Differential Regulation of Tumor Angiogenesis by Distinct ErbB Homo- and Heterodimers

Lily Yen,* Naciba Benlimame,* Zeng-Rong Nie,* Dingzhang Xiao,*
Taiqi Wang,* Ala-Eddin Al Moustafa,* Hiroyasu Esumi,[†] Julie Milanini,[‡]
Nancy E. Hynes,[§] Gilles Pages,[‡] and Moulay A. Alaoui-Jamali*^{||}

*Lady Davis Institute for Medical Research of the Sir Mortimer B. Davis Jewish General Hospital, Departments of Medicine, Oncology, Pharmacology/Therapeutics and McGill Centre for Translational Research in Cancer, McGill University, Montreal, Quebec, H3T 1E2, Canada; [†]Investigative Treatment Division, National Cancer Center Research Institute East, Chiba, 277-8577 Japan; [‡]Centre de Biochimie, Centre National de la Recherche Scientifique-Unité Mixte Recherche 6543, Université de Nice, 06108 Nice, France; and [§]Friedrich Miescher-Institute, CH-4002 Basel, Switzerland

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Interactions between cancer cells and their microenvironment are critical for the development and progression of solid tumors. This study is the first to examine the role of all members of the ErbB tyrosine kinase receptors (epidermal growth factor receptor [EGFR], ErbB-2, ErbB-3, or ErbB-4), expressed singly or as paired receptor combinations, in the regulation of angiogenesis both in vitro and in vivo. Comparison of all receptor combinations reveals that EGFR/ErbB-2 and ErbB-2/ErbB-3 heterodimers are the most potent inducers of vascular endothelial growth factor (VEGF) mRNA expression compared with EGFR/ErbB-3, EGFR/ErbB-4, ErbB-2/ErbB-4, and ErbB-3/ErbB-4. Immunohistochemistry of tumor xenografts overexpressing these heterodimers shows increased VEGF expression and remarkably enhanced vascularity. Enhanced VEGF expression is associated with increased VEGF transcription. Deletional analysis reveals that ErbB-mediated transcriptional up-regulation of VEGF involves a hypoxia-inducible factor 1-independent responsive region located between nucleotides –88 to –66 of the VEGF promoter. Mutational analysis reveals that the Sp-1 and AP-2 transcription factor binding elements within this region are required for up-regulation of VEGF by heregulin β 1 and that this up-regulation is dependent on the activity of extracellular signal-related protein kinases. These results emphasize the biological implications of cell signaling diversity among members of the ErbB receptor family in regulation of the tumor microenvironment.

INTRODUCTION

Under physiological conditions, the tissue microenvironment is regulated by a complex process involving autocrine and paracrine loops, whereby epithelial cells interact with

mesothelial cells, endothelial cells, and components of the extracellular matrix. Structural and functional changes in the tumor microenvironment have been associated with the development and progression of solid tumors. Among these, the induction of angiogenesis, a process that broadly refers to the formation of new capillary blood vessels from preexisting vessels, has been shown to be essential for tumor growth as well as tumor invasion to distant sites (Folkman, 1990). Indeed, it seems that many tumors initially lie dormant until a tumor-mediated switch in the local equilibrium between negative and positive regulators of angiogenesis occurs.

Several factors have been shown to contribute to the regulation of angiogenic switches, including the tumor suppressors p53 and von Hippel-Lindau; hypoxia; nitric oxide; and oncogenes such as ras, raf, and src (Grugel *et al.*, 1995;

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^{||}Corresponding author. E-mail address: malaou@po-box.mcgill.ca.

Abbreviations used: AR, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-related kinase; FGF, fibroblast growth factor; HIF1, hypoxia-inducible factor 1; HRG, heregulin; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PDGF, platelet-derived growth factor; TGF α , transforming growth factor- α ; VEGF, vascular endothelial growth factor.

Mukhopadhyay *et al.*, 1995; Rak *et al.*, 1995; Liekens *et al.*, 2001). We and others have reported previously that signaling through the epidermal growth factor receptor (EGFR) or ErbB-2 receptor tyrosine kinases play a role in the regulation of angiogenesis (Goldman *et al.*, 1993; Vitoria-Petit *et al.*, 1997; Yen *et al.*, 2000), at least in part via up-regulation of a potent angiogenic factor known as vascular endothelial growth factor (VEGF) (Eriksson and Alitalo, 1999). The ErbB family of receptor tyrosine kinases consists of four members, which includes EGFR (HER1/ErbB-1), ErbB-2 (HER2/Neu), ErbB-3 (HER3), and ErbB-4 (HER4). Aberrant expression of these receptors is commonly found in human cancers (Hynes and Stern, 1994; Salomon *et al.*, 1995; Yarden and Sliwkowski, 2001), and cooverexpression of multiple members of the ErbB family has been reported in a number of cancers, including breast and ovarian (Osaki *et al.*, 1992; Simpson *et al.*, 1995; deFazio *et al.*, 2000). Both the diversity and hence complexity of the ErbB signaling network are mediated by the existence of multiple ligands, each with specificity toward distinct members of the ErbB family. The ligands for the ErbB receptors can be sorted into three categories based on their binding specificities: 1) those that bind EGFR alone (epidermal growth factor [EGF], transforming growth factor- α [TGF α], and amphiregulin [AR]), 2) those that bind to ErbB-3 or ErbB-4 (heregulins/neuregulins), and 3) those that bind to ErbB-4 or EGFR (betacellulin, epiregulin, and heparin-binding EGF) (Riese and Stern, 1998). The ErbB receptors take part in a complex process of combinatorial interactions by the formation of ligand-induced heterodimers between the different family members (Carraway and Cantley, 1994; Alroy and Yarden, 1997; Riese and Stern, 1998), whose binding results in the formation of nine possible homo- and heterodimeric receptor combinations. Each of the dimeric receptor complexes in turn activates distinct signaling pathways (Carraway and Cantley, 1994; Alroy and Yarden, 1997).

