

Up-Regulation of Vascular Endothelial Growth Factor in Breast Cancer Cells by the Heregulin- β 1-activated p38 Signaling Pathway Enhances Endothelial Cell Migration¹

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ABSTRACT

Heregulin (HRG) belongs to a family of polypeptide growth factors that bind to receptor tyrosine kinases ErbB3 and ErbB4. HRG binding induces ErbB3 and ErbB4 heterodimerization with ErbB2, activating downstream signal transduction. Vascular endothelial growth factor (VEGF) is a primary regulator of physiological angiogenesis and is a major mediator of pathological angiogenesis, such as tumor-associated neovascularization. In this study, we demonstrate that HRG- β 1 increased secretion of VEGF from breast cancer cells in a time- and dosage-dependent manner and that this increase resulted from up-regulation of VEGF mRNA expression via transcriptional activation of the VEGF promoter. Deletion and mutational analysis revealed that a CA-rich upstream HRG response element located between nucleotide-2249 and -2242 in the VEGF promoter mediated HRG-induced transcriptional up-regulation of VEGF. While investigating the downstream signaling pathways involved in HRG-mediated up-regulation of VEGF, we found that HRG activated extracellular signal-regulated protein kinases, Akt kinase, and p38 mitogen-activated protein kinase (MAPK). However, only the specific inhibitor of p38 MAPK (SB203580), not extracellular signal-regulated kinase inhibitor PD98059 nor the inhibitor of phosphatidylinositol 3-kinase-Akt pathway (Wortmannin), blocked the up-regulation of VEGF by HRG. The HRG-stimulated secretion of VEGF from breast cancer cells resulted in increased migration of murine lung endothelial cells, an activity that was inhibited by either VEGF-neutralizing antibody or SB203580. These results show that HRG can activate p38 MAPK to enhance VEGF transcription via an upstream HRG response element, leading to increased VEGF secretion and angiogenic response in breast cancer cells.

INTRODUCTION

VEGF³, also known as vascular permeability factor, is an important stimulator of angiogenesis. Normal levels and appropriately timed expression of VEGF are essential for normal development of the vascular system (1–3). VEGF-induced angiogenesis is also essential for the growth of solid tumors (4–6). VEGF is highly expressed in solid tumors and is required for the maintenance of tumor blood vessels. Withdrawal of VEGF causes tumor regression (7–9). Recently, VEGF was recognized as a survival factor for endothelial cells (10). VEGF expression is regulated by many growth factors and

cytokines, such as insulin-like growth factor (11), interleukin-6 (12), transforming growth factor- β (13), basic fibroblast growth factor, epidermal growth factor, and platelet-derived growth factor (14). VEGF expression is increased by hypoxia (4, 15) and inhibited by p53 (16). It is conceivable that the expression and function of VEGF are regulated by many cellular factors during tumor progression. Revealing these factors and their mechanisms of action will enable us to better alter the detrimental consequence of VEGF.

HRG, also named neu differentiation factor (17), neuregulin (18), AchR-inducing activity (19), and glial growth factor (20), is a member of the epidermal growth factor-like growth factor family. HRGs are ligands of ErbB3 and/or ErbB4, which belong to the ErbB family of receptor tyrosine kinases. The binding of HRG to its receptors induces either ErbB3 or ErbB4 to form homodimers or to form heterodimers with ErbB2, thus triggering diverse signaling cascades (21). HRGs can induce a variety of cellular responses in different cell types, including proliferation, differentiation, survival, apoptosis, migration, and aggregation (22–26). Data from HRG gene knockout mice demonstrated that HRG is essential for the early development of the heart and central nervous system (27). HRG is also known to be involved in breast cancer metastasis and in ErbB2-related and hormone-independent breast cancer progression (28, 29). HRG has been shown to regulate invasive and metastasis-related properties in breast cancer cell lines (30). However, the role of HRG in breast cancer metastasis remains elusive.

HRG has also been implicated in the regulation of gene expression. HRG was reported to stimulate AchR and utrophin gene expression in muscle cells via GA-binding proteins by 2–3 fold compared with that in the absence of HRG (31–35). HRG also up-regulated AchR ϵ gene expression about 3-fold in p-19 teratocarcinoma cells and breast cancer cells through Sp1-containing complex (36). The mechanisms of HRG-induced transcriptional regulation in these studies remain unclear. After studying the role of HRG and ErbB2 in breast cancer progression and metastasis, we found that HRG up-regulated VEGF secretion from breast cancer cell lines through transcriptional up-regulation and that it required activation of the p38 MAPK signaling pathway. Furthermore, HRG- β 1-induced VEGF secretion enhanced mouse endothelial cell migration. Thus, transcriptional up-regulation of VEGF via activation of the p38 MAPK pathway may be one of the mechanisms that contribute to HRG-induced breast cancer metastasis.

MATERIALS AND METHODS

Cell Culture and Transfection. Breast cancer cell lines were cultured in DMEM/F12 containing 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). Transient transfection was performed following the manufacturer's instruction for LipofectAmine (Life Technologies, Inc.). Briefly, cells were seeded in 6-well plates overnight. Luciferase reporter gene (3 μ g/well) and pCMV- β -gal plasmid (0.2 μ g/well), used as an internal control, were mixed in 100 μ l of OPTI-MEM medium, to which 10 μ l of diluted LipofectAmine was added. The mixture was incubated for 30 min at room temperature. Then, 800 μ l of OPTI-MEM was added to the mixture, which was transferred onto the cells for 3–4 h. Cells were incubated in serum-free

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³ The abbreviations used are: VEGF, vascular endothelial growth factor; HRG, heregulin; nt, nucleotide; AchR, acetylcholine receptor; MAPK, mitogen-activated protein kinase; DMEM/F12, 1:1 mixture of Dulbecco's modified essential medium and Ham's F12 nutrient mixture; CMV, cytomegalovirus; HRE, heregulin response element; uHRE, upstream heregulin response element; WT, wild-type; MUT, mutant; EGF1d, epidermal growth factor-like domain; CM, conditioned media; ERK, extracellular signal-regulated kinase; ActD, actinomycin D; CMX, cycloheximide; MluE, murine lung endothelial; PI-3K, phosphatidylinositol 3-kinase.

