Int. J. Cancer: **100,** 642–653 (2002)

Publication of the International Union Against Cancer

HEREGULIN INHIBITS PROLIFERATION VIA ERKs AND PHOSPHATIDYL-INOSITOL 3-KINASE ACTIVATION BUT REGULATES UROKINASE PLASMINOGEN ACTIVATOR INDEPENDENTLY OF THESE PATHWAYS IN METASTATIC MAMMARY TUMOR CELLS

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Heregulin (HRG) and type I receptor tyrosine kinase (RTK) expression was investigated in the highly invasive and metastatic LM3 cell line, our previously described model of metastasis for mammary cancer (Bal de Kier Joffe *et al.* [1986] Invasion Metastasis 6:302–12; Urtreger *et al.* [1997] Int J Oncol 11:489–96). Although LM3 cells do not express HRG, they exhibit high levels of ErbB-2 and ErbB-3 as well as moderate expression of ErbB-4. Addition of exogenous HRGβ1 resulted in inhibition of both proliferation and migration of LM3 cells. HRGβ1 was also able to decrease the activity of urokinase-type plasminogen activator (uPA) and matrix metalloproteinase 9 (MMP-9), 2 key enzymes in the invasion and metastatic cascade. HRGβ1 treatment of LM3 cells induced tyrosine phosphorylation of ErbB-2, ErbB-3 and ErbB-4 as well as the formation of ErbB-2/ErbB-3 and ErbB-2/ErbB-4 heterodimers. Assessment of the signaling pathways involved in HRGβ1 action indicated that the addition of HRGβ1 to LM3 cells resulted in activation of phosphatidylinositol 3- kinase (PI-3K) and in strong induction of the association of the p85 subunit of PI-3K with ErbB-3. HRGβ1 also caused the rapid activation of ERK1/ERK2 and Stat3 and Stat5 (signal transducers and activators of transcription [STAT]). This is the first demonstration of the ability of HRGβ1 to activate STATs in mammary tumor cells. Blockage of PI-3K activity with its chemical inhibitor wortmannin, or of MEK1/ERKs activity with PD98059, resulted in suppression of the ability of HRGβ1 to inhibit LM3 cell growth. Notwithstanding the suppression of these 2 signaling pathways, HRGβ1 still proved capable of inhibiting uPA activity. Therefore, our results provide evidence that signaling pathways involved in HRGβ1-induced proliferation appear to be distinct from those involved in HRGβ1 regulation of uPA, a protease that plays a pivotal role in invasion and metastasis. © *2002 Wiley-Liss, Inc.*

Key words: *heregulin; ErbB receptors; metastatic mammary tumors; ERKs; phosphatidylinositol 3-kinase*

The high mortality rate associated with breast cancer is mainly due to its ability to invade and metastasize distant sites. Tumor invasion and the development of metastasis entails a complex and multistep process in which unique properties are acquired by tumor cells. These properties include limitless growth, alterations in cell communication, enhanced motility and the capacity to degrade basement membrane components, to invade tissues and to grow autonomously at secondary sites.[1] Accumulating evidence shows that growth factors (GFs) are able to regulate most of these biologic behaviors.[2]

The neu differentiation factor 1 (NDF1) or heregulin (HRG) family includes a series of polypeptides generated by differential splicing of a single primary transcript.[3,4] All HRG isoforms share an epidermal growth factor (EGF)-like motif that acts as the receptor binding domain.[3,4] The structure of this motif reveals 2 major subclasses of HRG variants, α and β.[5] Recently, 2 other families of heregulins have been described, NDF2 and NDF3, encoded by different genes.[6,7] NDF1/HRG exerts multiple physiologic actions through a unique combinatorial signaling resulting from dimerization of the members of the type I receptor tyrosine kinase family (RTKs).[8] Type I RTKs includes 4 members: epidermal growth factor receptor (EGFR/ErbB-1),[9] ErbB-2,[10] ErbB-3[11,12] and ErbB-4.[13] HRG binds to ErbB-3 and ErbB-4.[5] The remaining RTK-I family members, ErbB-1/EGF-R and ErbB-2, act as coreceptors.[8]

The biologic effects of HRG exhibit variations that depend on cell type, HRG isoform and cellular complements of RTK-I members available to make up functional heterodimers. Certain HRG isoforms are reported to induce growth arrest and differentiation of mammary epithelial cells, whereas other breast cells respond mitogenically.[14–16] Also, stimulatory[17,18] and inhibitory effects[19–21] of HRG on the proliferation of breast cancer cell lines, expressing different levels of ErbB-2, have been reported. At the moment, a consensus is emerging on the fact that HRG exerts a growth-inhibitory effect on ErbB-2-overexpressing breast tumor cells.[19–24] HRG treatment of breast cancer cells induces activation of the Erk/MAP kinases,[25,26] Jnk/SAP (stress-activated protein) kinases,[27] p70/p85 S6 kinase[25] and phosphatidylinositol 3-kinase (PI-3K).[26,27] However, the role that each of these signal transduction cascades plays in HRG regulation of proliferation or differentiation remains to be elucidated.

HRG has also been found to regulate several cellular responses associated with breast cancer cell progression to a metastatic phenotype. Thus, HRG was found to promote motility and invasion and to induce cytoeskeletal reorganization of breast cancer cells.[24,28,29] HRG regulation of the expression and activity of proteases capable of degrading the extracellular matrix, such as matrix metalloproteinase-9 (MMP-9) and urokinase-type plasmin-

Grant sponsor: National Scientific Council of Argentina (CONICET); Grant numbers: PID 4188/96 and PID 4199/96; Grant sponsor: National Agency of Scientific Promotion of Argentina; Grant numbers: IDB 802/OC-AR PICT 0503402, IDB 1201/OC-AR PICT 0506114; Grant sponsor: Centro Argentino Brasilero de Biotecnología (CABBIO); Grant sponsor: University of Buenos Aires; Grant sponsor: Beca Ramón Carrillo-Arturo Oñativia.

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Received 26 July 2001; Revised 2 May 2002; Accepted 3 May 2002

DOI 10.1002/ijc.10533

ogen activator (uPA), has also been reported in breast cancer cell lines.[24,28,30,31] Varying results have been found regarding the participation of different signaling pathways in HRG regulation of the metastatic phenotype. Particularly, PI-3K and MAPK activation were found to be involved in HRG regulation of cellular behaviors related to invasion and metastasis such as adhesion, aggregation, migration and regulation of cytoskeletal reorganization.[23,32,33]

The aim of our study was to investigate the expression of HRG and type I RTKs, within our well-characterized model of metastasis for mammary cancer. The LM3 cell line derives from the M3 mouse mammary adenocarcinoma. Like its parental tumor, LM3 cells exhibit a highly invasive behavior and 100% incidence of lung metastasis.[34] In the present study, we found that LM3 cells do not produce HRG and that they overexpress ErbB-2 and ErbB-3. Exogenous HRG-β1 inhibits LM3 cell proliferation as well as uPA and MMP-9 activities. Whereas ERKs and PI-3K signaling pathways are involved in HRGβ1 regulation of growth, they play no role in HRGβ1 inhibition of uPA activation.

MATERIAL AND METHODS

Lm3 cell line and proliferation assays

The LM3 cell line was established in our laboratory.[34] Briefly, it was obtained from primary cultures of the Balb/c transplantable mammary adenocarcinoma M3, metastatic to lung.[35] Upon s.c. injection, LM3 cells produced locally invasive, poorly differentiated adenocarcinoma highly metastatic to the lung.[34] LM3 cells were maintained in MEM supplemented with 2.5% FCS, 2 mM L-glutamine and 80 μg/ml gentamicin. For proliferation assays, cells were plated in multiwell plastic dishes at a density of 20×10^3 in MEM + 2.5% FCS and allowed to attach overnight before treatment with recombinant human HRGβ1 (Neomarkers, Freemont, CA) at concentrations ranging from 0.02 to 40 ng/ml.

In experiments assessing the role of ErbB-4 or ErbB-3 in HRG-induced proliferation, cells were preincubated with 10 μg/ml of either an ErbB-4 mouse monoclonal antibody (Oncoprotein Ab-3, clone H4.72.8, Neomarkers) or an ErbB-3 mouse monoclonal antibody (Oncoprotein Ab-5, clone H3.105.5, Neomarkers) prior to HRG treatment. As control, cells were also incubated with preimmunne mouse serum. After 24 hr of incubation, 50% of media was replaced by fresh media and cells were incubated for another 24 hr in the presence of 0.2 μCi of ^{3}H-thymidine (NEN, Dupont, Boston MA; specific activity: 70–90 Ci/mmol). Cells were then trypsinized and harvested. Assays were performed in octuplicate. The differences between control and experimental groups were analyzed by ANOVA followed by Tukey's *t*-test between groups.

In previous experiments we demonstrated that thymidine uptake correlates with the number of cells/well. In some experiments, cell growth was evaluated by protein content, using the MTS assay, as previously described.[34] We have already proved the existence of a direct correlation between cell number and protein content.[34] To study the effect of HRGβ1 on plating efficiency (clonogenic assay), 2×10^3 monodispersed LM3 cells were seeded on 60 mm plastic dishes in MEM medium with 2.5 % FCS. At 24 hr, cells were treated with different concentrations of HRGβ1 in the presence of 2.5% FCS. Medium was changed every 72 hr. After 8 days of culture, plates were washed, fixed with 5% acetic acid in methanol and stained with hematoxylin and the number of colonies was counted under inverted microscope. Clonogenic ability was defined as the percentage of cells able to grow as colonies of more than 10 cells.

