells (25), and cell aggregation was suggested to play an important role in cancer cell invasion/metastasis (26–28). Here, we asked whether HRG- $\beta$  may enhance breast cancer cell aggregation. To this end, 5 or 50 ng/ml of HRG- $\beta$  was added to the cell suspension of serum-starved MCF-7 human breast cancer cells. We found that HRG-treated MCF-7 cells formed dramatically more

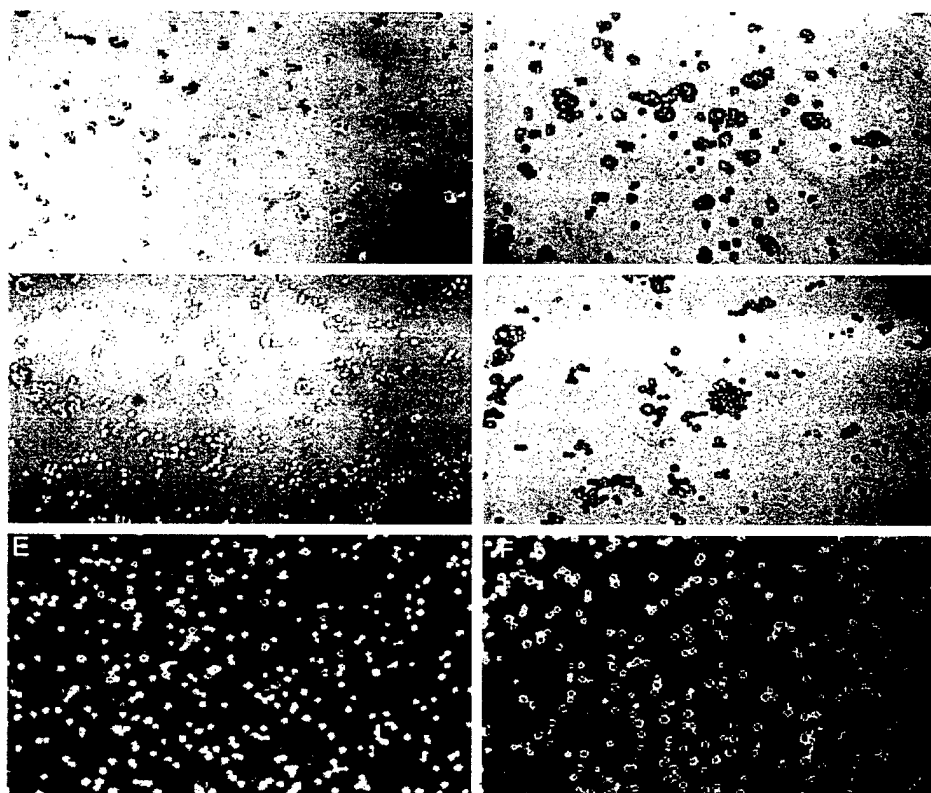
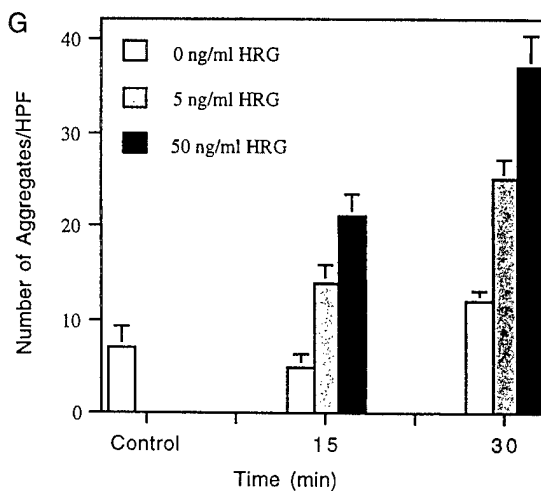