DMEM/F12 media overnight and then cultured in fresh medium containing 10 ng/ml HRG (Neomarker, Fremont, CA) for 24 h. Cell lysates were prepared, and luciferase and β -gal activities were assayed using a luciferase assay kit (Promega, Madison, WI).

Luciferase Reporter and HRE Mutants. VEGF promoter luciferase reporter constructs were prepared as described previously (16). The uHRE mutant was generated by PCR mutation. The upstream primers for the uHRE mutant were as follows: WT, AAGGTACCGCTTATGGGGGTGGGGGGT-GCCT; uHRE mutant, AAGGTACCGCTTATGGGGGTAAAGGGGTGCCT. The downstream primer was TGCCTAGTCTCTGCCACCAC. The PCR product was recovered and cloned back to full-length VEGF promoter reporter plasmid via *Acc65I/SpeI* sites. The mutation was confirmed by sequence analysis.

Generation of Stable Cell Lines Expressing EGFlid of HRG. The cDNA fragment of the EGFlid (37) was generated by PCR using the following primers: 5' primer, CGGGATCCGGGACAAGCCATCTTGTAAAAT; 3' primer, AGCGGCCGCTTCTGGTACAGCTCCTCCG. The EGFlid was cloned in pSecTag2B vector (Invitrogen, Carlsbad, CA). Both the EGFlid expression plasmid and the pSecTag2B vector were transfected into MCF7 cells. The stable transfectants were selected by Zeocin (Invitrogen).

Western Blot Analysis. Cells were starved in serum-free medium overnight before HRG treatment. Cells were then incubated either with 10 ng/ml HRG for different times (12, 24, or 48 h) or for 24 h with various concentrations of HRG (1, 2, 5, or 10 ng/ml). The CM were collected and concentrated by Centricon (Amicon, Inc., Beverly, MA). To examine signaling pathway involvement, cells were treated with 10 ng/ml HRG for 10, 30, or 60 min, and cell lysates were prepared as described previously (25). The proteins from the CM or cell lysates were then separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and probed by monoclonal VEGF antibody (R&D Systems, Minneapolis, MN) or specific antibodies against phosphorylated ERK, Akt, and p38 MAPK (New England Biolabs, Inc., Beverly, MA). Protein signals were detected using the enhanced chemiluminescence detection system (Amersham, Corp., Arlington Heights, IL). Horseradish peroxidase-conjugated antibody against mouse or rabbit IgG (Jackson ImmunoResearch Laboratories) was used as a secondary antibody.

Northern Blot Analysis. SKBr3 and MCF7 cells were starved overnight and incubated with 10 ng/ml HRG for 2, 6, or 24 h. For chemical inhibitor treatment assays, cells were incubated for 1 h with 5 μ g/ml ActD, 50 μ g/ml CMX, 20 μ M PD98059, 100 nM Wortmannin, or 10 μ M SB203580 (Calbiochem, San Diego, CA) and then treated with HRG (10 ng/ml) for 6 h. Total RNA was extracted from cells using Ultraspec RNA reagent (Biotech Laboratories, Inc., Houston, TX) and separated by electrophoresis in 1% denaturing formaldehyde-agarose gel. The RNA was transferred to Hybond-N⁺ nylon membrane (Amersham) overnight. The membrane was UV cross-linked with a UV Stratalinker 1800 (Stratagene, San Diego, CA). The membranes were prehybridized and hybridized with VEGF-specific and glyceraldehyde-3-phosphate dehydrogenase-specific cDNA probes (16) at 68°C in PerfectHyb plus hybridization buffer (Sigma Chemical Co., St. Louis, MO), washed, and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Endothelial Cell Migration Assay. Endothelial cell migration was assayed in 24-well, 6.5-mm-internal-diameter Transwell cluster plates (8.0- μ m pores; Costar Corp., Cambridge, MA) using MluE cells (American Type Culture Collection, Rockville, MD) and CM of HRG-treated and untreated SKBr3 cells. The CM were prepared by incubating SKBr3 cells with 10 ng/ml HRG in the presence or absence of 0.5 μ M SB203580. Three thousand MluE cells were suspended in 0.1 ml of serum-free DMEM/F12 with 0.1% BSA and loaded into the 0.1 mg/ml gelatin-coated upper chamber of a Transwell cluster plate. The lower chamber of the Transwell plate was filled with the 600- μ l CM or CM neutralized with anti-VEGF polyclonal antibody (50 pg/ml, mixed and stored at 4°C overnight; Santa Cruz Biotechnology, Santa Cruz, CA). A solution of DMEM/F12 plus 0.1% BSA was used as a negative control. The assay was allowed to proceed for 5.5 h at 37°C, after which the Transwell filters were fixed with methanol and stained with Giemsa (LabChem Inc., Pittsburgh, PA). The cells on the upper surface of the filter were removed by wiping with a cotton swab, and cell migration was determined by counting the number of cells/high-power field (\times 200) that had migrated to the lower side of the filter. Five high-power fields/filter and triplicate filters/sample were counted. Assays were repeated at least twice. The difference in migration rates was analyzed using the two-tailed Student *t* test.

RESULTS

Increase of VEGF Secretion in Breast Cancer Cells by HRG- β 1. Previous studies (25, 30) have shown that HRG can regulate metastasis and invasion-related properties in breast cancer cells. It is well known that angiogenesis is essential for tumor metastasis and invasion and that VEGF is a major proangiogenic factor in tumor and normal tissues (1–6). Therefore, we investigated whether HRG- β 1 regulates VEGF secretion in breast cancer cell lines. Six breast cancer cell lines were treated with or without 10 ng/ml recombinant HRG- β 1. CM were collected, and the levels of VEGF in CM were detected by Western blot analysis using anti-VEGF antibody. HRG- β 1 increased VEGF secretion dramatically in SKBr3, MCF-7, and MDA-MB-468 cell lines and slightly in the MDA-MB-453 cell line (Fig. 1A). However, MDA-MB-453 and BT-483 cell lines did not show significant VEGF induction by HRG- β 1 (Fig. 1A). Further study of MCF-7 and SKBr3 cells indicated that the VEGF secretion induced by HRG- β 1 was dosage- and time-dependent (Fig. 1B). The HRG- β 1-mediated VEGF induction appeared with 1 ng/ml HRG and occurred as early as 12 h at the 10 ng/ml HRG concentration. Similar data were obtained from MCF7 cells, but the induction was greater in the SKBr3 cell line than in the MCF7 cell line. The VEGF signal was detectable in both MCF7 and SKBr3 cells without HRG- β 1 treatment, suggesting that both cell lines secreted basal levels of VEGF (Fig. 1B, Lane 1).