Migration assay

A wound assay was employed to study the effect of HRGβ1 on LM3 cell migration. Wounds 400 μm wide were made in confluent LM3 monolayers. Cells were treated with varying concentrations of HRGβ1 in the presence of 2.5% FCS and allowed to migrate into the cell-free area. At 24 hr, cells were fixed and stained with Giemsa and the cell-free area was quantitated by densitometry. We also performed migration assays using Transwell cell culture chambers (8 μm membrane pore; Corning Costar, Corning, NY). Each filter was coated with 0.1% gelatin on the lower side and dried for 3 hr and then 2×10^5 LM3 cells, in 180 μl serum-free MEM, were seeded on the upper surface of the chamber. The wells contained 0.5 ml MEM and 8 μg/ml human fibronectin as a chemoattractant. Cells were incubated for 20 hr at 37°C in a CO_2 incubator in the presence or absence of 20 ng/ml of HRG.

At the end of the incubation period, only cells that had passed through the filter pores and attached on the lower surface of the filter were considered to have migratory ability. Cells on the upper surface of the filter were completely removed by wiping with a cotton swab. Membranes were fixed with Carnoy's fixative and stained with Hoescht 33258. The nucleus of those cells that migrated was counted under a fluorescence microscope. At least 20 fields of ×400 per membrane were counted and the mean value was employed to perform the statistical analysis using Student's *t*-test. Assays were done in triplicate.

Urokinase-type plasminogen activator and matrix metalloproteinase 9 activity

The effect of HRGβ1 on both uPA and MMP-9 activity was evaluated in conditioned media. Semiconfluent monolayers of LM3 cells cultured in 35 mm well plates were extensively washed with PBS and then incubated in serum-free MEM in the presence or absence of varying concentrations of HRGβ1 for 24 hr. When the effect of the kinase inhibitors wortmannin or PD98059 was evaluated, cells were pretreated with these inhibitors for 90 min prior to the addition of HRGβ1. Conditioned media were individually harvested and the remaining monolayers were lysed to measure protein content.[34] Samples were stored at −70°C and used only once after thawing.

A radial caseinolytic method of Saksela[36] was used to quantify uPA activity. uPA activities were referred to a urokinase standard curve ranging from 0.05 to 10 IU/ml and normalized to the original cell culture protein content. Specificity of uPA activity was determined by blocking sample activity with anticatalytic uPA antibodies (kindly provided by G. Hansen, Righospitalet, Copenhagen, Denmark) or with 1 μM amiloride. Plasminogen-free casein-agarose gels were used to test plasminogen-independent activity. MMP-9 activity was studied by measuring the collagenolytic activity secreted by LM3 cells by SDS-PAGE copolymerized with 0.1% gelatin, as previously reported.[37] After running, gels were washed in 2% Triton X-100 and incubated for 72 hr in 0.25 M Tris-HCl/1 M NaCl/25 mM $CaCl_2$ (pH 7.4) buffer for specific activity detection, or in the same solution plus 40 mM EDTA to detect nonspecific activity. Gels were fixed and stained with Coomassie blue. Activity bands were visualized by negative staining.

RNAse protection assay

The HRG probe was the 333 bp HRG cDNA fragment cloned in a pCRII vector (Invitrogen, La Jolla, CA) and linearized with *Hin*dIII to provide the antisense riboprobe.[38] The rpL32 human cDNA clone (encoding ribosomal protein L32)[39] was provided by Dr. R. Rochford (The Scripps Research Institute, La Jolla, CA). This recombinant clone, constructed in a pGem 4 vector (Promega, Madison, WI), was linearized with *Eco*IR, providing a template for the antisense riboprobe protecting a 76 bp mRNA fragment. Antisense ^{32}P-labeled RNA probes were transcribed with T7 RNA polymerase following the manufacturer's protocol (Promega). Thirty micrograms of total RNA, isolated as described,[40] were hybridized with 2×10^5 cpm of each probe in the conditions previously described,[38] followed by digestion with RNase A (Sigma, St. Louis, MO). All RNA samples were hybridized simultaneously with HRG and rpL32 probes (used to correct for small variations in the amount of RNA loaded). Samples were extracted with phenol/chloroform/isoamyl alcohol (20:20:1) and precipitated with 20 μg of tRNA (Sigma) and 2 vol of absolute ethanol. The pellets were resuspended in 5 μl of an 80% formamide loading buffer and run on a 7.5% polyacrylamide sequencing gel with 8 M

urea. Size markers were prepared by end labeling *Msp*I-digested fragments of pBR322 plasmid.

Type I RTK expression, tyrosine phosphorylation and heterodimerization

To study RTK expression, LM3 cells were lysed in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis (β-aminoethyl ether)-N-N-N′-N′ tetraacetic acid (EGTA), 10% glycerol, 0.5% Nonidet P-40, 1 mM $ClMg_2$ 1mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 1 mM sodium orthovanadate, 5 mM NaF, 20 mM sodium molybdate and 5 mM sodium pyrophosphate. Lysates were centrifuged at 40,000*g* for 40 min at 4°C and the protein content in the supernatant was determined using a Bio-Rad (Richmond, CA) kit. Proteins were solubilized in sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 0.01% bromophenol blue) and subjected to SDS-PAGE on a 6% gel. Proteins were electroblotted onto nitrocellulose. Membranes were blocked with PBS, 0.1% Tween 20 (PBST) and immunoblotted with the following antibodies: ErbB-2 rabbit polyclonal antibody Neu C-18 (Santa Cruz Biotechnology, Santa Cruz, CA), ErbB-3 rabbit polyclonal antibody C-17 (Santa Cruz Biotechnology), ErbB-4 rabbit polyclonal antibody C-18 (Santa Cruz Biotechnology) and ErbB-4/HER-4 Oncoprotein Ab-2 rabbit polyclonal antibody (Neomarkers). After washing, the membranes were incubated with HRP-conjugated secondary antibody (Amersham International, Buckinghamshire, UK). Enhanced chemiluminescence (ECL) was performed according to the manufacturer's instructions (Amersham). To perform RTK tyrosine phosphorylation analysis, lysates from LM3 cells treated or untreated with HRGβ1 were prepared as described above. In experiments assessing the role of blocking antibodies on HRG-induced tyrosine phosphoryation, cells were preincubated with 10 μg/ml of either an ErbB-4 mouse monoclonal antibody (Oncoprotein Ab-3, clone H4.72.8, Neomarkers) or am ErbB-3 mouse monoclonal antibody (Oncoprotein Ab-5, clone H3.105.5, Neomarkers) prior to HRG treatment. As control, cells were also incubated with preimmunne mouse serum. All lysates (1 mg protein) were precleared with Protein A-Agarose (Santa Cruz Biotechnology). Two to 5 μg of either ErbB-2 Neu C-18, ErbB-3 C-17 (Santa Cruz Biotechnology) or ErbB-4 Ab-2 (Neomarkers) were used in each immunoprecipitation, which was rocked for 2 hr at 4°C. Thereafter, the immunocomplexes were captured by adding Protein A-Agarose and rocked for an additional 2 hr. Beads were washed 3 times with lysis buffer and then boiled for 10 min in sample buffer and subjected to SDS-PAGE on a 6% gel. Proteins were electroblotted onto nitrocellulose and filters were probed with mouse monoclonal Anti-P-Tyr PY-99 (Santa Cruz Biotechnology). Proteins were visualized with HRP-conjugated secondary antibody, using ECL detection (Amersham). Identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with anti-ErbB-2, anti-ErbB-3 or anti-ErbB-4 antibodies to verify that nearly equal amounts of immunoprecipitated proteins were loaded. To study type I RTK association, 1 mg protein from LM3 cell lysates was immunoprecipitated with the ErbB-2 antibody as described. Immnunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose. Filters were probed with either the ErbB-3 or the ErbB-4 antibody. ErbB-1/EGF-R receptor binding assay was performed as previously described.[41]

PI-3K activation

Lysates (1 mg protein) from LM3 cells treated or untreated with HRGβ1 were immunoprecipitated with 4 μg of a mouse monoclonal anti-P-Tyr PY-99 antibody (Santa Cruz Biotechnology). Immunocomplexes were subjected to SDS-PAGE (7.5% gel) and analyzed by Western blotting with an anti-p85 antibody (Neomarkers, clone UB93-3). To study HRGβ1 activation of PI-3K enzymatic activity in intact LM3 cells, subconfluent cultures of LM3 cells treated or untreated with HRGβ1 were prelabeled for 24 hr in serum-free MEM containing 1 mg/ml albumin and 4 μCi/ml [2-^{3}H]inositol (Dupont-New England Nuclear, 14.6–15.2 Ci/mmol). At the end of the prelabeling period, cells were extensively washed and then incubated for 10 min in serum-free MEM containing 1 mg/ml albumin in the presence or absence of HRGβ1 (20 ng/ml) or insulin-like growth factor-I (IGF-I, 50 ng/ml). When appropriate, cells were preincubated with wortmannin (500 mM) or LY294002 for 90 min prior to stimulation with HRGβ1. At the end of the incubation with GFs, the dishes were plated on ice, the medium was aspirated and the cells were covered with cold methanol/concentrated HCl (100:1). Cells were scraped off the dishes and transferred to glass tubes. After washing the dishes with an additional aliquot of the extraction solution, the extract was mixed with an equal volume of chloroform and the phases were separated, washed and dried as described before.[42] Phosphatidylinositol was separated by thin-layer chromatography developed with $CHCl_3/CH_3OH$ / NH_4OH 9.15 M (40:40:15) as described.[42] Phosphorylated products were visualized by autoradiography. To study PI-3K association with ErbBs, protein extracts (1 mg) from LM3 cells treated with HRGβ1 for the indicated periods were immunoprecipitated with anti-ErbB-3, -ErbB-2 or -ErbB-4 antibodies as described above and Western blotting was performed with an anti-p85 antibody (Neomarkers). Protein extracts (100 μg total protein) were blotted in parallel with the anti-p85 antibody.