Previous studies, including our own, have shown that cell signaling associated with EGFR and ErbB-2 is implicated in angiogenesis (Goldman *et al.*, 1993; Vitoria-Petit *et al.*, 1997; Yen *et al.*, 2000). However, none of these studies have addressed the role of ErbB-3, ErbB-4, or ErbB receptors in the context of heterodimers. As yet, there is no understanding of the relative contribution each specific ErbB receptor or receptor combinations has on the induction of angiogenesis and which signaling pathway(s) are involved. Herein, we carried out a comprehensive study to examine the effects of EGFR, ErbB-2, ErbB-3, and ErbB-4 receptors, expressed singly or as paired combinations, on the regulation of angiogenesis in *in vivo* conditions. We report that the EGFR/ErbB-2 and ErbB-2/ErbB-3 heterodimers are the most potent inducers of VEGF expression and tumor vascularization compared with all other receptor combinations. We also demonstrate that activation of ErbB receptors by ErbB ligands regulates VEGF at the transcriptional level, and we have localized the region of the VEGF promoter that confers this responsiveness.

MATERIALS AND METHODS

Cell Culture

NIH3T3 mouse fibroblasts and the MCF7 human breast adenocarcinoma cells were obtained from American Type Culture Collection

(Manassas, VA). The MCF7 clone stably transfected with ErbB-2 (MCF7-HER218) and its neomycin control (MCF7-neo22) (Benz *et al.*, 1993) were graciously provided by Dr. C. Kent Osborne. The T47D-5R and control T47D-puromycin cells were described previously (Jannot *et al.*, 1996). Cancer cells were maintained in the appropriate media (Mediatech, Herndon, VA) described by the American Type Culture Collection supplemented with 10% fetal bovine serum (MCF7 and T47D) or calf serum (NIH3T3) and antibiotics.

Retrovector Construction and Stable Overexpression of Receptors in NIH3T3 and Breast Cancer Cells

We created bicistronic retrovectors to coexpress each ErbB receptor member with the enhanced green fluorescent protein (EGFP) as a marker for proviral transfer in the target cells. The AP2 retrovector, pJ6bleo plasmid, and 293GPG retroviral packaging cell line (Galipeau *et al.*, 1999) were generously provided by Dr. Jacques Galipeau (McGill University, Montreal, Quebec, Canada). The pLXSN-EGFR, pLXSN-ErbB-2, and pLXSN-ErbB-3 constructs (Riese *et al.*, 1995) were kind gifts from Drs. David J. Riese II (Purdue University, West Lafayette, IN) and David F. Stern (Yale University, New Haven, CT). The AP2-EGFR, AP2-ErbB-2, and AP2-ErbB-3 retrovectors were generated as follows: the 4.1-kb *XhoI* fragment of pLXSN-EGFR, the 4.1-kb *XhoI* fragment of pLXSN-ErbB-2, or the 4.14-kb *XhoI*-*BamHI* fragment of pLXSN-ErbB-3, and the 4.66-kb *NarI*-*BamHI* fragment of pLXSN-ErbB-4, each containing complete ErbB cDNA, were isolated. Each fragment was then subcloned into the corresponding sites of the AP2 retrovector, with the exception that the ErbB-4 cDNA was inserted into the *BstBI*-*BamHI* sites of AP2. Recombinant retroviral particles were generated by stable transfection of the 293GPG packaging cell line, with subsequent production of high-titer retrovirus as described previously (Galipeau *et al.*, 1999). MCF7 and NIH3T3 cells were transduced with singly or paired combinations of AP2-ErbB retroviral particles. Cells were plated at a density of 2×10^4 cells/well in a 24-well plate with 50 μ l of concentrated retrovirus added to the growth media. The following day, the media were replaced with fresh media containing virus. Transductions were repeated daily for three consecutive days. Stably transduced cells were expanded and flow cytometric analysis was performed with an Epics XL/MCL analyzer (Beckman Coulter, Fullerton, CA) to verify gene transfer efficiency as measured by green fluorescent protein (GFP) fluorescence.

VEGF Promoter Constructs

The -2279/+54, -1179/+54, -1014/+54, and -794/+54 VEGF promoter-luciferase reporter gene constructs cloned into the pGL2-basic vector (Promega, Madison, WI) were described previously (denoted as phVEGF1, phVEGF2, phVEGF3, and phVEGF4, respectively) (Minchenko *et al.*, 1994; Kimura *et al.*, 2000). The -416/+54 construct was obtained by ligating the 479-base pair *BglIII* fragment of the promoter into the *BglIII* site of pGL2-basic. The -272/+54 construct was prepared by digesting the -2279/+54 vector with *SacI/KpnI* to release the 5'-end, blunt-ending the vector, and then religating. The -136/+54 construct was obtained by digestion of the -2279/+54 vector with *ApaI/KpnI*, blunt-ending and then religating. The -88/+54 (p88-wt), p88-mutAP-2, p88-mutSp-1, p88-mutAP-2/Sp-1, and -66/+54, -52/+54 constructs have been described previously (Milanini *et al.*, 1998).