Fig. 1. Homophilic aggregation of MCF-7 and SKBR3 breast cancer cells was enhanced by HRG- $\beta$ , but not by EGF. Single-cell suspension (500  $\mu$ l;  $5 \times 10^4$  cells/ml in DMEM/F12 medium containing 0.5% BSA) in the presence or absence of HRG- $\beta$  or EGF were plated into each well of a 24-well low-binding plate. The plate was incubated at 4°C or 37°C on a rotating platform for 30 min. Photographs were taken using a Nikon N6006 camera (magnification,  $\times 200$ ). A and B, MCF-7 cells were treated with 0 or 5 ng/ml HRG- $\beta$ . C and D, SKBR3 cells were treated with 0 or 5 ng/ml HRG- $\beta$ . E and F, MCF-7 cells were treated with 0 or 5 ng/ml EGF. G, MCF-7 cells were serum-starved and detached from tissue culture dishes using 10 mM EDTA and 0.1% BSA in PBS. Single-cell suspensions (500  $\mu$ l;  $5 \times 10^4$  cells/ml in DMEM/F12 medium containing 0.5% BSA) in the absence or presence of 5 ng/ml or 50 ng/ml HRG- $\beta$  were plated into wells of a 24-well low-binding affinity plate. The plate was incubated at 37°C on a rotating platform for 30 min. Cells were then fixed and quantitated. Untreated control cells were fixed immediately after plating. The number of cell aggregates represents the average number determined from four random high-power fields.



aggregates than those of untreated cells (Fig. 1, A and B). The same effect was seen in the human breast cancer cell lines SKBR3 (Fig. 1, C and D), MDA-MB-435, and MDA-MB-231 (data not shown). To test whether the HRG- $\beta$ -enhanced aggregation is an energy-dependent process, we performed the aggregation assay at both 4°C and 37°C. MCF-7 cells formed aggregates at both temperatures (data not shown), indicating that the aggregation process is not energy-dependent. The quantitative measures of aggregation assays are shown in Fig. 1G, which demonstrated that the enhancement of cell aggregation by HRG- $\beta$  is concentration-dependent. This process is HRG- $\beta$ -specific, because EGF does not enhance MCF-7 and SKBR3 cell aggregation under the same conditions (Fig. 1, E and F). These results indicated that HRG- $\beta$  can enhance human breast cancer cell aggregation *in vitro* and that the effect is HRG- $\beta$ -specific.

**HRG- $\beta$  Enhances Tyrosine-phosphorylation of erbB2 and erbB3 Heterodimers and Their Association with the 85-kDa Subunit of PI3K.** To investigate the downstream signals involved in HRG- $\beta$ -enhanced aggregation, we examined HRG- $\beta$ -mediated activation of erbB2 and erbB3 in the MCF-7 and SKBR3 cell lines. The erbB2 and erbB3 receptors from HRG- $\beta$ -treated or -untreated MCF-7 and SKBR3 cells were immunoprecipitated with anti-erbB2 and anti-erbB3 antibodies; then, Western-blot analysis with antiphosphotyrosine antibody was performed to measure tyrosine phosphorylation of erbB2 and erbB3 heterodimers (Fig. 2, A and B). Treatment of MCF-7 and SKBR3 cells with HRG- $\beta$  dramatically increased tyrosine phosphorylation levels of anti-erbB3 immunoprecipitates (Fig. 2B) and moderately increased those of anti-erbB2 immunoprecipitates (Fig. 2A). To determine the association of the 85-kDa subunit of PI3K (p85) with erbB2 and erbB3 in these cells, we performed Western blot analysis of the anti-erbB2 and anti-erbB3 immunoprecipitates with anti-p85 antibody (Fig. 2, A and B). HRG- $\beta$  stimulation greatly increased the p85 association with anti-erbB3 immunoprecipitates in both cell lines (Fig. 2B), and a moderate increase of the p85 association was seen in anti-erbB2 immunoprecipitates (Fig. 2A). These results indicate that HRG- $\beta$  can activate erbB3 and erbB2 and, consequently, increase the association of p85 with erbB2 and erbB3 receptor dimers.