ERK1/2 and STATs activation

LM3 cells were left untreated or treated with 20 ng/ml of HRG-β for varying periods and 100 μg protein from cell lysates were electrophoresed on 12% SDS gels and immunoblotted with an anti-phospho ERK1/ERK2 monoclonal antibody (E-4, Santa Cruz Biotechnology). Membranes were stripped and hybridized with an antibody anti-total ERK1/ERK2 (C-14, Santa Cruz Biotechnology). When appropriate, LM3 cells were pretreated with PD98059 (10 μM) or with its solvent DMSO for 90 min before stimulation with HRGβ1 20 ng/ml for 10 min. To study Stat3 activation, 100 μg of protein from cells treated as described above were electrophoresed and immunoblotted with an anti-phospho Stat3 antibody (B-7, Santa Cruz Biotechnology). The membrane was stripped and hybridized with an anti-total Stat3 (C-20). For analysis of Stat5, 1 mg of protein from LM3 cells, treated as described above, was immunoprecipitated with an anti-total Stat5 antibody (C-17), followed by Western blotting with a mouse monoclonal Anti-P-Tyr PY-99 antibody. After stripping, the membrane was probed with the anti-total Stat5 antibody.

To explore the role of ErbB-4 or ErbB-3 in HRG-induced STAT activation, cells were preincubated for 90 min with 10 μg/ml of an ErbB-4 mouse monoclonal antibody (Oncoprotein Ab-3, clone H4.72.8, Neomarkers) or with 10 μg/ml of an ErbB-3 mouse monoclonal antibody (Oncoprotein Ab-5, clone H3.105.5, Neomarkers) prior to HRG treatment. As control, cells were also incubated with preimmune mouse serum.

RESULTS

Expression of HRG and type I RTKs in LM3 cells

HRG and type I RTK expression was investigated in LM3 cells, 1 of the tumor lines of our previously described model of metastasis for mammary cancer.[34,35] The LM3 cell line, obtained from the well-characterized transplantable M3 murine mammary adenocarcinoma,[35] evidences a highly invasive behavior *in vitro* and 100% incidence of lung metastasis when inoculated into Balb/c mice.[34]

To examine HRG expression at the mRNA level, we performed an RNAse protection assay using the homologous probe we previously obtained in cloning the mouse HRG.[38] This probe recognizes both HRGα and HRGβ isoforms. As can be seen in Figure 1, HRG message was not found in LM3 cells. As positive control for HRG expression, we used our well-characterized C4HD cells derived from a murine mammary adenocarcinoma induced by medroxyprogesterone acetate.[38,43]

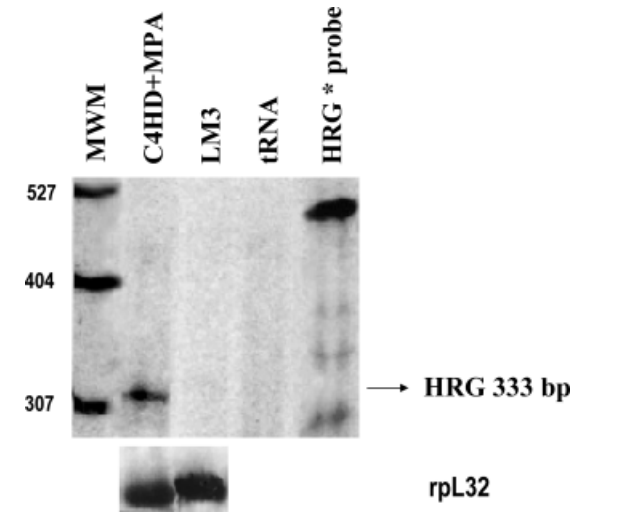


FIGURE 1 – Hereglulin (HRG) expression. HRG RNAse protection assay. Thirty micrograms of total RNA from LM3 cells were hybridized with HRG and rpL32 probes as described in Material and Methods. The HRG-protected fragment is 333 bp, and the rpL32-protected fragment is 76 bp. MWM, molecular weight markers. C4HD cells, derived from a murine mammary adenocarcinoma induced by medroxyprogesterone acetate, were used as positive control.

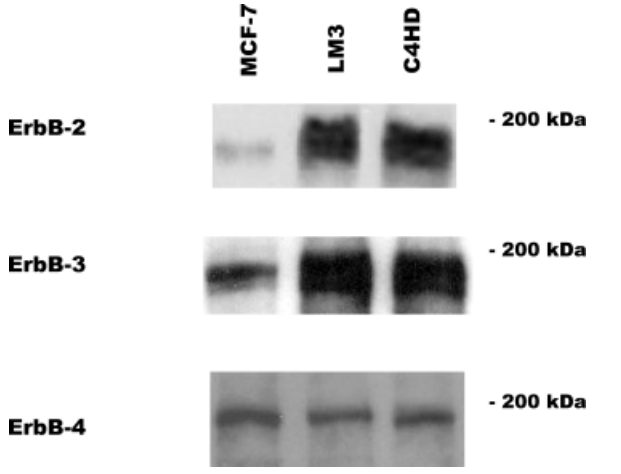


FIGURE 2 – Expression of type I RTKs at the protein level. Eighty micrograms of protein from LM3 cell lysates were electrophoresed and immunoblotted for ErbB-2, ErbB-3 and ErbB-4. MCF-7 human breast cancer cells and C4HD murine mammary adenocarcinoma cells were used as controls for RTK expression levels.

We then investigated ErbB-2, ErbB-3 and ErbB-4 expressions in LM3 cells by Western blot analysis. As control for low ErbB-2 levels, we used the human breast cancer MCF-7 cell line,[44] and for ErbB-2 overexpression, we used C4HD cells.[38,43] LM3 evidenced significantly higher levels of ErbB-2 than MCF-7 cells (3–4-fold), comparable to those present in C4HD cells and those considered as overexpression in human breast cancer cell lines[44] (Fig. 2). LM3 cells also expressed high levels of ErbB-3, comparable to those present in C4HD cells[38,43] and 3–5-fold higher than those expressed by MCF-7 cells (Fig. 2). Our analysis of ErbB-4 expression, using a panel of ErbB-4-specific antibodies, indicated the presence of low ErbB-4 levels in LM3 cells (Fig. 2), similar to those found in C4HD cells.[38,43] EGF-R (ErbB-1) was also present in LM3 cells (46.4 ± 5.1 fmol/mg protein).

HRGβ1 inhibits LM3 cell proliferation

Previous reports demonstrated that HRG growth effects were best observed on low-density cultures.[22] Therefore, we first studied the effect of exogenous HRGβ1 on the proliferation of LM3 cells seeded at low density, using a clonogenic assay. HRGβ1 significantly inhibited clonal LM3 cell growth (median effective dose [ED_{50}]: 0.8 ± 0.18 ng/ml), reducing the number of colonies with more than 10 cells after 8 days of treatment (Fig. 3*a*). We then seeded LM3 cells at higher density and treated them with HRGβ1 for 48 hr. Effects of HRGβ1 on cell growth were evaluated by using a [^{3}H]thymidine incorporation assay. Figure 3*b* shows that HRGβ1 was still able to inhibit LM3 cell proliferation, although the doses required to achieve ED_{50} (10.2 ± 1.5 ng/ml) were higher than those required when cells were seeded at low densities. After 48 hr of HRGβ1 treatment, complete inhibition of growth was observed with an HRGβ1 concentration of 20 ng/ml (Fig. 3*b*). Similar results were obtained when cell growth was evaluated by cell protein content or using an MTS assay (data not shown). HRGβ1 did not stimulate growth of LM3 cells at any of the doses tested (0.02–40 ng/ml).

It has recently been reported that ErbB-4 is both necessary and sufficient to trigger an antiproliferative response in human breast cancer cells.[45] We therefore assessed the role of ErbB-4 in transmitting HRGβ1 antiproliferative signals in LM3 cells. For this purpose, we blocked HRGβ1 binding to ErbB-4 by using a mouse monoclonal ErbB-4 antibody (Ab-3). As shown in Figure 3*c*, addition of the ErbB-4 antibody to HRGβ1-treated LM3 cells resulted in a dose-dependent inhibition of HRGβ1 antiproliferative effects. The ErbB-4 antibody, at the highest dose tested (10 μg/ml), inhibited HRGβ1 antiproliferative response by 60–70% (Fig. 3*c*). To investigate ErbB-3 involvement in HRGβ1 inhibition of LM3 cell growth, we used the same experimental approach. We found that blockage of HRGβ1 binding to ErbB-3, by using the monoclonal ErbB-3 antibody Ab-5, inhibited HRGβ1 antiproliferative response in a dose-dependent fashion (Fig. 3*c*). It is noteworthy that the highest dose of the ErbB-3 antibody used (10 μg/ml) completely abolished any HRGβ1 effect on LM3 cell proliferation (Fig. 3*c*).

HRGβ induces ErbB-2, ErbB-3 and ErbB-4 tyrosine phosphorylation and heterodimerization

HRGβ1-induced antiproliferative response in LM3 cells was inhibited when HRGβ1 binding to ErbB-4 or to ErbB-3 was abolished, suggesting that HRGβ1 activation of these receptors was involved in the antiproliferative effects. Therefore, we examined HRGβ1 effects on the level of tyrosine phosphorylation of ErbB-3 and ErbB-4, as well as on ErbB-2 tyrosine phosphorylation levels, as a marker of activation of these receptors Extracts from LM3 cells treated with HRGβ1 were immunoprecipitated with anti-ErbB-2, ErbB-3 or ErbB-4 antibodies and the phosphotyrosine content of these receptors was determined by performing Western blotting with an anti-phosphotyrosine antibody. A certain degree of ErbB-2 tyrosine phosphorylation was observed in LM3 cells growing in medium without the addition of HRGβ1 (Fig. 4*a*). This is in accordance with previous findings showing that ErbB-2 overexpression may result in its constitutive tyrosine phosphorylation.[46,47] HRGβ1 treatment of LM3 cells induced an increase in ErbB-2 tyrosine phosphorylation as early as 5 min after treatment (Fig. 4*a*). On the other hand, it is noteworthy that HRGβ1 dramatically increased tyrosine phosphorylation of ErbB-3 (Fig. 4*c*). Induction of ErbB-4 tyrosine phosphorylation was also observed after HRG treatment (Fig. 4*e*).