Transient Transfection and Luciferase Assay

Cells seeded in 12-well plates (10^5 cells/well) were transiently transfected by LipofectAMINE (Invitrogen, Carlsbad, CA) with 500 ng/well of reporter plasmid plus 250 ng/well of CMV β -galactosidase plasmid (control for transfection efficiency) in serum-free medium according to manufacturer's instructions. After 5 h of transfection, the cells were incubated with fresh serum-free medium with or

without growth factors or hormone stimulation (20 ng/ml heregulin [HRG] β 1, human recombinant heregulin β 1, Neomarkers, Fremont, CA; and 20 ng/ml human recombinant EGF, Invitrogen; and 20 ng/ml amphiregulin, R & D Systems, Minneapolis, MN). For inhibition experiments, 6 μ M UO126 (Alexis, Lufelfingen, Switzerland) was added 1 h before addition of growth factors. After 48 h, the cells were rinsed with cold phosphate-buffered saline (PBS), and extracts were collected and assayed for luciferase activity as per Promega protocols by using a Lumat LB9507 luminometer (PerkinElmer Life Sciences, Boston, MA). Luciferase activity was normalized for β -gal activity.

Preparation of Nuclear Extracts and Electromobility Shift Assays (EMSAs)

Preconfluent MCF7-neo22 cells were serum starved overnight followed by stimulation with or without 20 ng/ml HRG for 24 h. Nuclear extracts were prepared by the method of Andrews and Faller (1991). The VEGF probe was synthesized to span the –88 to –66 region of the human VEGF promoter (5'-TTTCCGGGCGG-GCCGGGGCGGGGAT-3', with random sequences added to each end of the wild-type sequence denoted in italics). The probe was end labeled with T4 PNK and [γ -³²P]ATP. The binding reaction was performed in a final volume of 20 μ l. Nuclear extracts (3 μ g) were preincubated for 10 min in binding buffer [20 mM HEPES, pH 7.9, 5% glycerol, 100 mM KCl, 200 mM EDTA, pH 8.0, 200 mM EGTA, pH 8.0, 0.05 mg/ml poly(dI-dC), 2 mM dithiothreitol] with or without 100-fold excess unlabeled probe, consensus oligonucleotide for AP-2 (5'-GATCGAACTGACCGCCCGCGGCCGT-3'; Santa Cruz Biotechnology, Santa Cruz, CA), or consensus oligonucleotide for Sp-1 (5'-ATTGATCGGGGCGGGCGAGC-3'; Santa Cruz Biotechnology). Labeled probe (0.1 ng) was then added and the reaction incubated for 20 min. Samples were resolved in a 5% polyacrylamide gel.

Western Blot and Immunoprecipitation Analysis

Cells at 60–70% confluence were starved for 24 h in serum-free medium and then treated with HRG or EGF when indicated. Cell extracts were prepared (Yen *et al.*, 2000), blotted, and then detected with antibody against ErbB-2 (antibody-3, clone 3B5; Oncogene Science, Cambridge, MA), EGFR (clone13; Transduction Laboratories, Lexington, KY), ErbB-3 (clone C-17; Santa Cruz Biotechnology), or ErbB-4 (clone C-18; Santa Cruz Biotechnology). Blots were subsequently stripped and immunoblotted with monoclonal anti-GAPDH antibody (clone 6C5; Cedarlane Laboratories, Oakville, Ontario, Canada). For immunoprecipitation, 200 μ g of protein was immunoprecipitated with anti-EGFR (clone13), anti-ErbB-2 (antibody-3), anti-ErbB-3 (clone C-17), or anti-ErbB-4 (clone H4.77.16, Neomarkers) as described previously (You *et al.*, 1998). Immunoprecipitated samples were then blotted on nitrocellulose and detected with antibody against phosphotyrosine (4G10; Upstate Biotechnology). Blots were then stripped and immunoblotted with antibodies specific for each immunoprecipitated receptor, as described above. To determine extracellular signal-regulated kinase (ERK) activation, extracts from cell stimulated with HRG or EGF (as described above) were immunoblotted with antibody specific for phosphorylated ERK1 and ERK2 (12D4; Upstate Biotechnology). Blots were then stripped and reprobed with antibody that recognizes total ERK1 and ERK2 (ERK1/2-CT; Upstate Biotechnology).

Immunofluorescence

NIH3T3 cells overexpressing ErbB receptors were seeded on coverslips for 2 d at a density of 50,000 cells/35-mm dish. The cells were rinsed with PBS and fixed with 3% (wt/vol) paraformaldehyde in PBS for 5 min., followed by an incubation in precooled methanol (–20°C) for 15 min. The cells were then rinsed with PBS and blocked with 2% bovine serum albumin (BSA), 2% normal goat

serum, and 0.2% gelatin in PBS. The cells were then incubated with the following primary antibodies: monoclonal anti-EGFR antibody (antibody-1; Calbiochem, San Diego, CA); monoclonal anti-ErbB-2 antibody (antibody-3), polyclonal anti-ErbB-3 (C-17), or polyclonal anti-ErbB-4 (clone H4.77.16) for 1 h at room temperature. All antibody dilutions were made in blocking solution. After three washes with 0.2% BSA in PBS, the cells were incubated with appropriate secondary antibodies conjugated to Texas Red (Jackson Immuno-research Laboratories, West Grove, PA) for 30 min. The coverslips were then washed with PBS and mounted with Airvol (Air Products and Chemicals). Confocal analyses were performed using an LSM 410 confocal microscope (Zeiss, Jena, Germany).