**Activation of ERK and PI3K by HRG- $\beta$ .** Both ERK and PI3K have been reported to be involved in cell adhesion of several cell types (22, 29). Therefore, we examined whether HRG- $\beta$  can activate ERK and PI3K in MCF-7 cells. MCF-7 cells were starved and treated with HRG- $\beta$  before kinase assays were performed. We found that ERK activity can be dramatically activated by different concentrations of HRG- $\beta$  (Fig. 3A). We also tested the kinetics of ERK activation by Western blot analysis using phospho-ERK-specific antibodies. The results revealed that ERK activity increased within 5 min and was sustained at least for 30 min (Fig. 3B). Next, we examined whether HRG- $\beta$  can activate PI3K in these cells. As shown in Fig. 3C, PI3K activity was greatly increased by HRG- $\beta$  stimulation. Kinetic studies of PI3K activation revealed that PI3K activity began to increase 5 min after the addition of HRG- $\beta$  and reached its highest level at 10 min, which was sustained for at least 30 min (Fig. 3D). These results indicate that HRG- $\beta$  can activate both ERK and PI3K in MCF-7 cells and that both ERK and PI3K activation precede the induction of aggregation.

**ERK Activation Is Not Required for HRG- $\beta$ -stimulated Cell Aggregation.** Using PD98059, a specific inhibitor of MEK1 (30, 31), we investigated whether interfering with the MEK1-ERK signaling pathway would inhibit HRG- $\beta$ -enhanced cell aggregation. As demonstrated by both ERK assay (Fig. 4A) and Western analysis using phospho-ERK-specific antibodies (Fig. 4B), activation of ERK by HRG- $\beta$  was inhibited in a concentration-dependent manner when the cells were treated with different concentrations of PD98059. We next

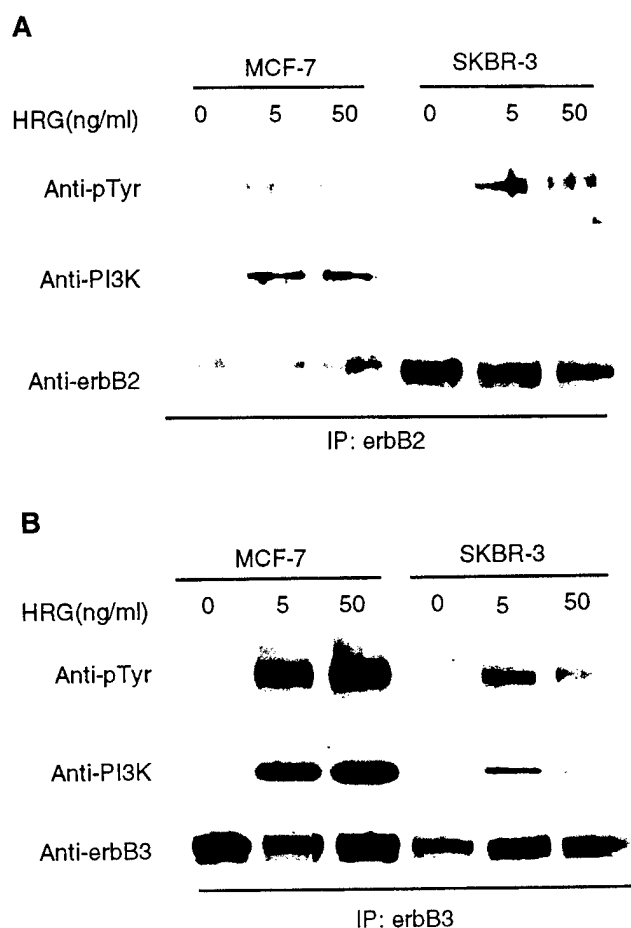


Fig. 2. HRG- $\beta$  increases erbB2 and erbB3 heterodimer tyrosine-phosphorylation and their association with PI3K. Serum-starved MCF-7 and SKBR3 cells were treated with 0, 5, and 50 ng/ml HRG- $\beta$  for 5 min. Cell lysates containing equal amounts of proteins were immunoprecipitated with antibodies against erbB2 (A) and erbB3 (B). Immunoprecipitates were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies against phosphotyrosine (top), stripped, and rehybridized with antibodies against the p85 PI3K subunit (middle), then stripped again, and rehybridized with antibodies against erbB2 (A, bottom) and erbB3 (B, bottom).