To confirm that inhibition of HRGβ1 binding to ErbB-3 or ErbB-4, by the use of the respective blocking antibody, resulted in the abrogation of HRGβ1 ability to phosphorylate these receptors, we assessed their degree of tyrosine phosphorylation. Therefore, LM3 cells were preincubated with anti-ErB-3 (Ab-5) or ErbB-4 (Ab-3) blocking antibodies prior to HRGβ1 treatment. As seen in Figure 4*c* (lane 5), abolishment of HRG-ErbB-3 binding resulted in the abrogation of HRGβ1-induced ErbB-3 phosphorylation.

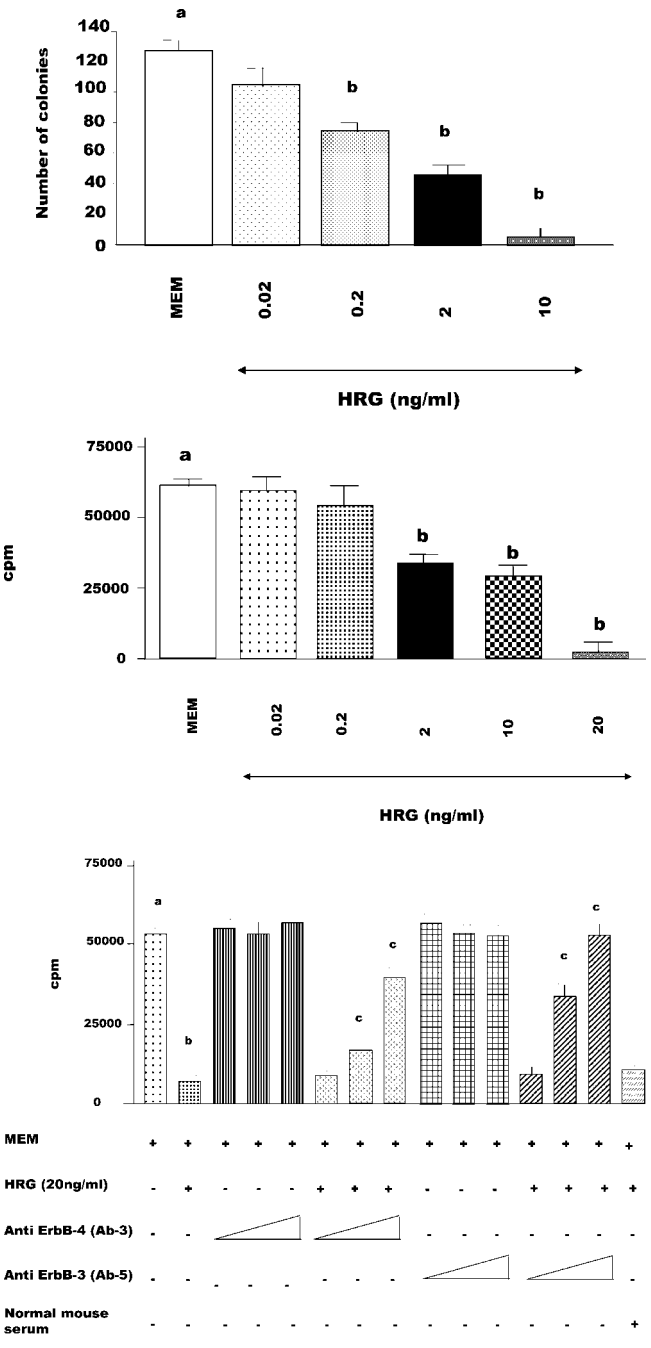


FIGURE 3 – Effects of exogenous hererugulin (HRG)β1 on the proliferation of LM3 cells. (*a*) Clonogenic assay. Monodispersed LM3 cells (2×10^3) were incubated in medium with 2.5% FCS supplemented with HRGβ1 at various concentrations. After 8 days of culture the number of colonies was counted. *b* vs *a*: $p < 0.001$. Data are presented as mean ± SD. The experiment shown is representative of a total of 3. (*b*) LM3 cells (20×10^3) were incubated for 48 hr in medium with 2.5% FCS supplemented with HRGβ1 at various concentrations. Incorporation of [^{3}H]thymidine was used as a measure of DNA synthesis. *b vs. a*: $p < 0.001$. Data are presented as mean ± SD. The experiment shown is representative of a total of 6. (*c*) LM3 cells (20×10^3) were incubated for 48 hr in medium with 2.5% FCS in the presence or absence of 20 ng/ml of HRGβ1. In the indicated lanes, cells were preincubated with increasing concentrations (1, 5 and 10 μg/ml) of either ErbB-4 or ErbB-3 monoclonal antibodies for 90 min before HRGβ1 addition. Incorporation of [^{3}H]thymidine was measured. *b vs. a*; *c vs. b*: $p < 0.001$. As control we used preimmune mouse serum. Data are presented as mean ± SD. The experiment shown is representative of a total of 3.

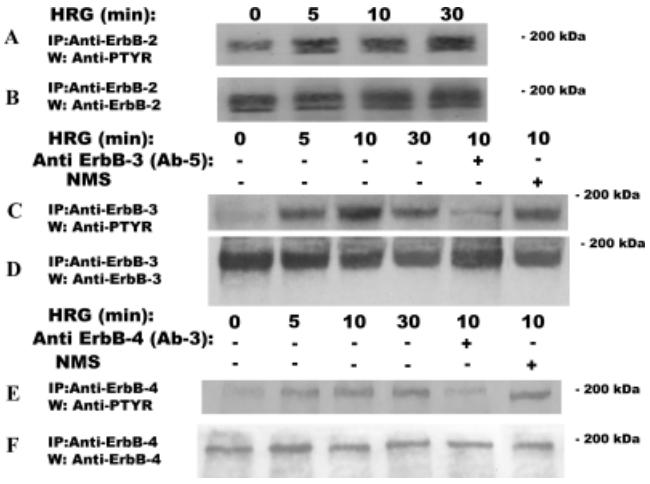


FIGURE 4 – ErbB-2, ErbB-3 and ErbB-4 tyrosine phosphorylation. ErbB-2, ErbB-3 and ErbB-4 were immunoprecipitated from extracts of LM3 cells treated and untreated with HRGβ1 for the indicated periods. To evaluate the role of blocking antibodies on HRGβ1-induced tyrosine phosphorylation, cells were preincubated with 10 μg/ml of either an ErbB-3 mouse monoclonal antibody (Ab-5) or an ErbB-4 mouse monoclonal antibody (Ab-3) prior to HRGβ1 treatment. As control, cells were also incubated with preimmunne mouse serum (NMS). Immunocomplexes were subjected to SDS-PAGE (7.5% gel) and analyzed by Western blotting with an anti-P-Tyr mAb (*a*, *c* and *e*). Identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with anti-ErbB-2 (*b*), anti-ErbB-3 (*d*) or ErbB-4 (*f*) antibodies to verify that nearly equal amounts of immunoprecipitated proteins were loaded. W, Western blot, IP, immunoprecipitation.

Similarly, blockage of HRGβ1 binding to ErbB-4, resulted in inhibition of the ability of HRGβ1 to phosphorylate ErbB-4 (Fig. 4*e*, lane 5). As a whole, results shown in Figures 3*c* and 4 (*c*, lane 5 and *e*, lane 5) indicate that HRGβ1 phoshorylation and therefore activation, of both ErbB-3 and ErbB-4 is involved in mediating HRGβ1 antiproliferative effects in LM3 cells.

We then studied the capacity of HRGβ1 to induce the formation of heterodimers between ErbB-2 and ErbB-3. Although a certain level of complexes between these receptors was found in cells growing in serum-free medium, HRGβ1 was able to promote an increase in the abundance of the complex between ErbB-2 and ErbB-3 within 5 min (Fig. 5). The highest level of complexes was seen at 10 min. HRGβ1 treatment of LM3 cells also resulted in the formation of ErbB-2/ErbB-4 heterodimers with kinetics similar to those found with the ErbB-2/ErbB-3 dimers (Fig. 5).

HRGβ1 inhibits LM3 cell migration

To determine the specific effects of HRGβ1 on the invasive phenotype, we first studied HRGβ1 regulation of LM3 cell motility. An *in vitro* wound assay was performed to analyze the effect of HRGβ1 on LM3 cell migration. As shown in Figure 6*a*, a larger cell-free area in the wound was observed when cells were treated with HRGβ1, indicating its ability to inhibit LM3 cell migration. To confirm this result further, we performed another experimental approach. Thus, we examined HRGβ1 capacity to regulate LM3 cell migration in a Transwell chamber assay. As shown in Figure 6*b*, HRGβ1 treatment of LM3 cells resulted in a significant inhibition of LM3 cell migration.

HRGβ inhibits uPA and MMP-9 activity

Increased expression of proteases capable of degrading the extracellular matrix is a critical component of the invasion and metastasis cascade. Therefore, we investigated HRGβ1 capacity to regulate uPA and MMP-9 activity. We measured the activity of uPA by using a radial caseinolysis assay and the activity of MMP-9 by zymography, in the conditioned medium of LM3 cells.