Northern Blot Analysis

Cells at 70% confluence were starved for 24 h in serum-free medium and then treated with 20 ng/ml HRG for 24 h. Cells were lysed directly and RNA was extracted using RNA-Plus reagent (Quantum Biotech). Total RNA (25 μ g) was resolved by electrophoresis through a 1% denaturing formaldehyde gel and transferred to nylon membrane. Equal loading of RNA in each lane was evaluated by ethidium bromide staining before transfer. The cDNA probe for VEGF₁₆₅ (Yen *et al.*, 2000) was ³²P-labeled (Oligolabelling kit; Amersham Biosciences, Piscataway, NJ) and used to hybridize overnight at 42°C, and then autoradiographed. Autoradiograms were digitized by scanning and densitometric analysis was performed using Scion Image version 4.02 (Scion, Fredrick, MD) software.

Growth of Tumor Xenografts in Nude Mice

Subconfluent MCF7 and NIH3T3 cells were suspended in PBS and injected into the mammary fat pads or subcutaneously into the flanks (5 $\times$ 10⁵/50 μ l or 10⁶/0.2 ml, respectively) of BALB/c (nu/nu) mice. Experimental animals were cared for in accordance with institutional and federal guidelines.

Immunohistochemistry of Tumor Xenografts

For tumor histology, tumors were either snap frozen in liquid nitrogen or fixed in 10% buffered formalin and embedded in paraffin. Antibodies used for immunohistochemistry were as follows: rat monoclonal anti-mouse CD31 (Mec 13.3; BD PharMingen, San Diego, CA); mouse monoclonal against ErbB-2 (antibody-3); mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (Novacastra); and rabbit polyclonal anti-VEGF (147; Santa Cruz Biotechnology). Cryostat sections were used for CD31 staining, whereas paraffin-embedded material was used for the remaining antibodies. All sections were analyzed by conventional light microscopy and photographed using color slides (Eastman Kodak, Rochester, NY). For CD31 staining, 7- μ m cryosections of tumors were air-dried and fixed in –20°C acetone for 10 min. Sections were rehydrated in PBS and then blocked with 5% normal goat serum for 1 h. The sections were then incubated overnight at 4°C with CD31 antibody diluted 1:25 in 3% BSA-PBS. After several PBS rinses, sections were incubated for 30 min with a biotinylated secondary anti-rat antibody (BD PharMingen), followed by a 30-min incubation with avidin-biotin-horseradish peroxidase complex, and then developed with DAB kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with Harris' hematoxylin and mounted. VEGF, ErbB, and PCNA immunostaining was performed as described previously (Jaitovich-Groisman *et al.*, 2001), with primary antibodies diluted (ErbB-2, 1:100; PCNA, 1:100; and VEGF, 1:200) in 5% normal goat serum. After digitization, color images were processed using specialized functions from Photoshop 5.5 imaging software (Adobe Systems, Mountain View, CA). Briefly, specific CD31 staining of the vessels was semiautomatically amplified, measured, and reported as an area per square millimeter.

Figure 1. Characterization of receptor expression in NIH3T3 control and ErbB-transduced cells overexpressing receptor combinations. Immunofluorescence was performed using antibodies for the respective receptors and Texas Red secondary antibodies. Immunofluorescence of NIH3T3 cells transduced with control AP2 (GFP) retrovirus (A), transduced to overexpress single ErbB receptors (B), or transduced with paired ErbB receptor combinations (C). DIC, differential interference contrast.

RESULTS

Expression of ErbB Receptor Combinations in NIH3T3 Cells Reconstitutes Ligand-activated Signaling

Activation of distinct ErbB receptor dimers results in the initiation of specific downstream signaling events. Thus, to delineate the relative contribution of each ErbB receptor to angiogenesis, we developed an NIH3T3 model of ErbB overexpression whereby polyclonal populations of cells overexpress ErbB receptor members, either singly or in paired combinations (Figure 1). NIH3T3 murine fibroblast cells with undetectable endogenous ErbB receptor

expression were stably transduced with a bicistronic retrovirus that coexpresses both a specific ErbB receptor and the enhanced green fluorescent protein. The use of polyclonal populations of transduced cells overcomes artifacts associated with clonal cell selection. As shown in Figure 1, >95% of cells were GFP positive in all transduced cells. Immunofluorescence was used to verify the receptor expression in cells transduced with control AP2 virus (Figure 1A), cells overexpressing single ErbB receptors (Figure 1B), or cells overexpressing receptor pairs (Figure 1C). The immunofluorescence shows that >95% of cells were positive for either single (Figure 1B) or double (Figure 1C) ErbB receptor expression, as evidenced by positive stain-

Figure 2. Characterization of receptor expression in ErbB-transduced NIH3T3 cells. Western blot analysis was performed on NIH3T3 cells using antibodies for the respective receptors. (A) Western blot analysis of the control NIH3T3-AP2 cells and NIH3T3 cells expressing ErbB receptor combinations. (B and C) Activation of the receptors in response to ligand. NIH3T3 cells expressing ErbB homo- (B) or heterodimers (C) were stimulated for 10 min with no growth factor (control) or the ErbB ligands EGF or heregulin $\beta 1$, and then the receptors were immunoprecipitated with receptor-specific antibodies followed by immunoblotting to detect phosphotyrosine. Blots were subsequently stripped and reprobed for with the appropriate ErbB-specific antibodies.