assessed the role of ERK in stimulating cell aggregation by HRG- $\beta$ . As expected, 5 ng/ml HRG- $\beta$  effectively enhanced aggregation of MCF-7 cells (Fig. 5, A and B), and PD98059 alone had no effect on MCF-7 cell aggregation (data not shown). It is notable that PD98059 had no inhibitory effect on HRG- $\beta$ -enhanced MCF-7 cell aggregation (Fig. 5, C and D), indicating that induction of cell aggregation by HRG- $\beta$  does not require activation of MEK1-ERK.

**PI3K Activation Is Required for MCF-7 Cell Aggregation Enhanced by HRG- $\beta$ .** To examine the involvement of PI3K in HRG- $\beta$ -enhanced MCF-7 cell aggregation, we tested whether a specific chemical inhibitor of PI3K, wortmannin (32, 33), would block HRG- $\beta$ -enhanced MCF-7 cell aggregation by inhibiting PI3K activity. We treated the MCF-7 cells with varying concentrations of wortmannin. As shown in Fig. 4C, PI3K activity was inhibited by wortmannin in a concentration-dependent manner. Moreover, activation of PI3K by HRG- $\beta$  at 4°C can also be inhibited by wortmannin (Fig. 4D). To determine the role of PI3K in enhancing cell aggregation by HRG- $\beta$ , we examined the effect of wortmannin on HRG- $\beta$ -mediated enhancement of MCF-7 cell aggregation. Wortmannin alone had no discernible effect on MCF-7 cell aggregation (data not shown), but it led to a concentration-dependent inhibition of HRG- $\beta$ -enhanced MCF-7 cell aggregation (Fig. 5, E and F) compared with that without wortmannin (Fig. 5B). To confirm the PI3K requirement in HRG- $\beta$ -enhanced

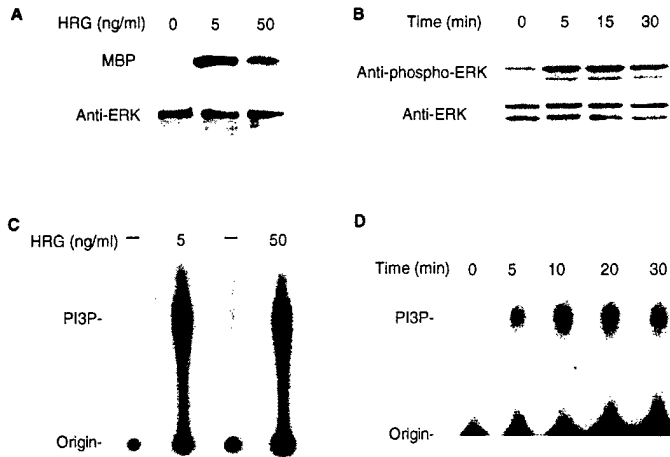


Fig. 3. Activation of ERK and PI3K by HRG- $\beta$ . Serum-starved MCF-7 cells were treated with HRG- $\beta$  at the indicated concentrations and time intervals. **A**, cell lysates were immunoprecipitated with anti-ERK2 antibodies, and the immunocomplexes were subjected to an ERK assay. Phosphorylation of myelin basic protein (*MBP*) by ERK was visualized by autoradiography (*top*). The ERK protein level was determined by Western blotting using anti-ERK antibody (*bottom*). **B**, cell lysates containing equal amounts of proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies against phospho-ERK (*top*), stripped, and rehybridized with antibodies against ERK (*bottom*). **C** and **D**, cell lysates were immunoprecipitated with the anti-erbB3 antibodies and the immunocomplexes subjected to PI3K assay. The products of the reaction were analyzed by thin-layer chromatography and visualized by autoradiography. The product of PI3K, PI3P, is indicated on the left.

aggregation, we also tested the ability of LY294002 (34), a competitive PI3K inhibitor, to inhibit HRG- $\beta$ -enhanced cell aggregation. Like wortmannin, LY294002 alone had no effect on MCF-7 cell aggregation (data not shown) but inhibited HRG- $\beta$ -enhanced MCF-7 cell aggregation in a concentration-dependent manner (Fig. 5, *G* and *H*). These results indicate that PI3K is required for transducing HRG- $\beta$  signals that result in MCF-7 cell aggregation.