Induction of VEGF mRNA Expression by HRG- β 1. To test whether increased VEGF secretion occurs through mRNA up-regulation, Northern blot analysis was performed on HRG- β 1-treated and untreated MCF-7 and SKBr3 cells using a labeled VEGF probe. As shown in Fig. 2A, the VEGF mRNA level increased in a time-dependent manner in both the SKBr3 and the MCF7 cell lines after 10 ng/ml of HRG- β 1 treatment. The VEGF mRNA induction appeared as soon as 2 h after HRG- β 1 treatment, continuously increased up to 6 h, and then decreased at the 24-h time point. Compared with levels in untreated cells, the level of VEGF mRNA induction by HRG- β 1 at 6 h were 2.4 and 2.7 times higher in MCF7 and SKBr3 cell lines, respectively. The HT1080 cell line, which was used as a positive control, showed a much higher level of basal VEGF signal. VEGF

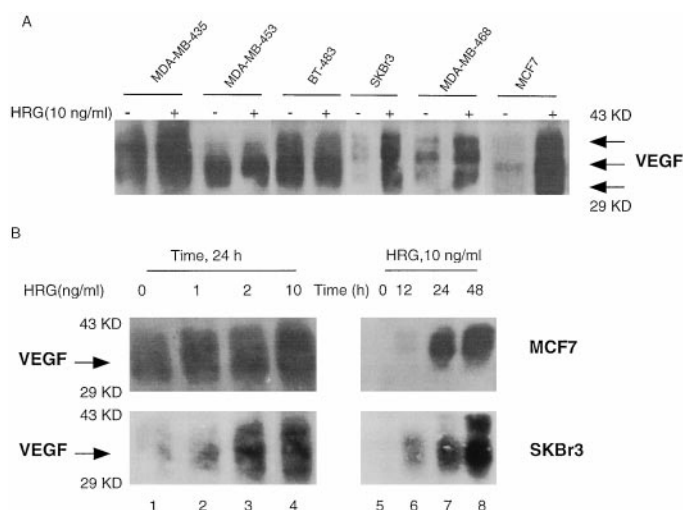


Fig. 1. HRG up-regulates VEGF secretions from various breast cancer cell lines. CM were collected from serum-starved breast cancer cells treated with various dosages of HRG- β 1 for various times. Western blot analysis was performed as described in "Materials and Methods." A, up-regulation of VEGF in multiple breast cancer cell lines treated with 10 ng/ml recombinant HRG- β 1 for 24 h. B, HRG- β 1 up-regulation of VEGF secretion in MCF7 and SKBr3 cells is concentration- and time-dependent. CM were collected from MCF7 and SKBr3 cells treated with 0, 1, 2, or 10 ng/ml HRG for 24 h or with 10 ng/ml HRG- β 1 for 0, 12, 24, or 48 h.

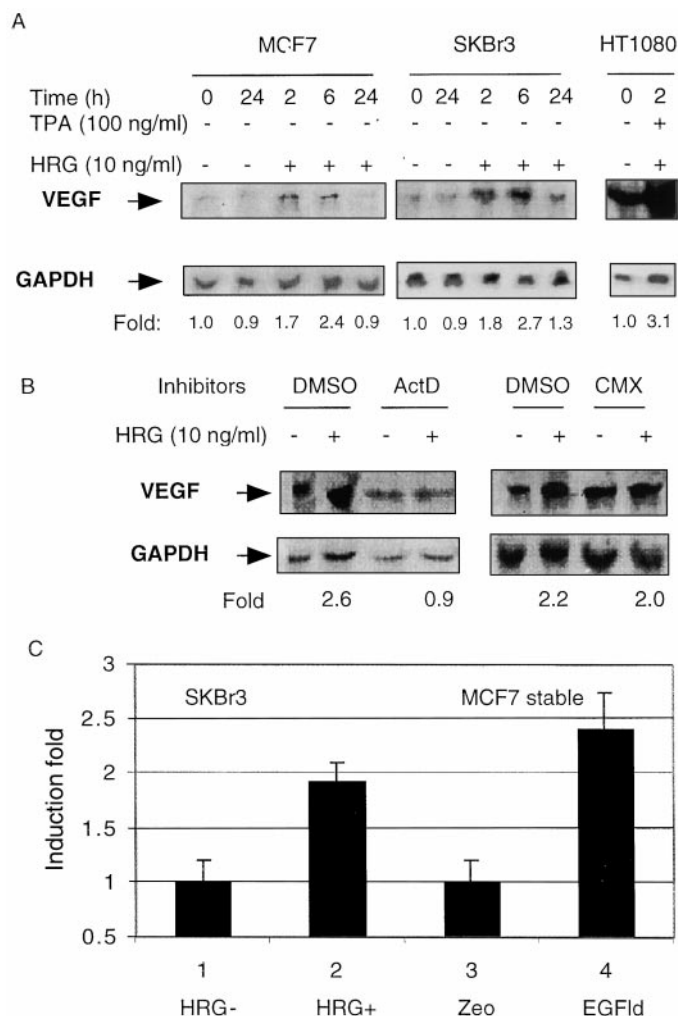


Fig. 2. VEGF mRNA up-regulation by HRG- β 1 is time-dependent through transcriptional regulation. *A*, Northern blot analysis of VEGF mRNA in MCF7 and SKBr3 cells treated with 10 ng/ml HRG for 2, 6, or 24 h and in HT1080 cells treated with 100 ng/ml 12-*O*-tetradecanoyl-phorbol-13-acetate for 2 h. *B*, Northern blot analysis of VEGF mRNA in SKBr3 cells pretreated with either 5 μ g/ml ActD or 50 μ g/ml CMX for 1 h and then incubated with 10 ng/ml HRG- β 1 for 6 h. Total RNA (20 μ g) was separated on 1% denaturing formaldehyde-agarose gel, and Northern blot analysis was performed. *C*, HRG- β 1 activates VEGF-luciferase activity. VEGF full-length luciferase reporter and internal control *CMV*- β -gal plasmids were transfected either in SKBr3 cells and incubated with 10 ng/ml HRG for 24 h after transfection or in MCF7 stable transfectants that constitutively expressed and secreted EGFlid. Luciferase and β -galactosidase activities in cell lysates were then tested.

mRNA expression was further induced by 12-*O*-tetradecanoyl-phorbol-13-acetate about 3-fold more than the level in untreated cells.