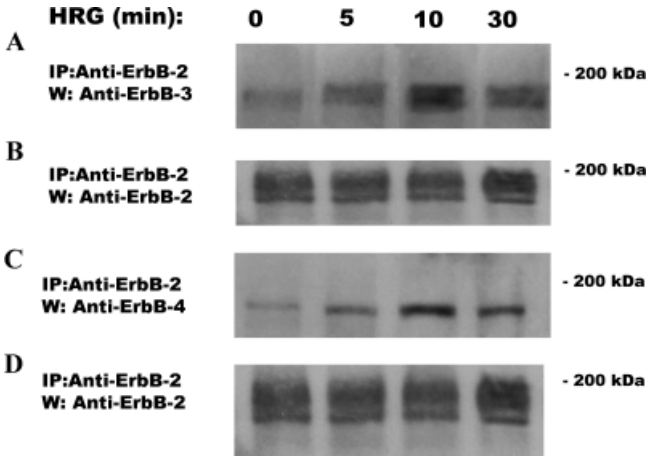


FIGURE 5 – ErbB-2, ErbB-3 and ErbB-4 heterodimerization. ErbB-2 was immunoprecipitated from LM3 cell extracts, and immunocomplexes were subjected to SDS-PAGE (7.5% gel) and analyzed by Western blotting with anti-ErbB-3 (*a*) or -ErbB-4 (*c*) antibodies. Identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with anti-ErbB-2 (*b* and *d*) antibody to verify that nearly equal amounts of immunoprecipitated protein were loaded. W, Western blot, IP, immunoprecipitation. HRG, heregulin.

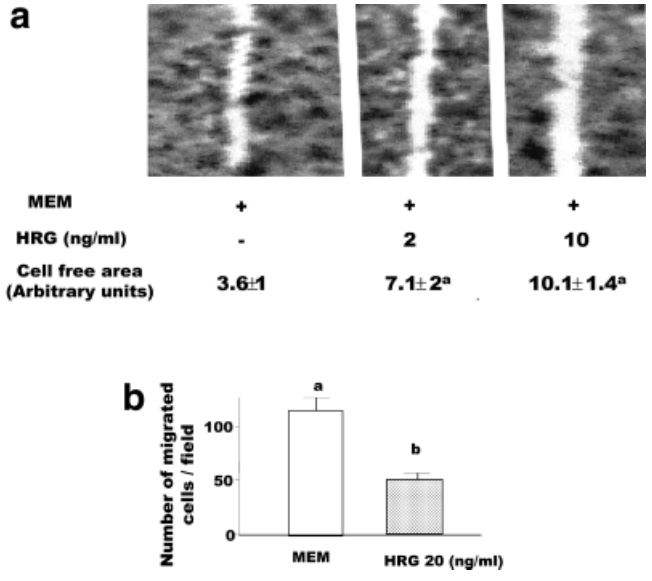


FIGURE 6 – Effects of heregulin (HRG)β1 on LM3 cell migration. Wounds 400 μM wide were made in confluent LM3 cell monolayers. Cells were treated with HRGβ1 at various concentrations and allowed to migrate into the cell-free area for 24 hr. The cell-free area was measured by densitometry and expressed as arbitrary units. (*a*) $p < 0.01$ with respect to cells growing in MEM without HRGβ1 supplementation. Shown is a representative experiment of a total of 3 that gave similar results. (*b*) Migration assay using Transwell cell culture chambers. At least 20 fields of ×400 per membrane were counted. *b vs. a*: $p < 0.001$. Data are presented as mean ± SD. Assay was done in triplicate. The experiment shown is representative of a total of 3.

Figure 7*a* and *b* shows that HRGβ1 induced a dose-dependent inhibition of the activity of both enzymes.

Activation of PI-3K, ERK1/ERK2 and STATs by HRGβ

Activation of PI-3K has emerged as a critical component in a variety of cellular functions regulated by HRG such as proliferation,[23] cytoskeletal reorganization,[28,29] induction of cell aggregation,[48] adhesion and migration.[28,49] We therefore investigated whether PI-3K plays any role in HRGβ1 signaling pathways in our experimental system. Stimulation of LM3 cells with HRGβ1 resulted in a rapid recruitment of the p85 subunit of PI-3K to the phosphotyrosine-containing cellular fraction, consistent with previous reports of growth factor-stimulated PI-3K activation in other cell lines (Fig. 8*a*).[49,50] To assess the effect of HRGβ1 on PI-3K activation in intact cells, we prelabeled LM3 cells for 24 hr in MEM containing [2-^{3}H]inositol and then incubated them in the presence or absence of HRGβ1 (20 ng/ml). Because it has already been shown that IGF-I activates PI-3K,[51] we also treated LM3 cells with IGF-I as a positive control. Figure 8*b* shows that PI-3K activity was greatly increased by 10 min of HRGβ stimulation of LM3 cells, revealed by the appearance of Ptdlns-3-P, Ptdlns-3,4-P_2 and Ptdlns-3,4,5-P_3. Wortmannin, a specific chemical inhibitor of PI-3K, at a concentration of 500 nM, completely blocked HRGβ1-induced PI-3K activation (Fig. 8*b*).

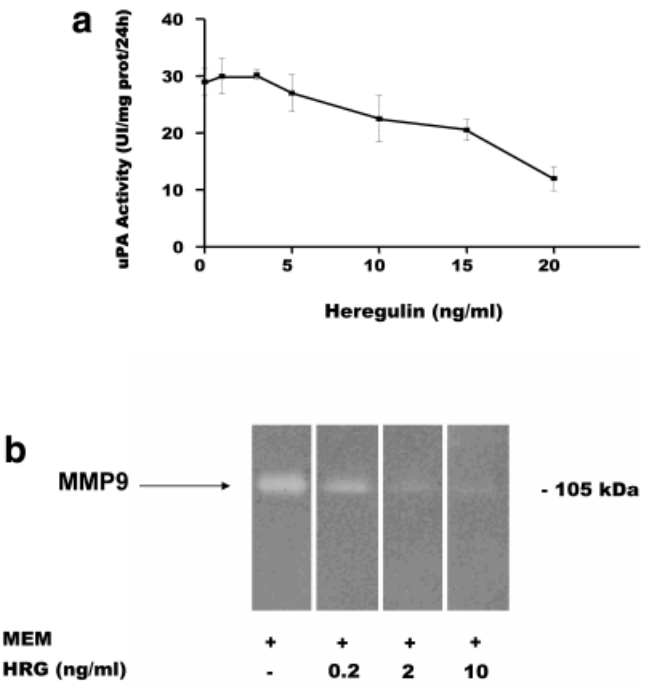
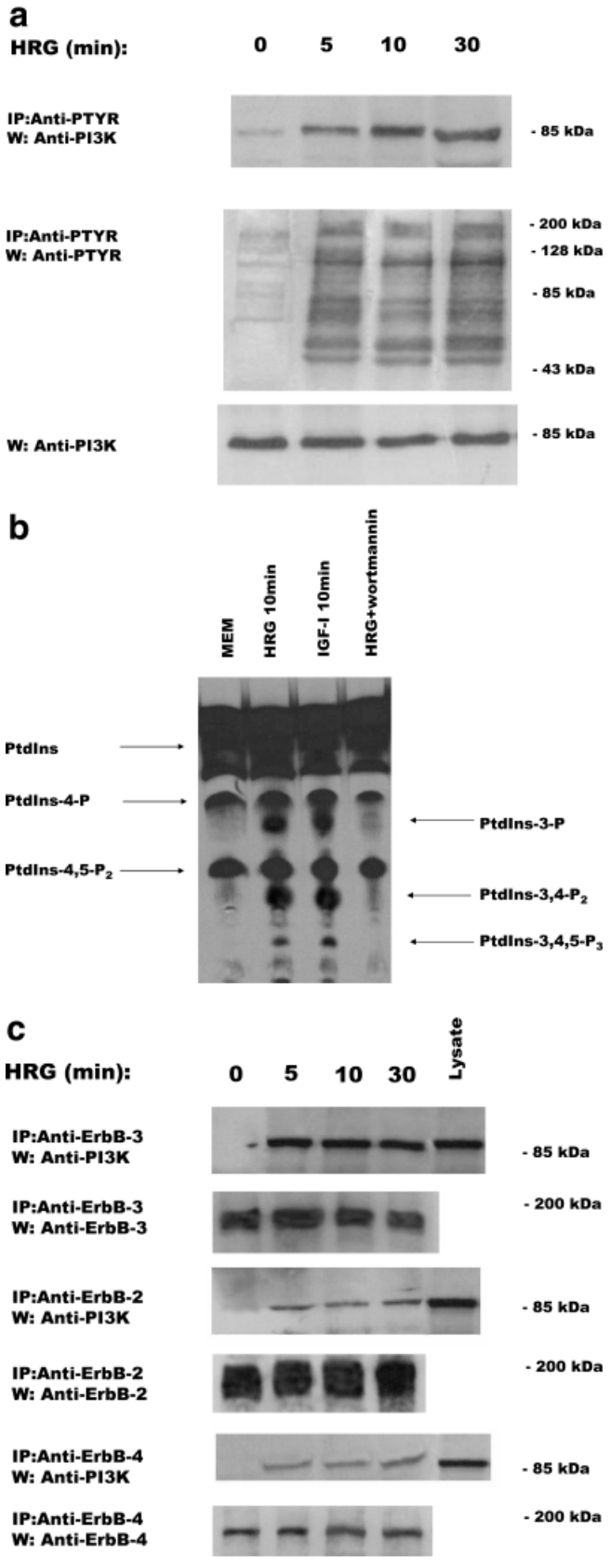


FIGURE 7 – Effect of heregulin (HRG)β1 on uPA and MMP-9 activity. Semiconfluent monolayers of LM3 cells were incubated in serum free-medium for 24 hr with and without the addition of HRGβ1 at various concentrations. Conditioned media were collected, and the remaining monolayers were lysed to measure protein content. (*a*) uPA activity was quantified by a radial caseinolytic assay. Data are presented as mean ± SD. Shown is a representative experiment of a total of 4. (*b*) MMP-9 activity was detected by zymography. The experiment shown is representative of a total of 4.

We then investigated the association of the p85 subunit of PI-3K with ErbB-2, ErbB-3 and ErbB-4. Extracts from LM3 cells treated with HRGβ1 were immunoprecipitated with anti- ErbB-2, -ErbB-3 or -ErbB-4 antibodies and Western blotting was performed with an anti-p85 antibody (Fig. 8*c*). HRG-β stimulation greatly induced p85 association with ErbB-3 (Fig. 8*c*). A moderate induction of the association of p85 with ErbB-2 and ErbB-4 was also seen (Fig. 8*c*).