Figure 3. Effect of ErbB overexpression on VEGF mRNA levels. Northern blot analysis of NIH3T3 cells transduced with control AP2, single ErbB receptors, or paired ErbB receptors. Total RNA was isolated and used to determine VEGF mRNA expression. Loading was controlled by staining the gel with ethidium bromide. Bottom, quantitation of VEGF mRNA is shown. The results are representative of three separate experiments.

ing for Texas Red. Therefore, it is evident that in the case of doubly transduced cells, both receptors are coexpressed within the same cell. Western blot analysis of the different receptors within each group (Figure 2A) shows that all receptors are expressed at similar levels within all cell groups. The activation profile of the receptor combinations in response to the ErbB ligands EGF or HRG was determined, as measured by receptor tyrosine phosphorylation (Figure 2, B and C). NIH3T3 cells expressing ErbB homo- and heterodimers were stimulated by EGF or HRG, and then the receptors were immunoprecipitated with receptor-specific antibodies followed by immunoblotting to detect phosphotyrosine. Receptor activation in response to ligand for cells expressing one ErbB receptor family member (Figure 2B) revealed that EGFR was partially active in the absence of ligand and was greatly stimulated upon addition of EGF. As expected, overexpression of ErbB-2 resulted in constitutive activation of the receptor (Wallasch *et al.*, 1995; Zhang *et al.*, 1996). Cells expressing ErbB-4 responded modestly to stimulation by HRG. ErbB-3 homodimers showed weak response to HRG, consistent with the notion that ErbB-3 has an impaired kinase domain (Guy *et al.*, 1994). In cells expressing pairwise receptor combinations, EGF activated all EGFR-containing heterodimers, EGFR/ErbB-2, EGFR/ErbB-3,

Figure 4. Differential VEGF expression and angiogenesis in NIH3T3 cells overexpressing of distinct ErbB receptor combinations. Transduced NIH3T3 cells, expressing single ErbB receptors (A) or paired ErbB receptor combinations (B), were injected subcutaneously into BALB/c nude mice and resulting tumor xenografts were analyzed immunohistochemically. Paraffin sections from NIH3T3-ErbB tumors were stained with hematoxylin and eosin to analyze the tumor morphology or immunolabeled with anti-VEGF antibody. Cryostat sections were immunolabeled with anti-CD31 antibody. The primary antibody was omitted for negative control staining (-). Bar, 400 μ m. (C) Quantitation of vascularization in NIH3T3-ErbB tumors. NIH3T3-ErbB tumors were quantitated for vessel staining as described in MATERIALS AND METHODS. Results were obtained from three independent tumor sections. Values represent the mean $\pm$ SD. * p < 0.05, compared with EGFR or ErbB-2; ** p < 0.05, compared with ErbB-2 or ErbB-3.

and EGFR/ErbB-4 (Figure 2C). HRG activated EGFR/ErbB-3, EGFR/ErbB-4, and ErbB-3/ErbB-4 heterodimers weakly, whereas ErbB-2/ErbB-3 heterodimers show robust activation upon stimulation with HRG. Interestingly, in cells overexpressing ErbB-2/ErbB-4 (Figure 2C, bottom right), the ErbB-2 remains constitutively active in the absence of ligand, whereas the ErbB-4 in these cells becomes phosphorylated only upon stimulation with its cognate ligand, HRG; This activation profile has been

previously shown (Cohen *et al.*, 1996) and is consistent with the notion that heterodimerization of ErbB-2/ErbB-4 occurs only in the presence of ligand, whereas in the absence of ligand, only ErbB-2 displays constitutive ligand-independent receptor phosphorylation. Taken together, these results show that in cells expressing receptor combinations, stimulation of the receptors with EGF or HRG results in the formation of active heterodimer complexes.

Figure 4 (legend on facing page).

Up-Regulation of VEGF Is Mediated by Discrete ErbB Receptor Combinations In Vitro

Northern blot analysis on NIH3T3 cells overexpressing the different receptor combinations reveals that levels of VEGF mRNA are moderately increased upon overexpression of EGFR alone (Figure 3, lane 2) and ErbB-2 alone (lane 3), compared with control-transduced cells. Overexpression of EGFR/ErbB-2 and ErbB-2/ErbB-3 yielded even greater induction of VEGF mRNA expression (lanes 6 and 9, respectively), with a slight down-regulation in all cells expressing ErbB-4 combinations: ErbB-4, EGFR/ErbB-4, ErbB-2/ErbB-4, and ErbB-3/ErbB-4 (bottom).

In Vivo Angiogenesis Is Regulated by Distinct ErbB Heterodimers

To determine whether the in vitro up-regulation of VEGF mRNA by specific ErbB receptor dimers correlates with in vivo angiogenesis, we injected the transduced NIH3T3 cells subcutaneously into nude mice. In all cases, the expression of these receptors in the tumors was confirmed using immunohistochemistry (our unpublished data). As expected, the control AP2 virus-transduced cells were unable to form tumors. Interestingly, cells overexpressing ErbB-3 alone formed small local tumors after 2 mo of growth. Tumor formation of the remaining receptor combinations is in agreement with previous studies showing that overexpression of ErbB-2 (Di Fiore *et al.*, 1987b) or EGFR (Di Fiore *et al.*, 1987a) results in a ligand-independent and -dependent transformation of NIH3T3 cells, respectively, and that ErbB-2 has a synergistic transforming effect with EGFR, ErbB-3, and ErbB-4 (Kokai *et al.*, 1989; Alimandi *et al.*, 1995).