We next examined whether VEGF mRNA up-regulation by HRG- β 1 is because of increased transcription or increased RNA stability and sought to determine whether nascent RNA or protein synthesis is required for HRG- β 1-mediated VEGF mRNA up-regulation. To this end, we pretreated SKBr3 cells with 5 μ g/ml ActD, a RNA synthesis inhibitor, or 25 μ g/ml CMX, a protein synthesis inhibitor, for 1 h before HRG- β 1 was added to the culture medium. ActD completely abolished HRG- β 1-induced VEGF up-regulation, whereas CMX had no effect on VEGF induction by HRG- β 1 (Fig. 2*B*). This finding suggests that HRG-induced VEGF up-regulation requires new RNA synthesis but not new protein synthesis. Because the induction of VEGF by HRG- β 1 requires new RNA synthesis, it most likely occurs through transcriptional up-regulation. Furthermore, because VEGF induction by HRG- β 1 is independent of protein synthesis, it is probably mediated by the activation of existing transcrip-

tion factor(s) through translocation or modification, such as phosphorylation of transcription factor(s).

To further determine whether HRG- β 1-mediated VEGF up-regulation occurs through transactivation of the VEGF promoter, the full-length VEGF promoter was fused to the luciferase reporter gene, which was transfected into SKBr3 cells. HRG- β 1 treatment increased the luciferase activity driven by full-length VEGF promoter by approximately 2-fold. In addition, the CMV-driven EGFlid of HRG was transfected into MCF7 cells from which stable transfectants that constitutively expressed and secreted EGFlid of HRG were generated. When the full-length VEGF promoter-luciferase reporter gene was transfected into these HRG EGFlid stable transfectants, the luciferase activity was 2.4 times higher than that in the control cells (Fig. 2*C*). The fold of induction of promoter activity is comparable with the fold of induction of VEGF mRNA observed in Northern blot analysis (Fig. 2, *A* and *B*) and is consistent with HRG- β 1-mediated transcriptional up-regulation of other target genes reported previously (31–36). These data demonstrate that VEGF up-regulation by HRG- β 1 occurs through transcriptional up-regulation of VEGF promoter activity. Because the VEGF promoter was activated both by the recombinant full-length extracellular domain of HRG- β 1 and by the EGFlid of HRG- β 1 in MCF7 cells, the posttranslational modification of HRG- β 1 may not be required for VEGF up-regulation in SKBr3 and MCF7 cell lines, and the EGFlid of HRG- β 1 must be sufficient to induce VEGF transcriptional up-regulation.

Activation of VEGF Promoter Activity by HRG- β 1 via Upstream CA-rich HRE. To determine the HRE in the VEGF promoter, 5'-VEGF promoter deletion constructs were fused to the luciferase reporter gene and were transfected into either HRG- β 1-treated SKBr3 cells or MCF7 HRG- β 1 EGFlid stable transfectants. Luciferase assays showed that the functional HRE responsible for

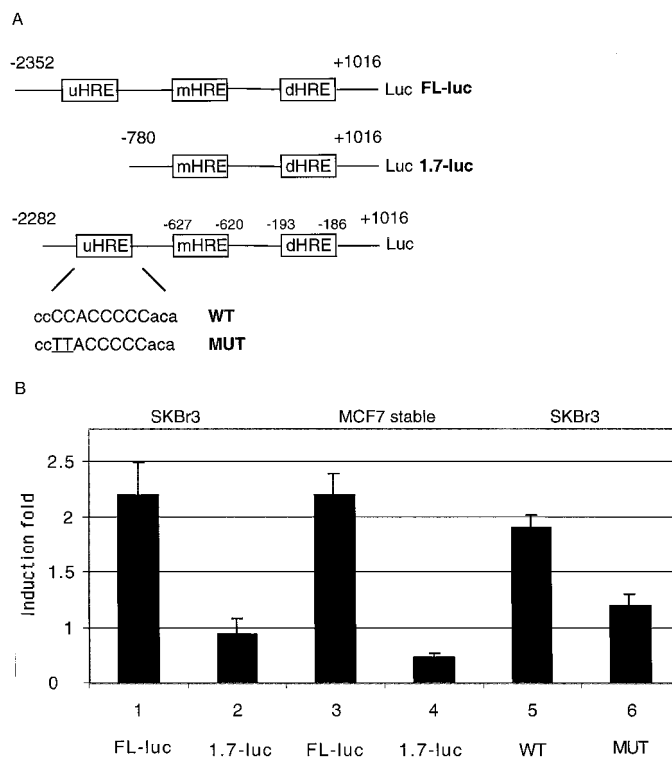


Fig. 3. Identification of HRE in VEGF promoter. SKBr3 cells or MCF7 stable transfectants were transfected with full-length luciferase reporter, 1.7-luc, WT, and MUT VEGF promoter-driven reporter plasmids, in which the complementary uHRE sequence was mutated from ccCCACCCCaca in WT to ccTTACCCCaca in MUT, together with *cmv*- β -gal. SKBr3 cells were then incubated with 10 ng/ml HRG for 24 h, and luciferase and β -galactosidase activities from transfected cells were measured.