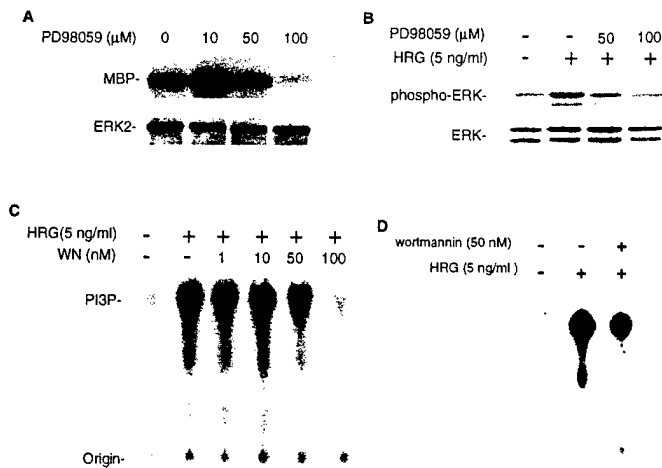


Fig. 4. Inhibition of ERK and PI3K by kinase inhibitors. **A**, serum-starved MCF-7 cells were pretreated with the indicated concentrations of PD98059 or its solvent DMSO for 2 h at 37°C before stimulation with 5 ng/ml HRG- $\beta$  for 5 min. ERK2 was immunoprecipitated with anti-ERK2 antibodies and assayed *in vitro*. *MBP*, myelin basic protein. **B**, cell lysates containing equal amounts of proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies against phospho-ERK (*top*), stripped, and rehybridized with antibodies against ERK (*bottom*). **C**, serum-starved MCF-7 cells were pretreated with the indicated concentrations of wortmannin (*WN*) or its solvent (DMSO) for 1 h at 37°C, then stimulated with 5 ng/ml HRG- $\beta$  for 5 min at 37°C. PI3K was immunoprecipitated with anti-erbB3 antibodies and assayed *in vitro*. **D**, serum-starved MCF-7 cells were pretreated with indicated concentrations of wortmannin or its solvent (DMSO) for 1 h at 37°C, then stimulated with 5 ng/ml HRG- $\beta$  for 5 min at 4°C. PI3K was immunoprecipitated with anti-erbB3 antibodies and assayed *in vitro*.

## DISCUSSION

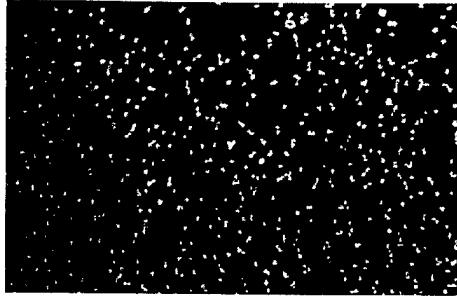
We report here that HRG- $\beta$  can enhance aggregation of MCF-7 and SKBR3 human breast cancer cells. We have further investigated the molecular mechanisms underlying HRG- $\beta$ -enhanced aggregation. Our results indicated that HRG- $\beta$  induces phosphorylation of the erbB2 and erbB3 receptors and rapidly activates ERK and PI3K in MCF-7 cells. Although MEK1 inhibitor PD98059 effectively reduced HRG- $\beta$ -mediated ERK enzyme activity, it failed to inhibit HRG- $\beta$ -enhanced cell aggregation, indicating that HRG- $\beta$ -activated ERK does not contribute to HRG- $\beta$ -enhanced MCF-7 cell aggregation. However, blocking of PI3K by PI3K-specific chemical inhibitors wortmannin and LY294002 effectively inhibited HRG- $\beta$ -enhanced MCF-7 aggregation, indicating that PI3K is required for HRG- $\beta$ -enhanced cell aggregation.