To determine whether the overexpression of different ErbB receptor combinations were associated with a change in tumor vascularity, we performed a histological analysis of the tumor sections from the NIH3T3-ErbB

xenografts (Figure 4). Consistent with the Northern blot analysis, VEGF immunohistochemistry reveals increased VEGF protein production in tumors overexpressing ErbB-2 (Figure 4A). Strikingly, tumors overexpressing ErbB-2/EGFR and ErbB-2/ErbB-3 heterodimers are the most potent inducers of VEGF expression (Figure 4B, 1st and 3rd rows, respectively). It is evident that the same tumors from the same cells that exhibited enhanced VEGF expression in vitro also show remarkably enhanced vascularity, as determined by immunohistochemical analysis for CD31, which stains the endothelial cells lining the blood vessels (Figure 4, A and B, last column). In particular, tumors derived from cells overexpressing ErbB-2 (Figure 4A, 2nd row), EGFR/ErbB-2 (Figure 4B, 1st row), or ErbB-2/ErbB-3 (Figure 4B, 3rd row) develop a greater number of blood vessels, with extensive branching and lumen formation. Quantitation of the vascularization (Figure 4C) confirms the assertion that signaling from specific ErbB receptor heterodimers is important in the in vivo regulation of tumor angiogenesis and highlights the strong up-regulation of angiogenesis by EGFR/ErbB-2 and ErbB-2/ErbB-3 heterodimers. Interestingly, tumors overexpressing EGFR/ErbB-4, ErbB-2/ErbB-4, and ErbB-3/ErbB-4 also show some increased vascularization, but no apparent induction of VEGF (Figure 4, B and C).

Overexpression of ErbB-2 Results in Enhanced Tumor Angiogenesis

To further confirm the importance of ErbB-2 heterodimer signaling in the induction of angiogenesis, we broadened our study to include two model systems of the MCF7 human mammary carcinoma cell line. MCF7 cells express all of the members of the ErbB receptor family, none of which are significantly overexpressed (Beerli *et al.*, 1995). We used the MCF7-HER218 clone stably transfected with ErbB-2 (and its MCF7-neo22 control) (Benz *et al.*, 1993), as well as a polyclonal population of MCF7 cells stably overexpressing ErbB-2 via transduction with retrovirus (MCF7-ErbB-2 and its control MCF7-AP2). As shown in Figure 5, overexpression of ErbB-2 results in constitutive activation of ErbB-2, as shown by phosphotyrosine content of the receptor. Enhanced activation of the receptor is observed after stimulation by HRG. To examine the effects of ErbB-2 overexpression on angiogenesis in vivo, MCF7-AP2 and -ErbB-2 cells

Figure 5. Overexpression of ErbB-2 in human MCF7 breast cancer cells. Serum-starved MCF7-neo22, -HER218, -AP2, and -ErbB-2 cells were stimulated with 50 ng/ml EGF or 100 ng/ml HRG for 10 min. ErbB-2 was immunoprecipitated, and tyrosine phosphorylation of the receptor was determined with anti-phosphotyrosine antibody. The blot was then stripped and probed with anti-ErbB-2 antibody.

were injected into the mammary fat pad (to mimic the mammary gland microenvironment) and the resulting tumors were analyzed. Immunohistochemical analysis using an antibody against ErbB-2 confirmed that the tumor cells maintain ErbB-2 overexpression (Figure 6A, 4th row). Immunohistochemistry with antibody specific for VEGF revealed an elevation of VEGF staining in ErbB-2-overexpressing tumors compared with their AP2 controls (5th row). Consistent with the increased VEGF expression, CD31 immunohistochemistry reveals a significant increase in blood vessel density in MCF7-ErbB-2 tumors (bottom row), with ErbB-2-overexpressing tumors creating a larger and denser network of vessels. The MCF7-ErbB-2 tumors were found to have ~65.3% more vessels compared with the controls (Figure 6B).

Heregulin β 1 Stimulation Activates Transcription of the VEGF Promoter

To determine the molecular mechanisms responsible for the up-regulation of VEGF expression in response to stimulation by HRG, we performed a detailed examination of VEGF gene regulation in paired cell lines expressing the ErbB receptors. VEGF up-regulation was confirmed after HRG stimulation of ErbB receptors without a significant change in VEGF mRNA stability (our unpublished data). To determine whether the up-regulation of VEGF by the ErbB receptors is controlled at the level of transcription, we transiently transfected a luciferase reporter gene construct driven by the full-length VEGF promoter into MCF7-HER218 cells overexpressing ErbB-2, as well as its

Figure 6. Overexpression of ErbB-2 results in enhanced neovascularization. Immunohistochemical analysis of MCF7-ErbB-2 and -AP2 mammary fat pad tumor sections. Tumors were fixed, embedded in paraffin, and stained with hematoxylin and eosin or immunolabeled for ErbB-2, VEGF, or PCNA. To visualize the blood vessel endothelial cells, cryosections of tumors were stained with anti-CD31 antibody by using DAB as the chromogen. Sections were counterstained with hematoxylin. For negative controls (-), the primary antibodies were omitted. Bar, 400 μ m. (B) Quantitation of vascularization in MCF7-AP2 and -ErbB-2 tumors. Tumors were quantitated for vessel staining as described in MATERIALS AND METHODS. Results were obtained from three independent tumor sections. Values represent the mean $\pm$ SD of quadruplicate determinations. * $p < 0.01$, compared with AP2.