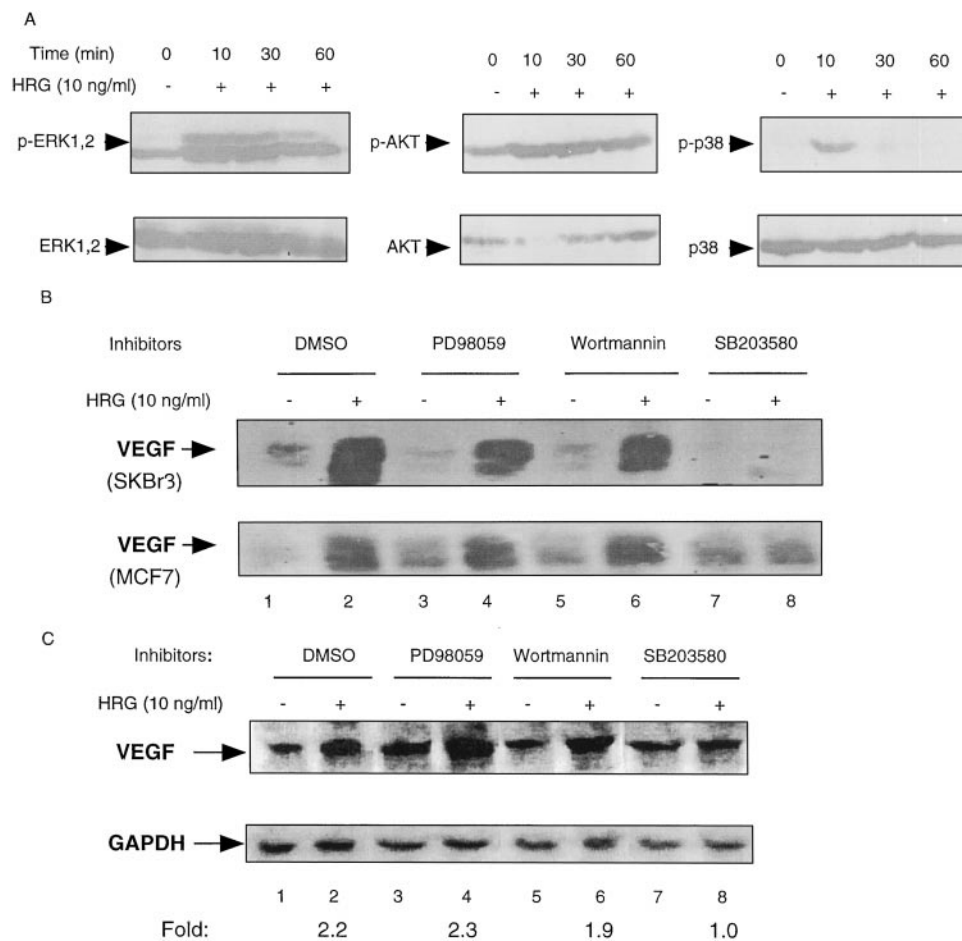


Fig. 4. The p38 MAPK kinase pathway is required for the induction of VEGF secretion by HRG- β 1. **A**, multiple signal pathways were activated by HRG- β 1 in SKBr3 cells. SKBr3 cells were treated by HRG- β 1 for 10, 30, or 60 min. Cell lysates were prepared, and Western blots were performed with antibodies against phosphorylated ERK (*p-ERK*), phosphorylated Akt (*p-AKT*), and phosphorylated p38 MAPK (*p-p38*). The ERK, Akt, and p-38 protein levels were determined by Western blot using respective antibodies. **B** and **C**, MCF7 or SKBr3 cells were incubated with or without 20 μ M PD98059, 0.1 μ M Wortmannin, or 10 μ M SB203580. HRG (10 ng/ml) was added to the media, CM were collected after 24 h for Western blot analysis of VEGF secretion, and total RNA was prepared from cells for Northern blot analysis of VEGF mRNA after 6 h of HRG- β 1 treatment.

VEGF up-regulation was located in the region between nt -2352 and -780 (uHRE; Fig. 3A). Two types of HREs have been reported in muscle cells (31–35) and in T47D breast cancer cells (36), and both types of HRE sequences are in the nt -2352 to -780 region of the VEGF promoter. On the basis of the previous report on T47D breast cancer cells (36), we reasoned that CA-rich HRE may mediate VEGF up-regulation in breast cancer cells. When the CA-rich uHRE sequence within the nt -2352 to -780 region was mutated from CCACCC to TTACCC, the VEGF promoter activation in SKBr3 by HRG- β 1 was reduced to an almost basal level (Fig. 3B, Lane 6). Similar data were also obtained when the WT and MUT constructs were transfected into MCF7 cells expressing HRG EGfId (data not shown). Thus, this CA-rich uHRE was functionally required for VEGF up-regulation by HRG- β 1 in both MCF7 and SKBr3 breast cancer cells. Although two additional CA-rich consensus HRE sequences, middle HRE and downstream HRE, are located downstream of uHRE, they do not seem to mediate VEGF up-regulation by HRG- β 1. Thus, although HRE consensus sequence is required, the HRE consensus sequence alone might not be sufficient to mediate induction of VEGF in SKBr3 cells by HRG- β 1. HRE function probably requires neighboring sequences and may be cell-type dependent.

Requirement of the p38 MAPK Pathway for the VEGF Up-Regulation by HRG- β 1. HRG- β 1 is a ligand for ErbB3 and ErbB4 membrane receptor tyrosine kinases and can activate multiple signaling pathways in a variety of cell types (25, 38, 39). To determine which HRG- β 1-activated signaling pathways in SKBr3 cells might contribute to HRG- β 1-mediated VEGF up-regulation, Western blot analysis was performed on HRG- β 1-treated and untreated SKBr3 cells using antibodies recognizing phosphorylated and activated ERK,

Akt, and p38 MAPK (Fig. 4A). HRG- β 1 effectively activated the MAP/ERK kinase pathway, the PI-3K/Akt pathway, and the p38 MAPK pathway in SKBr3 cells. Interestingly, the p38 MAPK pathway was only transiently activated by HRG- β 1. Considering that the VEGF mRNA signal was induced by HRG- β 1 as early as 2 h after treatment, further increasing up to 6 h, and later decreasing at 24 h, it would be intriguing to determine whether HRG- β 1-induced VEGF up-regulation requires the p38 MAPK pathway. Addition of the p38 MAPK pathway-specific inhibitor SB203580 to SKBr3 and MCF7 cells before HRG- β 1 treatment completely abolished the induction of VEGF secretion from these two cell lines (Fig. 4B, Lanes 7 and 8). In contrast, neither the MAP/ERK kinase pathway inhibitor (PD98059) nor the PI-3K/Akt pathway inhibitor (Wortmannin) effectively inhibited HRG- β 1-mediated VEGF induction (Fig. 4B, Lanes 3–6), although PD98059 and Wortmannin effectively inhibited ERK1/2 activation and Akt activation, respectively (data not shown). Additionally, HRG- β 1-mediated up-regulation of VEGF mRNA was also blocked by SB203580 (Fig. 4C, Lanes 7 and 8), whereas neither PD98059 nor Wortmannin abolished VEGF mRNA up-regulation by HRG- β 1 (Fig. 4C, Lanes 3–6). Therefore, both Western and Northern blot analyses demonstrated that HRG- β 1-mediated activation of the p38 MAPK pathway is required for VEGF up-regulation.