Previous reports indicated that among the members of the erbB receptor family, erbB3 is a potent activator of PI3K (35–37). In MCF-7 and SKBR3 cells, erbB2 and erbB3 can be activated by HRG- $\beta$ , and the resulting heterodimers of erbB2 and erbB3 can associate with the 85-kDa subunit of PI3K. However, the level of p85 associated with anti-erbB3 immunoprecipitates was dramatically higher than that of anti-erbB2 immunoprecipitates (Fig. 2, *A* and *B*), a result consistent with the previous studies. Compared with the SKBR3 cell line, MCF-7 cells expressed more erbB3, but less erbB2, and responded more strongly to HRG- $\beta$ -enhanced aggregation (Fig. 1), indicating that erbB3 may play an important role in HRG- $\beta$ -induced PI3K activation and cell aggregation.

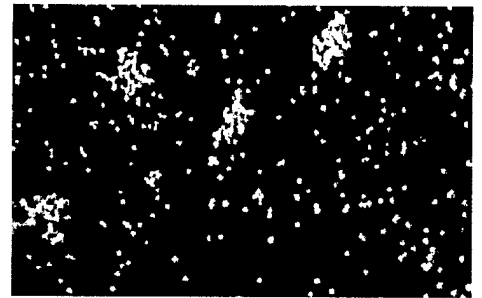
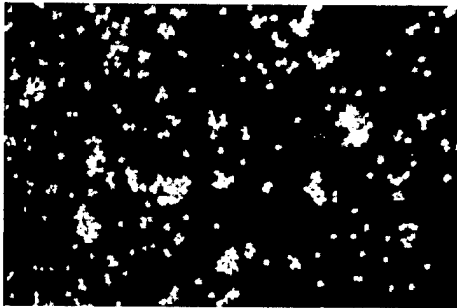
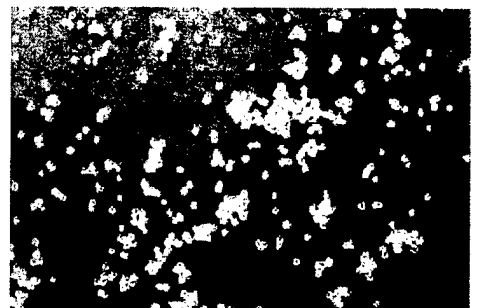
Regulation of cell adhesion may occur at several levels, including affinity modulation, clustering, coordinated interactions with the actin cytoskeleton, and up-regulation of adhesion molecule expression (22). HRG has been reported to induce expression of integrin (38) and intercellular adhesion molecule 1 in human cancer cells (19). However, the time required for HRG- $\beta$ -enhanced cell aggregation is shorter than the time needed for up-regulation of adhesion molecule expression. Therefore, functional activation of adhesion molecules and its consequences such as affinity modulation, clustering, and coordinated interactions with the actin cytoskeleton are more likely to be involved in the HRG- $\beta$ -enhanced cell aggregation. By activating erbB receptors, HRG- $\beta$  may send its signals through the PI3K pathway to activate these adhesion molecules, thereby inducing cell aggregation. Further investigation is needed to clarify which adhesion molecules are involved in this process. Another question that arises from these data are which downstream signaling molecules of PI3K are responsible for activating the adhesion molecules. Previous studies indicated that the small guanosine 5' triphosphate-binding protein Rac is involved in cell adhesion and is downstream of PI3K (39–41). A recent study found that, in epithelial Madin-Darby canine kidney cells, Tiam1, an exchange factor for Rac, is localized to adherens junctions (42). These findings suggested an attractive notion that Rac may also play a role in the HRG- $\beta$ -enhanced cell aggregation.