Figure 7. VEGF up-regulation by ErbB-2 is controlled at the level of transcription in breast cancer cells. The VEGF-luciferase reporter gene construct driven by the full-length VEGF promoter (−2279/+54) was transiently transfected into MCF7-neo22 and MCF7-HER218 cells (A) or T47D-puro and T47D-5R cells (B), with or without HRG treatment, and then extracts were collected and measured for luciferase activity. Values represent the mean $\pm$ SD of quadruplicate determinations.

control. As shown in Figure 7A, overexpression of ErbB-2 results in an increased basal transcription of the VEGF promoter, and treatment with HRG further increases the level of VEGF transcription. An additional indication of the importance of ErbB-2 signaling in the regulation of VEGF transcription was shown using the T47D-5R breast cancer cell model, where ErbB-2 expression at the cell surface was abolished by expression of a single-chain antibody against ErbB-2 (Graus-Porta *et al.*, 1995). T47D-5R cells retain expression of the remaining ErbB receptor family members (Beerli *et al.*, 1995). As shown in Figure 7B, loss of functional ErbB-2 in T47D-5R results in a decrease in the basal level of VEGF transcription, as well as a decrease in the responsiveness to HRG.

The −88 to −66 Region of the VEGF Promoter Mediates Responsiveness to Heregulin

To localize the VEGF promoter region involved in HRG responsiveness, we used luciferase reporter gene constructs harboring a series of VEGF promoter 5' deletions (Figure 8A). The various constructs have 5' ends corresponding to regions −2279 to −52 (relative to the transcription start site), with a common 3' end corresponding to +54. Because the overexpression of ErbB-2 seems to be

Figure 8. Responsiveness to HRG resides in the −88 to −66 region of the VEGF gene promoter. (A) Schematic representation of the luciferase reporter gene constructs harboring a series of VEGF promoter 5' deletions with 5' ends corresponding to regions −2279 to −52, with a common 3' end corresponding to +54. (B) MCF7-neo22 and MCF7-HER218 cells were transiently transfected with the various VEGF-luciferase deletion constructs with or without (control) HRG treatment. The cells were then lysed and luciferase activity was measured. Values represent the mean $\pm$ SD of quadruplicate determinations.

very important in mediating the up-regulation of VEGF (Figure 6), we decided to focus on regions of the promoter that are crucial to HRG responsiveness in both MCF7-neo22 and MCF7-HER218 cells. Transient transfection into MCF7 cells with or without HRG treatment revealed that although the −2279 to −88/+54 constructs displayed constitutive baseline activity, which was increased by HRG treatment (Figure 8B), further deletion of the pro-

Figure 9. Cooperative effect between AP-2 and Sp-1 on HRG-mediated transcriptional activation of the VEGF promoter. (A) Schematic representation of the putative binding sites for the Sp-1 and AP-2 transcription factors within the -88 to -66 region of the VEGF promoter. The AP-2 consensus sequence is boxed, whereas the Sp-1 binding sequence is underlined. Mutations in the AP-2 and Sp-1 binding sites are underlined. (B) MCF7-neo22 and -HER218 cells were transiently transfected with p88-wt, p88-mutAP-2, p88-mutSp-1, or p88-mutAP-2/Sp-1 and then treated with HRG and measured for luciferase activity. (C) MCF7-neo22 and -HER218 cells were transiently transfected with the p88-wt, p88-mutAP-2, p88-mutSp-1, or p88-mutAP-2/Sp-1 VEGF promoter-luciferase construct followed by stimulation with HRG, EGF, or AR, and then collected for luciferase assay. Values represent the mean $\pm$ SD of quadruplicate determinations. (D) EMSA with nuclear extracts from HRG-unstimulated and -stimulated MCF7-neo22 cells in the absence or presence of unlabeled cold probe competition (lanes 3 and 7), unlabeled double-stranded AP-2 oligonucleotides (lanes 4 and 8), or Sp-1 oligonucleotides (lanes 5 and 9). Two constitutively bound complexes (A and B) are shown by arrows.

motor resulted in loss of both basal and HRG-induced VEGF transcription in both MCF7-neo22 and -HER218 cells. This suggests that responsiveness resides in a region of the VEGF promoter between -88 and -66. This region

contains putative binding sites for Sp-1 and AP-2 transcription factors (Figure 9A). To determine the effects of Sp-1 and AP-2 on HRG-mediated transcriptional activation of the VEGF (Figure 9B), we transfected MCF7-neo22

Figure 9 (cont).

and -HER218 cells with either wild-type -88/+54 construct (p88-wt), or with constructs containing mutations in the AP-2 consensus site (p88-mutAP-2), mutations in the two Sp-1 binding sites (p88-mutSp-1), or mutations in all three sites (p88-mutAP-2/Sp-1) (Milanini *et al.*, 1998). Mutation of either the AP-2 or Sp-1 consensus sequences resulted in decreased activity of the VEGF promoter in ErbB-2-overexpressing MCF7-HER218 cells but did not completely prevent the responsiveness to HRG treatment. However, upon mutation of AP-2 and Sp-1 sites combined, the basal levels of promoter activity were decreased, and the HRG-dependent transcriptional activation was abolished, supporting a cooperative effect between AP-2 and Sp-1.