Increased Endothelial Cell Migration by HRG- β 1-mediated VEGF Secretion. Angiogenesis is critical for tumor growth and metastasis. VEGF is a well-known stimulator of angiogenesis (10). Because HRG- β 1 increases VEGF secretion from MCF7 and SKBr3 cells, we sought to determine whether VEGF up-regulation by HRG- β 1 might increase endothelial cell migration, which is an indicator of angiogenic response. CM were collected from SKBr3 cells

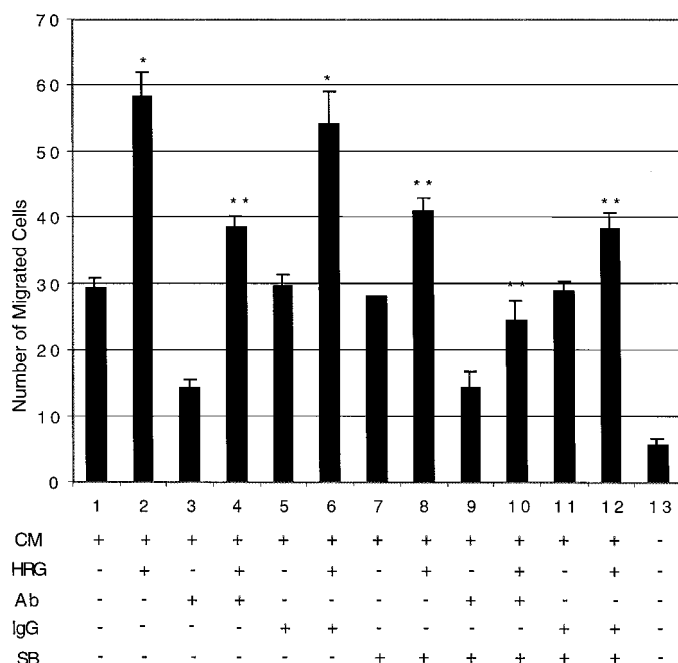


Fig. 5. Endothelial cell migration was increased in CM from HRG-treated cells. CM from SKBr3 cells were prepared as indicated at the bottom of the figure and in "Materials and Methods." MluE cells were suspended in SKBr3 cell CM with or without 0.1 ml of 50 pg/ml anti-VEGF antibody (Ab) or IgG. The endothelial cell migration assay was performed as described in "Materials and Methods," and the difference in migration rates was analyzed using the two-tailed Student *t* test. *, $P < 0.01$; **, $P < 0.05$.

treated with or without HRG- β 1 in the presence or absence of SB203580, added with or without VEGF antibody, and used to stimulate MluE cell migration in endothelial cell migration assays. Incubation of MluE cells with the CM from HRG- β 1-treated SKBr3 cells increased the number of migrated MluE cells by about 2-fold that of the level in untreated cells (Fig. 5, Lanes 1 and 2). Because VEGF antibody (50 pg/ml) inhibited the migration of MluE cells in CM from HRG- β 1-treated SKBr3 cells, whereas nonspecific IgG had no effect (Fig. 5, Lanes 3–6), this HRG- β 1-mediated increase of migration is most likely because of VEGF in the CM. Notably, VEGF antibody reduced the basal level of cell migration by inhibiting the basal level of VEGF in the CM (Fig. 5, Lanes 1, 3, and 5). MluE cell migration was reduced in CM collected from SKBr3 cells that were pretreated with 0.5 μ M SB203580, an inhibitor of the p38 MAPK pathway, before the cells were activated by HRG- β 1 (Fig. 5, compare Lanes 2 and 8), although SB203580 had no effect on MluE basal level migration (Fig. 5, Lanes 1 and 7). Furthermore, addition of VEGF antibody (50 ng/ml) to CM from SKBr3 cells that were pretreated with SB203580 and then stimulated with HRG- β 1 further decreased migration of MluE cells to below the basal level (Fig. 5, Lanes 1, 9, and 10), whereas the control IgG had no similar effect (Fig. 5, Lane 11 and 12). The data suggest that combination of VEGF antibody and SB203580 has an additive inhibitory effect to block endothelial migration induced by HRG- β 1-mediated VEGF secretion.

DISCUSSION

The data given here provide clear evidence that secretion of VEGF from MCF7 and SKBr3 breast cancer cell lines was induced by HRG- β 1 in a time- and dosage-dependent manner and that this induction was because of up-regulation of VEGF mRNA. Notably, the secretion of VEGF protein was increased after 24 h, whereas the induction of VEGF mRNA was increased to peak levels by 6 h but decreased by 24 h. This result may partly be because of the time

delays for either protein synthesis or protein secretion. Blockade of HRG- β 1-induced VEGF up-regulation by ActD pretreatment and analyses of VEGF promoter activity by HRG- β 1 treatment indicated that the induction of VEGF secretion by HRG- β 1 occurred through transcriptional up-regulation of VEGF. The HRG- β 1-mediated 2–3-fold of induction for VEGF is consistent with previous reports on HRG-induced transcriptional up-regulation of other target genes (31–36). The uHRE in the VEGF promoter responsible for HRG- β 1-mediated transcriptional up-regulation is identical to a previously reported HRE (36). It is interesting that not every CA-rich HRE is functional; the middle HRE and downstream HRE consensus sequences located downstream of uHRE did not seem to mediate VEGF up-regulation by HRG- β 1. These data suggest that the functionality of CA-rich HRE sequences may require other factor(s) or some neighboring element(s).