Cell-cell homophilic adhesion plays important roles in determining the structural organization and behavior of cells in tissues. Homophilic adhesion or aggregation is also important in tumor cell invasiveness and metastasis (26–28). Although reduced homotypic adhesion may contribute to dissemination of cells from the primary tumor, increased homotypic adhesion observed in circulating multicellular aggregates, also known as emboli, is required for lodgment, attachment, and growth of metastatic cells (43, 44). Positive correlations have been demonstrated between the propensity of tumor cells to undergo homotypic aggregation *in vitro* and their metastatic potential *in vivo* (45–47). Although only latter events of tumor cell metastasis (after tumor cell penetration into the blood

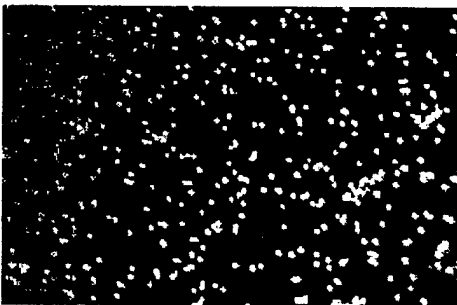
A. HRG 0 ng/ml



B. HRG 5 ng/ml

C. HRG 5 ng/ml + PD 50  $\mu$ MD. HRG 5 ng/ml + PD 100  $\mu$ M

E. HRG 5 ng/ml + WN 50 nM



F. HRG 5 ng/ml + WN 100 nM

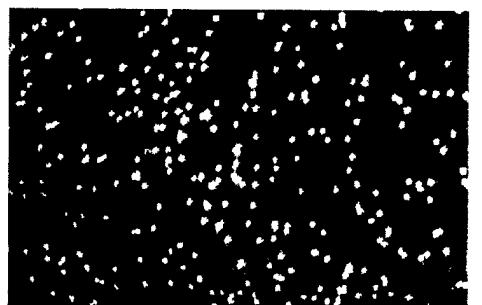
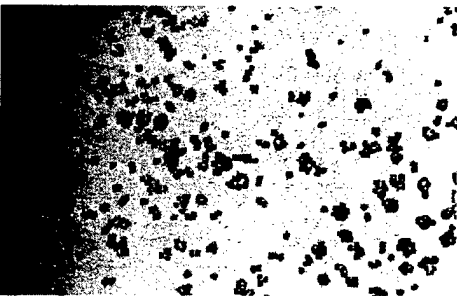
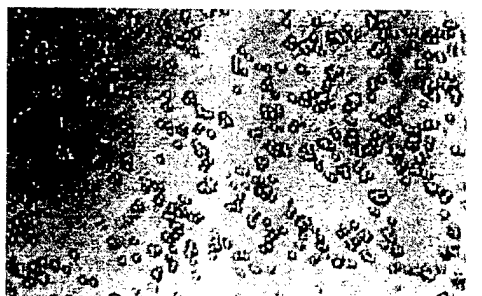
G. HRG 5 ng/ml + LY 30  $\mu$ MH. HRG 5 ng/ml + LY60  $\mu$ M

Fig. 5. HRG- $\beta$ -enhanced MCF-7 cell aggregation was inhibited by wortmannin and LY294002, but not by PD98059. Cells were pretreated with indicated concentrations of PD98059 for 2 h or wortmannin or LY294002 for 1 h, detached from tissue culture dishes using 10 mM EDTA and 0.1% BSA in PBS, and washed with DMEM/F12 serum-free medium. Single-cell suspensions (500  $\mu$ l;  $5 \times 10^4$  cells/ml in DMEM/F12 medium containing 0.5% BSA) in the presence or absence of 5 ng/ml HRG- $\beta$  were plated into wells of the 24-well low-binding affinity plate. The plate was incubated at 4°C on a rotating platform for 30 min. PD, PD98059; WN, wortmannin; LY, LY294002. Photographs were taken using a Nikon N6006 camera (magnification,  $\times 200$ ).

vessels) were tested in these previous studies, the work provided evidence that homophilic cell aggregation has an important role in tumor cell invasion and metastasis. The overall net effect of HRG- $\beta$  on human breast cancer cell invasion/metastasis remains unclear, the ongoing studies in our laboratory will continue to focus on this issue.

Our finding that PI3K mediates MCF-7 cell aggregation enhanced by HRG- $\beta$  identified PI3K as a new target in modulating human breast cancer cell aggregation. It may provide another clue to the control of human breast cancer cell invasion and metastasis.

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