HRG is also known to stimulate ERK1/ERK2.[25,26] Therefore, we next examined the effect of HRGβ on the activity of ERK1/ERK2, using antisera specific for the dually phosphorylated, active form of this kinase. As shown in Figure 9*a*, HRGβ treatment resulted in the rapid activation of ERK1/ERK2 that began to increase after 5 min, reached its highest levels at 10 min and remained sustained for at least 30 min. Activation of ERKs by



HRGβ was suppressed when cells were treated with PD98059 (10 μM), a specific inhibitor of MEK1 (Fig. 9b*)*.

The involvement of the different members of the type I RTK family in the activation of STAT proteins remains poorly studied. We therefore sought to determine whether HRGβ1 treatment of LM3 cells induced activation of STATs. As can be seen in Figure 10*A* (*a*), HRGβ1 induced a strong tyrosine phosphorylation of Stat3 that was maximal after 10 min of treatment and remained at the same high level after 30 min. To investigate the effect of HRGβ1 on Stat5 activation, we immunoprecipitated LM3 cell extracts with a polyclonal antibody that reacts with both Stat5a and 5b and performed Western blot with an anti-phosphotyrosine antibody. Figure 10*A* (*c*) shows that HRGβ1 induced an increase in Stat5 phosphotyrosine content. It has been previously shown that ErbB-4 expression is required for Stat5b activation in response to HRG.[52] Therefore, to explore the role of ErbB-4 in HRGβ1-induced activation of Stat3 and Stat5, we blocked HRGβ1 binding to ErbB-4 by using the monoclonal antibody Ab-3. Disruption of HRGβ1 binding to ErbB-4 dramatically inhibited HRGβ1-induced phosphorylation of both Stat3 (Fig. 10*A*, *a*) and Stat5 (Fig. 10*A*, c*)*.

The same experimental approach was performed to explore ErbB-3 involvement in the mechanism of HRGβ1 activation of Stat3 and Stat5. We found that blockage of HRGβ1 binding to ErbB-3, by using the anti-ErbB-3 monoclonal antibody Ab-5, resulted in inhibition of the ability of HRGβ1 phosphorylate Stat3 (Fig. 10*A*, *a*) and Stat5 (Fig. 10*A*, *c*). To determine that inhibition of HRGβ1 binding to ErbB-4 resulted in the abolishment of the HRG-ErbB-4 signaling pathway, we assessed the degree of tyrosine phosphorylation of ErbB-4 in LM3 cells in which HRGβ1 binding to ErbB4 had been blocked by the anti-ErbB-4 monoclonal antibody Ab-3. As seen in Figure 10*B*, *a*, abolishment of HRG-ErbB-4 binding resulted in abrogation of HRGβ1-induced ErbB-4 phosphorylation. Similarly, blockage of HRGβ1 binding to ErbB-3 by the ErbB-3 antibody Ab-5 resulted in the abrogation of HRGβ1 capacity to phosphorylate ErbB-3 (Fig. 10*B*, *c*). Therefore, our findings show that HRGβ1 binding to and activation of both ErbB-3 and ErbB-4 are required for HRGβ1-induced activation of Stat3 ands Stat5 in LM3 cells.

FIGURE 8 – Heregulin (HRG)β1 induces PI-3K activation and association with ErbBs. (*a*) HRGβ1 induces recruitment of the p85 subunit of PI-3K to the phosphotyrosine cellular fraction. LM3 cells incubated in the presence or absence of 20 ng/ml HRGβ1 for the indicated periods were lysed, and phosphotyrosine-containing proteins were immunoprecipitated with an anti-P-Tyr (PTYR) MAb. Immunocomplexes were subjected to SDS-PAGE (7.5% gel) and analyzed by Western blotting with anti-p85 antibody . In the middle panel, identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with an anti-P-Tyr MAb. In the lower panel, 100 μg protein from cell lysates was subjected to SDS-PAGE and analyzed by Western blotting with an anti-p85 antibody to show identical levels of the p85 PI-3K subunit in lysates from cells treated and untreated with HRGβ1. Shown is a representative experiment of a total of 3. IP, immunoprecipitation; W, Western blot. (*b*) HRG activates PI-3K enzymatic activity *in vivo.* Subconfluent cultures of LM3 cells were prelabeled for 24 hr in serum-free MEM containing 4 μCi/ml [2-^{3}H]inositol. At the end of the prelabeling period, cells were extensively washed and then incubated for 10 min in serum-free MEM in presence or absence of HRG (20 ng/ml) or insulin-like growth factor-I (IGF-I; 50 ng/ml, used as positive control for PI-3K activation). In addition, cells were preincubated with wortmannin (500 mM) for 90 min prior to stimulation with HRGβ1 (fourth lane). Phospholipids were extracted and separated by TLC chromatography as described in Material and Methods. The position of commercial standards (left) and D3-phosphoinositides (right) are indicated. Shown is a representative experiment of a total of 3. (*c*) HRG induces the association of the p85 subunit of PI-3K with ErbB-2, ErbB-3 and ErbB-4. Extracts from LM3 cells treated with HRGβ1 for the indicated periods were immunoprecipitated with anti- ErbB-3, -ErbB-2 or -ErbB-4 antibodies, and Western blotting was performed with an anti-p85 antibody. The lanes marked "lysate" show LM3 cell lysates blotted in parallel with anti-p85 antibody.

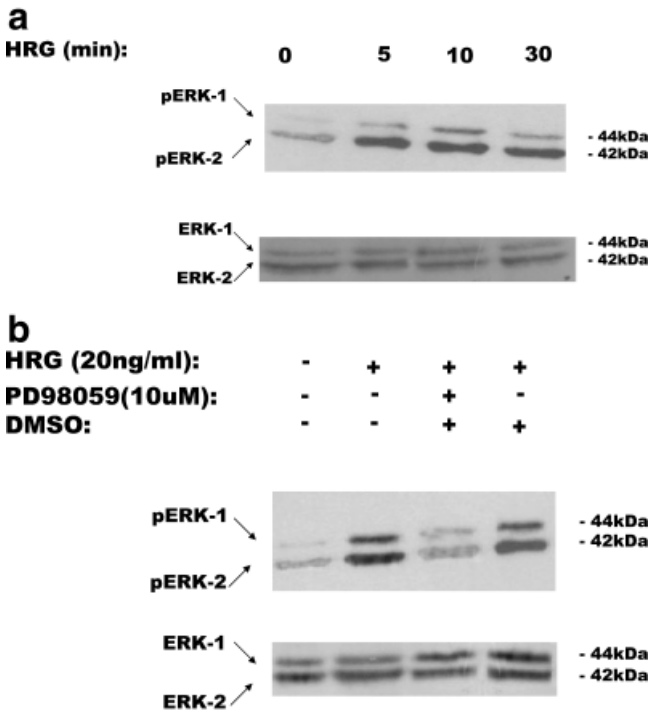


FIGURE 9 – Activation of ERKs by heregulin HRGβ1 (*a*) LM3 cells were treated with HRGβ for the indicated periods, and 100 μg protein from cell lysates was electrophoresed on 12% SDS gels and immunoblotted with an anti-phospho ERK1/ERK2 antibody (upper panel). The membrane was stripped and hybridized with anti-total ERK1/ERK2 antibody (lower panel). (*b*) LM3 cells were pretreated with PD98059 or its solvent DMSO for 90 min before stimulation with HRG 20 ng/ml for 10 min. Cell lysates were electrophoresed and immunoblotted with an anti-phospho ERK1/ERK2 antibody (upper panel). The membrane was stripped and hybridized with an anti-total ERK1/ERK2 antibody (lower panel).