HRG- β 1-induced VEGF up-regulation does not require protein synthesis, suggesting that it may be mediated by activation of certain existing transcription factor(s) by posttranslational modification or relocation. Previous reports indicated that protein phosphorylation is required for HRG-induced transcriptional up-regulation of AchRe (36) and that protein-tyrosine phosphatase has the same response element as neuregulin (a member of the HRG family) response element (33). These findings are reasonable given that HRGs can bind to ErbB3 or ErbB4 to activate signal cascades, at least partly, by inducing protein phosphorylations that lead to various cellular responses. Consistent with this notion, HRG- β 1-activated p38 MAPK signal pathway is shown here to be required for up-regulation of VEGF in SKBr3 and MCF7 cells, although HRG- β 1 activated multiple pathways in these cells. Currently, we are trying to identify the transcription factor(s) that are phosphorylated by HRG- β 1-activated p38 MAPK and to decipher the regulatory mechanisms of the VEGF promoter (direct or indirect activation or derepression) by phosphorylated transcription factor(s). It is likely that the transcription factor(s) mediating induction by HRG may vary for different promoters in different cell types. Because HRG function is mediated by signaling pathways, it is possible that different signal pathways can be activated in different cell types in response to the HRG signal. Therefore, the downstream transcription factor(s) activated by HRG- β 1 may vary in different cell types for different target genes.

HRG induction of gene up-regulation is generally weak in that all of the previous reports and our current study showed only 2–3-fold induction of promoter activities for every induced promoter examined in stable transfectants or under transient transfection conditions. Notably, the p38 MAPK pathway was only transiently activated by HRG- β 1 for less than 30 min, whereas ERK1/2 and PI-3K/Akt activations were prolonged. This transient activation of p38 MAPK may be one of the reasons for weak induction of VEGF promoter activities. Because previous reports did not identify the signal pathway(s) mediating HRG-induced gene up-regulation, it is not clear whether the p38 MAPK pathway may be a common pathway for gene up-regulation by HRG- β 1. Although HRG-induced gene regulation is relatively weak, HRG-mediated transcriptional up-regulation is essential for development and normal cellular functions (27, 37). In addition, HRG- β 1 was shown to increase motility of MCF7 cells by 2–3-fold through up-regulation of autocrine motility factor (40). In particular, VEGF induction by HRG- β 1 reported here may contribute to breast cancer angiogenesis, given that the VEGF expression level is tightly controlled (1–3). In support of this notion, our data from endothelial cell migration assays demonstrated that the HRG- β 1-mediated increase of VEGF secretion leads to increased migration ability of endothelial cells, which is an important indicator of angiogenic response.

Our study shows for the first time that activation of the p38 MAPK

pathway by HRG- β 1 leads to transcriptional up-regulation of VEGF in breast cancer cells via uHRE in the VEGF promoter. In addition, we have demonstrated that VEGF antibody and p38 MAPK inhibitor SB203580 each can inhibit endothelial cell migration induced by HRG- β 1-mediated VEGF secretion. Furthermore, the combination of VEGF antibody and SB203580 has an additive inhibitory effect to block endothelial cell migration induced by HRG- β 1-mediated VEGF secretion. These promising results reveal the potential of using VEGF antibody in combination with chemical inhibitors of the p38 MAPK to inhibit HRG- β 1-mediated tumor angiogenesis in breast cancers.