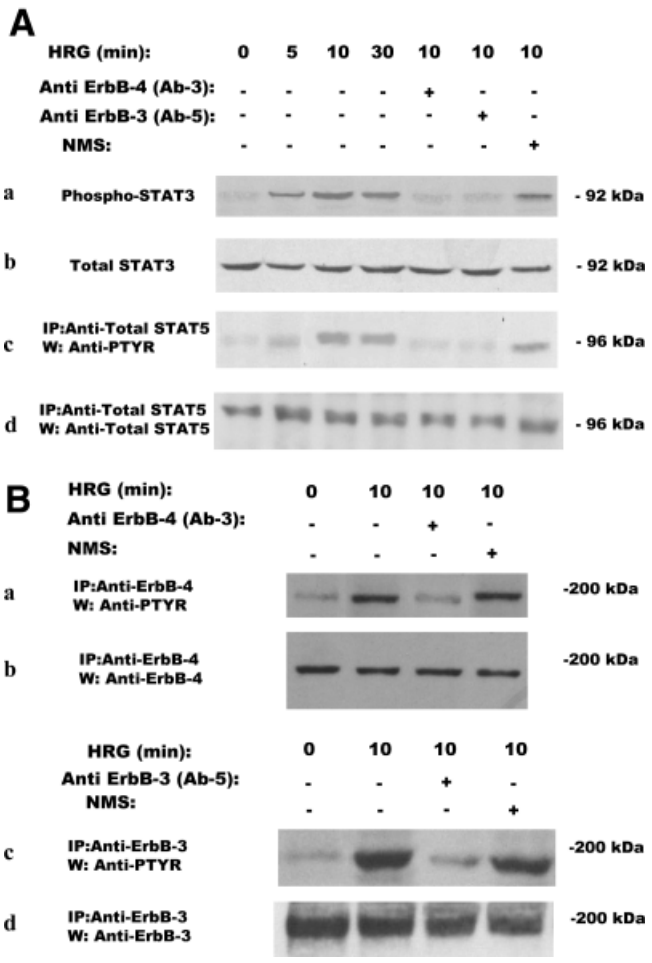


FIGURE 10 – Heregulin (HRG)β1 activates Stat3 and Stat5. (*A*) LM3 cells were treated with 20 ng/ml HRGβ1 for the indicated periods or were preincubated with 10 μg/ml of mouse monoclonal ErbB-4 (Ab-3) or ErbB-3 (Ab-5) antibodies or normal mouse serum for 90 min and then were treated with HRG for 10 min. (*a*) Proteins from cell lysates (100 μg) were electrophoresed and immunoblotted with an anti-phospho Stat3 antibody. (*b*) The membrane was stripped and hybridized with an anti-total Stat3. (*c*) Proteins from cell lysates (1 mg) were immunoprecipitated with an anti-total Stat5 antibody followed by Western blotting with an anti-phosphotyrosine antibody. (*d*) After stripping, the membrane was probed with the anti-total Stat5 antibody. (*B*) (*a*) Inhibition of HRG-mediated tyrosine phosphorylation of ErbB-4 by the ErbB-4 monoclonal antibody Ab-3. Protein (1 mg) from lysates of LM3 cells, treated with HRG or preincubated with the ErbB-4 monoclonal antibody Ab-3, or with normal mouse serum prior to HRG treatment, was immunoprecipitated with a rabbit polyclonal antibody to ErbB-4, and immunocomplexes were analyzed by Western blotting with an anti-P-Tyr MAb. (*b*) Identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with the anti-ErbB-4 rabbit polyclonal antibody to verify that nearly equal amounts of immunoprecipitated proteins were loaded. (*c*) Inhibition of HRG-mediated tyrosine phosphorylation of ErbB-3 by the ErbB-3 monoclonal antibody Ab-5. Protein (1 mg) from lysates of LM3 cells, treated with HRG or preincubated with the ErbB-3 monoclonal antibody Ab-5, or with normal mouse serum prior to HRG treatment, was immunoprecipitated with a rabbit polyclonal antibody to ErbB-3, and immunocomplexes were analyzed by Western blotting with an anti-P-Tyr MAb. (*d*) Identical aliquots of each immunoprecipitate were subjected to immunoblot analysis with the anti-ErbB-3 rabbit polyclonal antibody. W, Western blot, IP, immunoprecipitation.

HRGβ1 effect on LM3 cell proliferation requires PI-3K and ERK activation although HRGβ1 effect on uPA synthesis is independent of these 2 pathways

There are few works comparing the role that different signaling pathways play in HRG regulation of either proliferation or of cellular responses associated with the metastatic phenotype. We focused here on the role of PI-3K and ERK1/ERK2 signaling cascades because they are strongly activated by HRGβ1 treatment of LM3 cells. In addition, although it is firmly acknowledged that these pathways are involved in some of the biologic responses to HRG in breast cancer cells, their differential involvement in HRG modulation of growth and of properties related to invasion and metastasis remains elusive. To understand the involvement of PI-3K in HRGβ1 effects on LM3 cell proliferation, we tested whether wortmannin would block HRGβ1-induced inhibition of LM3 cell growth. As shown in Figure 11*a*, wortmannin alone had no detectable effect on LM3 cell proliferation during 48 hr addition, but led instead to the blockage of HRGβ1 capacity to inhibit LM3 cell proliferation. Similar results were obtained using another PI-3K chemical inhibitor, LY294002 (data not shown).

We then investigated whether blockage of the MEK1-ERK signaling pathway would inhibit the ability of HRGβ1 to regulate LM3 cell growth by using PD98059. Treatment of LM3 cells with PD98059 alone had no effect on their proliferation (Fig. 11*a*). However, HRGβ1 inhibition of LM3 cell growth was blocked by PD98059 (Fig. 11*a*).

As a way of assessing the role of PI-3K and ERKs on HRGβ1 regulation of LM3 metastatic phenotype, we chose to study uPA activity. Neither wortmannin or LY294002, nor PD98059 resulted in a significant abolishment of the ability of HRGβ1 to inhibit uPA activity (Fig. 11*b*).

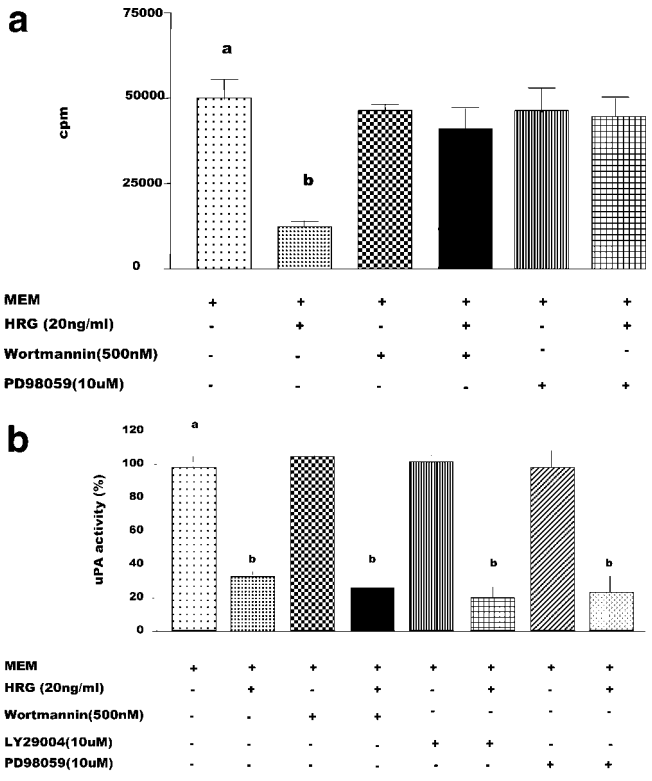


FIGURE 11 – Effect of PI-3K and MEK1/ERKs inhibitors on heregulin (HRG)β1 regulation of LM3 cell growth and uPA activity. (*a*) LM3 cells were incubated for 48 hr in MEM with 2.5% FCS containing vehicle DMSO (1:2,000) or in MEM (containing DMSO 1:2,000) + 20 ng/ml HRGβ1. To study the effects of wortmannin or PD98059, these 2 inhibitors were added 90 min before the 48 hr incubation with HRGβ1. Incorporation of [^{3}H]thymidine was used as a measure of DNA synthesis. *b vs. a*: $p < 0.001$. Data are presented as mean ± SD. The experiment shown is representative of a total of 5. (*b*) Semiconfluent monolayers of LM3 cells growing in serum-free MEM were left untreated or preincubated for 90 min with wortmannin, LY294002 or PD98059. Cells were then either treated with HRGβ1 or given vehicle alone and were incubated for 24 hr. Conditioned media were collected, and uPA activity was quantified by a radial caseinolytic assay. Data are presented as means ± SD. Shown is a representative experiment of a total of 4.

DISCUSSION

HRG inhibits LM3 cell proliferation and migration

Our results showing that HRGβ1 inhibited LM3 cell growth are in line with accumulating evidence indicating that HRG exerts a growth-inhibitory effect on ErbB-2-overexpressing breast tumor cells.[19–24] Accordingly, transfection of HRG cDNA to SKBR3 cells resulted in pronounced inhibition of anchorage-dependent and -independent growth.[53] To date, there is consensus in that the different cellular responses to HRG are very likely dependent on the array of type I RTKs activated and the intracellular signaling engaged. However, the influence that the levels of expression of each of the ErbBs receptors has on the growth effects of HRG remains poorly understood. For example, cells with elevated levels of ErbB-2 but little or no ErbB-3 and ErbB-4 are not growth-inhibited by HRG.[22] Recent work by Daly *et al.*[22,23] has confirmed the importance of ErbB-3 in HRG inhibition of SKBR3 cell growth. These authors generated 3 different pools of SKBR3 cells, 1 of them ectopically expressing ErbB-4 and the others expressing enhanced levels of ErbB-1 or ErbB-3. Only ErbB-3 overexpression markedly enhanced growth inhibition by HRG.[23] On the other hand, it has recently been reported that expression of ErbB-4 is both necessary and sufficient to trigger an antiproliferative response to HRG in human breast cancer cells.[45] In the present work, the importance of both ErbB-3 and ErbB-4 in HRGβ1 inhibition of LM3 cell growth was demonstrated by the finding that blockage of HRG binding to either ErbB-3 or ErbB-4 resulted in significant abrogation of the ability of HRGβ1 to inhibit LM3 cell growth. It is noteworthy that at the highest concentration of blocking antibodies used, only the anti-ErbB-3 antibody resulted in a complete abolishment of HRGβ1 biologic effect. In addition, HRGβ1 induced a dramatically higher degree of tyrosine phosphorylation of ErbB-3 than those of ErbB-2 and ErbB-4. Taken together, these results suggest that that ErbB-3 could be a major target of HRGβ1 in LM3 cells.

Notably, we found here that HRGβ1 growth-inhibitory effects on LM3 cells were highest when cells were plated at low density, in accordance with previous work by Daly *et al.*,[22] who showed that conditioned medium from high-density cultures protected SKBR3 cells from HRG-induced growth inhibition. A likely explanation for the density effect may be that SKBR3 cells secrete GFs that accumulate in dense cultures and protect them from HRG. Of the GFs that Daly *et al.*[23] examined, only IGF-I and insulin showed a protective effect. Interestingly, we had already found expression of IGF-I and its receptor, namely, IGF-IR, in M3 tumors and in primary cultures,[54] as well as in LM3 cells (Elizalde *et al.*, unpublished data). In addition, we have recently demonstrated that a functional IGF-IR is required for HRG mitogenic activity in mammary tumor cells.[38] Furthermore, we have also already shown the existence of a hierarchical interaction between IGF-IR and ErbB-2, by means of which IGF-IR directs ErbB-2 phosphorylation in breast cancer cells.[43] Therefore, our results suggest that IGF-I accumulated in dense LM3 cultures could be interfering with HRGβ1 action on LM3 cells through the ability of IGF-I to transmodulate ErbB-2 activity.

Compatible with its antiproliferative effects on LM3 cells, HRGβ1 also inhibited migration of these cells at all doses tested. Although there is no consensus on HRG role on migration, results similar to ours have been reported in SKBR3 cells in which high doses of HRG resulted in inhibition of motility and invasion.[29] Conversely, HRG has been found to induce migration and invasiveness and to regulate actin cytoskeleton in the nonmetastatic SKBR3 cells, in striking contrast to its growth-inhibitory effect on these cells.[24] HRG also promotes cell migration and regulates cytoskeletal reorganization of noninvasive MCF-7 cells.[28] In addition, HRG treatment of the metastatic MDA-MB-435 line exhibiting high levels of HRG expression also resulted in stimulation of cell adhesion and migration.[49] Interestingly, the phenotypic characteristics of all the cell lines mentioned above, in which HRG promotes invasion and metastasis, are different from those of LM3 cells. First, whereas MCF-7 and SKBR3 cells are nonmetastatic breast cancer cells, LM3 cells are highly metastatic. Second, MDA-MB-435 cells, which, like LM3, are highly metastatic and do not produce HRG, express an array and level of type I RTKs different from those of LM3 cells. These differences between LM3 cells and the other cells described confirm the hypothesis that HRG regulation of breast cancer cell activities may depend on the coexpression of type I RTKs as well as on the relative levels of expression of these receptors, which in turn might result in the differential activation of signaling pathways.

HRG inhibits MMP-9 and uPA activity

The role of proteases capable of degrading the extracellular matrix in HRG regulation of motility and invasiveness also remains poorly explored. Ours is the first evidence to show that HRGβ1 inhibition of growth and migration of mammary tumor cells correlates with its capacity to inhibit MMP-9 and uPA activity. Varying results have been reported in the literature. Thus, it was found that HRG treatment of SKBR3 cells increased expression of MMP-9, despite inducing growth inhibition.[24] However, another study reported that exposure of SKBR3 and MCF-7 cells to HRG had no effect on MMP-9 induction.[55] It has recently been noted that stimulation of MCF-7 cells with HRG leads to a sig-

nificant increase in expression of MMP-9 protein, as well as its gelatinolytic activity.[28] In regard to uPA, recent work has shown that stimulation by HRG of noninvasive MCF-7 cells induced expression of uPA at mRNA and protein levels and also its plasminogenic activity.[30] Similarly, HRG stimulates uPA expression and regulates its temporal distribution in colon cancer cells.[30] In conclusion, in our model system, HRGβ1 abolishment of growth and migration involves inhibition of uPA and MMP-9 activity, whereas in MCF-7 cells, HRG promotion of migration and invasiveness correlates with the activation of uPA and MMP-9. It could be inferred, from these apparently conflicting results, that HRG regulation of the metastatic phenotype, either to promote or to inhibit it, might be mediated by modulation of MMP-9 and uPA activities.

HRG activates PI-3K, ERKs and STATs

It is well acknowledged that HRG treatment of breast cancer cells results in activation of several signal transduction cascades such as the Erk/MAP kinases,[25,26] the Jnk/SAP kinases,[27] p70/p85 S6 kinase,[25] PI-3K[26,27] and p38 MAPK.[23] However, there are very few works comparing the role that these signaling pathways play in HRG regulation of either proliferation or cellular responses associated with the metastatic phenotype. Therefore, the present study was designed to compare the involvement of 2 signal transduction cascades, PI-3K and ERK1/ERK2, in HRG regulation of growth and activation of uPA. We focused on these pathways because HRGβ1 treatment of LM3 cells strongly activated both of them. In addition, although PI-3K and ERK1/ERK2 are probably the most exhaustively studied pathways in HRG modulation of breast cancer development and progression, their differential involvement in HRG regulation of growth and cell properties related to invasion and metastasis remains elusive.

We found here that HRGβ1 treatment of LM3 cells induced the rapid activation of PI-3K and its association with ErbB-2, ErbB-3 and ErbB-4. Interestingly, HRG induced a much higher association of PI-3K with ErbB-3 than with either ErbB-2 or ErbB-4, showing that, as happened with tyrosine phosphorylation of these receptors, ErbB-3 plays a role in HRGβ1 action on LM3 cells. Pharmacologic inhibition of PI-3K with wortmannin resulted in the suppression of the ability of HRGβ1 to inhibit LM3 cell growth, indicating that PI-3K plays a role in HRGβ1 regulation of LM3 cell growth. Activation of PI-3K in HRG-regulated proliferation or differentiation of breast cancer cells has been acknowledged.[23,32] Nevertheless, conflicting results were found. Thus, whereas PI-3K activation was critical to HRG-induced S-phase entry in SKBR3 cells,[23] it played a minor role in HRG-induced growth of T47D cells.[32] We found in our study that blockage of PI-3K activity did not affect the ability of HRGβ1 to inhibit uPA activity. This result is in accordance with previous reports showing that PI-3K does not play any role in HRG regulation of cell properties related to invasion and metastasis. For example, HRG stimulation of paxillin, a major protein of focal adhesion, resulted in PI-3K activation, but selective inhibition of PI-3K did not prevent HRG action in MCF-7 cells.[54] Similarly, HRG induction of autocrine motility factor expression was not affected by PI-3K inhibition.[33] Conversely, other studies have found that PI-3K participated in HRG regulation of cytoskeletal reorganization,[28] adhesion,[49] aggregation,[48] and migration[28,49] of breast cancer cells.

In the present report, we found that HRGβ1 treatment of LM3 resulted in the rapid activation of ERK1/ERK2. It is noteworthy that blockage of the MEK1/ERK signaling pathway suppressed the ability of HRGβ1 to inhibit LM3 cell proliferation but did not affect the ability of HRG to inhibit uPA activity. Activation of ERKs by HRG treatment of breast cancer cells has been demonstrated in numerous studies.[25,26] However, the role that ERKs play in signaling pathways activated by HRG that result in the regulation of growth, differentiation or progression to a metastatic phenotype remains elusive. For example, administration of HRGβ to growth-arrested T-47D cells activated the ERK signaling pathway and potently stimulated cell cycle progression, whereas treatment with PD98059 led to a complete block of HRG-induced growth.[32] In contrast, inhibition of ERK1/ERK2 had no effect on cell cycle progression or apoptosis caused by HRG in SKBR-3 cells.[22] Activation of ERKs in MCF-7 cells was found when HRG induced cell aggregation[48] and when HRG stimulated serine phosphorylation of paxillin.[56] However, blockage of the MEK1/ERK pathway with PD98509 had no effect on HRGβ1-mediated aggregation[48] or on paxillin phosphorylation.[56] On the contrary, HRG-induced expression of the autocrine motility factor in MCF-7 cells was blocked by specific inhibitors of ERKs.[33] We found here that blockage of 2 distinct signaling pathways, PI-3K and ERK1/ERK2, resulted in the complete abrogation of the ability of HRGβ1 to inhibit LM3 cell growth.

This result is in accordance with previous reports indicating that independent signaling pathways are involved in HRG regulation of a particular cell response. Thus, HRG-induced stimulation of autocrine motility factor in MCF-7 cells was blocked by specific inhibitors of ERKs and p38MAPK.[33] In addition, HRG-induced acetylcholine receptor (AchR) genes required activation of both Ras/MAPK and PI-3K signal transduction pathways.[57] The cell type-specific response to HRG appears to depend on the expression, level and activation of ErbB-2, ErbB-3 and ErbB-4, which in turn link these receptors to distinct signal transduction pathways. Our results, showing that both PI-3K and ERK1/ERK2 activation mediates HRGβ1 antiproliferative response, indicate that activation of a single signaling pathway is probably not sufficient for HRGβ1 inhibition of cell growth and the more likely scenario is that a combination of pathways is used to this end.

We found here that HRGβ1 treatment of LM3 cells induced activation of Stat3 and Stat5. Therefore, this is to our knowledge the first report to show that HRG is able to activate STATs in mammary cancer cells. In addition, we demostrated that both ErbB-3 and ErbB-4 are involved in HRG-induced activation of Stat3 and Stat5 in LM3 cells. The involvement of the different members of the type I RTK family in the activation of STAT proteins remains poorly studied. Better elucidated is the role of EGF-R/ErbB-1 in the activation of Stat1, Stat3 and Stat5, as well as its capacity to promote STAT activation via a Jak kinase-independent, EGF-R intrinsic tyrosine kinase-dependent and Src-dependent pathway.[52] Recently, Hynes and co-workers have found that HRG was not able to induce Stat5b activation in NIH3 cells expressing ErbB-2 and ErbB-3 but that it strongly promoted its phosphorylation in NIH3 cells expressing ErbB-2 and ErbB-4 receptors, suggesting that ErbB-2/ErbB-4 dimers were required for Stat5b activation in response to HRG.[52] Our present findings also showed a role for ErbB-3 in transmitting HRG signaling pathways that result in Stat3 and Stat5 phosphorylation. A crucial role of Stat3 in transducing signaling required for migration was recently demonstrated in experiments in which *in vitro* migration in response to EGF, to hepatocyte growth factor and to interleukin-6 was inhibited in primary cultures of keratinocites from Stat3 gene-disrupted mice.[58] Accordingly, the results presented here, indicating that HRG inhibition of LM3 cell migration correlates with HRGβ1 activation of Stat3, suggest that Stat3 may be involved in HRGβ1 regulation of migration.

In summary, we have demonstrated that HRG was able to inhibit proliferation and migration of LM3 mammary tumor cells, as well as the activity of 2 key enzymes of the metastatic cascade, uPA and MMP-9. These results clearly indicate that HRG can act as an antimetastatic factor in LM3 cells and they further open the question of whether this is a general phenomenon in mammary cells with a phenotype similar to LM3 cells. Therefore, our findings identified HRG as a likely new therapeutic agent to control the progression of breast cancer exhibiting a particular phenotype.

ACKNOWLEDGEMENTS

Tthe Beca Ramón Carrillo-Arturo Oñativia-2000 was awarded to E. Bal de Kier Joffé and the Beca Ramón Carrillo-Arturo Oñativia-2001 was awarded to P.V. Elizalde.

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