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REFERENCES

- Carmeliet, P., Mackman, N., Moons, L., Luther, T., Gressens, P., Van Vlaenderen, I., Demunck, H., Kasper, M., Breier, G., Evrard, P., Muller, M., Risau, W., Edgington, T., and Collen, D. Role of tissue factor in embryonic blood vessel development. *Nature (Lond.)*, **383**: 73–75, 1996.
- Carmeliet, P., Ng, Y. S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V. V., Stalmans, I., Mattot, V., Perriard, J. C., Dewerchin, M., Flameng, W., Nagy, A., Lupu, F., Moons, L., Collen, D., D'Amore, P. A., and Shima, D. T. Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Nat. Med.*, **5**: 495–502, 1999.
- Carmeliet, P., and Collen, D. Role of vascular endothelial growth factor and vascular endothelial growth factor receptors in vascular development. *Curr. Top. Microbiol. Immunol.*, **237**: 133–158, 1999.
- Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth *in vivo*. *Nature (Lond.)*, **362**: 841–844, 1993.
- Shweiki, D., Itin, A., Soffer, D., and Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature (Lond.)*, **359**: 843–845, 1992.
- Shweiki, D., Neeman, M., Itin, A., and Keshet, E. Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumor angiogenesis. *Proc. Natl. Acad. Sci. USA*, **92**: 768–772, 1995.
- Plate, K. H., Breier, G., Weich, H. A., and Risau, W. Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas *in vivo*. *Nature (Lond.)*, **359**: 845–848, 1992.
- Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science (Washington DC)*, **246**: 1306–1309, 1989.
- Benjamin, L. E., and Keshet, E. Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. *Proc. Natl. Acad. Sci. USA*, **94**: 8761–8766, 1997.
- Carmeliet, P., Lampugnani, M. G., Moons, L., Breviaro, F., Compernelle, V., Bono, F., Balconi, G., Spagnuolo, R., Oostuyse, B., Dewerchin, M., Zanetti, A., Angellilo, A., Mattot, V., Nuyens, D., Lutgens, E., Clotman, F., de Ruiter, M. C., Gittenberger-de Groot, A., Poelmann, R., Lupu, F., Herbert, J. M., Collen, D., and Dejana, E. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell*, **98**: 147–157, 1999.
- Akagi, Y., Liu, W., Zebrowski, B., Xie, K., and Ellis, L. M. Regulation of vascular endothelial growth factor expression in human colon cancer by insulin-like growth factor-I. *Cancer Res.*, **58**: 4008–4014, 1998.
- Cohen, T., Nahari, D., Cerem, L. W., Neufeld, G., and Levi, B. Z. Interleukin 6 induces the expression of vascular endothelial growth factor. *J. Biol. Chem.*, **271**: 736–741, 1996.
- Dolecki, G. J., and Connolly, D. T. Effects of a variety of cytokines and inducing agents on vascular permeability factor mRNA levels in U937 cells. *Biochem. Biophys. Res. Commun.*, **180**: 572–578, 1991.
- Wang, D., Huang, H. J., Kazlauskas, A., and Cavenee, W. K. Induction of vascular endothelial growth factor expression in endothelial cells by platelet-derived growth factor through the activation of phosphatidylinositol 3-kinase. *Cancer Res.*, **59**: 1464–1472, 1999.
- Richard, D. E., Berra, E., and Pouyssegur, J. Angiogenesis: how a tumor adapts to hypoxia. *Biochem. Biophys. Res. Commun.*, **266**: 718–722, 1999.
- Zhang, L., Yu, D., Hu, M., Xiong, S., Lang, A., Ellis, L., and Pollock, R. E. Wild-type p-53 suppresses angiogenesis in human leiomyosarcoma and synovial sarcoma by transcriptional suppression of VEGF expression. *Cancer Res.*, **60**: 3655–3661, 2000.
- Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W.-J., Wood, W. I., Goeddel, D. V., and Vandlen, R. L. Identification of Heregulin, a specific activator of p185^{erbB2}. *Science (Washington DC)*, **256**: 1205–1210, 1992.
- Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Birmingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J. B., Hsuan, J. J., Totty, N. F., Otsu, M., McBurney, R. N., Waterfield, M. D., Stroobant, P., and Gwynne, D. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature (Lond.)*, **362**: 312–317, 1993.
- Burden, S., and Yarden, Y. Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron*, **18**: 847–855, 1997.
- Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. *Cell*, **72**: 801–815, 1993.
- Riese, D. J., II, and Stern, D. F. Specificity within the EGF family/ErbB receptor family signaling network. *BioEssays*, **20**: 41–48, 1998.
- Daly, J. M., Jannot, C. B., Beerli, R. R., Graus-Porta, D., Maurer, F. G., and Hynes, N. E. Neu differentiation factor induces erbB2 down-regulation and apoptosis of erbB-2-overexpressing breast tumor cells. *Cancer Res.*, **57**: 3804–3811, 1997.
- Bacus, S. S., Gudkov, A. V., Zelnick, C. R., Chin, D., Stern, R., Stancovski, I., Peles, E., Ben-Baruch, N., Farbstein, H., and Lupu, R. Neu differentiation factor (heregulin) induces expression of intercellular adhesion molecule 1: implications for mammary tumors. *Cancer Res.*, **53**: 5251–5261, 1993.
- Staehler, A., Sommers, C., Mueller, S., Bayers, S., Thompson, E. W., and Lupu, R. Modulation of breast cancer progression and differentiation by the gp30/hergulin. *Breast Cancer Res. Treat.*, **31**: 175–182, 1994.
- Tan, M., Grijalva, R., and Yu, D. Heregulin B1-activated phosphatidylinositol 3-kinase enhances aggregation of MCF7 breast cancer cells independent of extracellular signal-regulated kinase. *Cancer Res.*, **59**: 1620–1625, 1999.
- Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben Levy, R., and Yarden, Y. Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell*, **69**: 205–216, 1992.
- Meyer, D., and Birchmeier, C. Multiple essential functions of neuregulin in development. *Nature (Lond.)*, **378**: 386–390, 1995.
- Lewis, G. D., Lofgren, J. A., McMurtry, A. E., Nuijens, A., Fendly, B. M., Bauer, K. D., and Sliwkowski, M. X. Growth regulation of human breast and ovarian tumor cells by heregulin: evidence for the requirement of ErbB2 as a critical component in mediating heregulin responsiveness. *Cancer Res.*, **56**: 1457–1465, 1996.
- Tang, C. K., Perez, C., Grunt, T., Waibel, C., Cho, C., and Lupu, R. Involvement of heregulin- β 2 in the acquisition of the hormone-independent phenotype of breast cancer cells. *Cancer Res.*, **56**: 3350–3358, 1996.
- Lupu, R., Cardillo, M., Cho, C., Harris, L., Hijazi, M., Perez, C., Rosenberg, K., Yang, D., and Tang, C. The significance of heregulin in breast cancer tumor progression and drug resistance. *Breast Cancer Res. Treat.*, **38**: 57–66, 1996.
- Altiok, N., Altiok, S., and Changeux, J. P. Heregulin-stimulated acetylcholine receptor gene expression in muscle: requirement for MAP kinase and evidence for a parallel inhibitory pathway independent of electrical activity. *EMBO J.*, **16**: 717–725, 1997.
- Schaeffer, L., Duclert, N., Huchet-Dymanus, M., and Changeux, J. P. Implication of a multisubunit Ets-related transcription factor in synaptic expression of the nicotinic acetylcholine receptor. *EMBO J.*, **17**: 3078–3090, 1998.
- Sapru, M. K., Florance, S. K., Kirk, C., and Goldman, D. Identification of a neuregulin and protein-tyrosine phosphatase response element in the nicotinic acetylcholine receptor epsilon subunit gene: regulatory role of an Rts transcription factor. *Proc. Natl. Acad. Sci. USA*, **95**: 1289–1294, 1998.
- Khurana, T. S., Rosmarin, A. G., Shang, J., Krag, T. O., Das, S., and Gammeltoft, S. Activation of utrophin promoter by heregulin via the ets-related transcription factor complex GA-binding protein α/β . *Mol. Biol. Cell*, **10**: 2075–2086, 1999.
- Gramolini, A. O., Angus, L. M., Schaeffer, L., Burton, E. A., Tinsley, J. M., Davies, K. E., Changeux, J. P., and Jasmin, B. J. Induction of utrophin gene expression by heregulin in skeletal muscle cells: role of the N-box motif and GA binding protein. *Proc. Natl. Acad. Sci. USA*, **96**: 3223–3227, 1999.
- Alroy, I., Soussan, L., Seger, R., and Yarden, Y. Neu differentiation factor stimulates phosphorylation and activation of the Sp1 transcription factor. *Mol. Cell. Biol.*, **19**: 1961–1972, 1999.
- Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Koski, R. A., Lu, H. S., and Yarden, Y. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell*, **69**: 559–572, 1992.
- Sepp-Lorenzino, L., Eberhard, I., Ma, Z., Cho, C., Serve, H., Liu, F., Rosen, N., and Lupu, R. Signal transduction pathways induced by heregulin in MDA-MB-453 breast cancer cells. *Oncogene*, **12**: 1679–1687, 1996.
- Daly, J. M., Olayioye, M. A., Wong, A. M., Neve, R., Lane, H. A., Maurer, F. G., and Hynes, N. E. NDF/hergulin-induced cell cycle changes and apoptosis in breast tumor cells: role of PI3 kinase and p38 MAP kinase pathways. *Oncogene*, **18**: 3440–3451, 1999.
- Talukder, A. H., Adam, L., Raz, A., and Kumar, R. Heregulin regulation of autocrine motility factor expression in human tumor cells. *Cancer Res.*, **60**: 474–480, 2000.