The -88 to -66 Region of the VEGF Promoter Mediates Responsiveness to Other ErbB Ligands

To determine whether the same proximal region of the VEGF promoter was also important in mediating up-regulation of VEGF transcription in response to other ErbB li-

Figure 10. Involvement of ERK1/2 in heregulin stimulation of VEGF expression. (A) Serum-starved MCF7-neo22 and -HER218 cells were exposed to no growth factor (control), EGF, or HRG for 10 min and analyzed for ERK1/2 activity by using antibodies specific for phosphorylated ERK1/2 (top) or total ERK1/2 protein (bottom). (B) Effect of MEK inhibitor on HRG induction of VEGF transcription. Serum-starved cells transiently transfected with the -88/+54 VEGF promoter reporter plasmid were pretreated with vehicle or U0126 for 1 h before stimulation with HRG or no treatment (control). Cell lysates were measured for luciferase activity 48 h after transfection.

gands, we transiently transfected MCF7-neo22 and -HER218 cells with the -88/+54 (p88-wt), p88-mutAP-2, p88-mutSp-1, or p88-mutAP-2/Sp-1 VEGF-luciferase constructs, followed by stimulation with HRG or two other ErbB ligands, namely, EGF and AR (Figure 9C). EGF and AR stimulation of ErbB-2-overexpressing MCF7-HER218 cells increased transcriptional activity of p88-wt VEGF by approximately twofold, compared with unstimulated cells. Consistent with our HRG results, dual mutation of the AP-2 and Sp-1 sites within the -88 to -66 region of the promoter

results in abolishment of EGF and AR responsiveness. Thus, responsiveness to a number of EGF-like ligands seems to reside in the proximal region of the VEGF promoter, with major involvement of the AP-2 and Sp-1 transcription factors. This was further shown by electromobility shift assay (Figure 9D), whereby we used a double-stranded probe corresponding to nucleotides -88 to -66 of the VEGF promoter to determine whether the AP-2 and Sp-1 transcription factors bind to this region. We identified a constitutive DNA-binding complex in both unstimulated and stimulated nuclear extracts from MCF7-neo22 cells (Figure 9D, complex A). Interestingly, upon stimulation with HRG, the binding of complex A became stronger and more pronounced (Figure 9D, compare lane 2 vs. 6). The binding specificity was determined by competition studies. Excess unlabeled probe readily competed off binding (Figure 9D, lanes 3 and 7), and formation of complex A was inhibited by oligonucleotides specific for either AP-2 or Sp-1 (Figure 9D, lanes 4, 5, 8, and 9). Western blot analysis of ERK1/2 (p44/p42 mitogen-activated protein kinase [MAPK]) phosphorylation after stimulation with EGF or HRG (Figure 10A) shows increased ERK1/2 activation upon HRG stimulation (Figure 10A, lane 3), which is further enhanced upon overexpression of ErbB-2 (Figure 10A, lane 6). The -88 to -66 region of the VEGF gene promoter was previously shown to be essential in the ERK1/2 regulation of the VEGF transcription (Milanini *et al.*, 1998). Under our conditions, we observed that induction of VEGF promoter activity by HRG, as measured by transient transfection of the p88-wt construct, was MAPK-dependent because it was blocked in cells treated with nontoxic doses of U0126, a specific inhibitor of mitogen-activated protein kinase kinase 1/2 and MAPK activity (Figure 10B).

DISCUSSION

Extensive progress has been made in identifying components of signal transduction pathways that are associated with the activation and oncogenicity of the various members of the ErbB receptor family. Of significance is the broad biological diversity of the ErbB receptors, attributed to their propensity to form various heterodimer combinations, which have been shown to activate distinct signaling pathways (Olayioye *et al.*, 2000; Yarden and Sliwkowski, 2001). In *in vivo* systems, including transgenic mice, overexpression of members of this family, particularly EGFR, ErbB-2, and ErbB-2/ErbB-3, has been associated with tumor growth and tumor progression (Siegel *et al.*, 1999; Brandt *et al.*, 2000), consistent with clinical studies in humans correlating overexpression of these receptors with aggressive disease (Yarden and Sliwkowski, 2001). The functional basis for the association of ErbB receptor signaling with tumor progression has not been fully established.

Epithelial cells express the four ErbB family members, and simultaneous cooverexpression of these receptors is common in human cancers. Thus, to address the role of specific ErbB receptor dimerization in angiogenesis, we expressed ErbB receptors, as single and paired combinations, in NIH3T3 cells with no detectable levels of EGFR, ErbB-2, ErbB-3, and ErbB-4 receptors. We have shown that tumors overexpressing ErbB-2, either alone or in combination with EGFR or ErbB-3 (but not ErbB-4), demonstrated enhanced

VEGF mRNA and protein expression. This increased VEGF expression in the EGFR/ErbB-2 and ErbB-2/ErbB-3 tumors correlates with a potent induction of tumor vascularization. Analysis of blood vessel morphology at high resolution revealed no clear differences between tumors expressing various heterodimers; however, vessels were larger in EGFR/ErbB-2- and ErbB-2/ErbB-3-overexpressing tumors. The ErbB ligands have the ability to bind specific receptors. For example, EGF, TGF α , and AR are specific ligands for EGFR; betacellulin (BTC), heparin-binding-EGF-like growth factor, and epiregulin bind both EGFR and ErbB-4, whereas the HRGs are the ligands for ErbB-3 and ErbB-4 (Riese and Stern, 1998). Although ErbB-2 has no known ligand, it can be activated by constitutive autophosphorylation when overexpressed (Pierce *et al.*, 1991) or via heterodimerization and transphosphorylation with ligand-bound EGFR, ErbB-3, and ErbB-4. All ErbB ligands readily activate ErbB-2 via dimerization with the appropriate high-affinity ErbB coreceptor, and receptor complexes containing ErbB-2 have a higher ligand affinity and signaling potency (Wada *et al.*, 1990; Sliwkowski *et al.*, 1994; Olayioye *et al.*, 2000). Indeed, ErbB heterodimeric receptor combinations have been found to be more mitogenic and transforming than dimeric combinations, with ErbB-2-containing heterodimers being the most potent (Kokai *et al.*, 1989; Riese *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996). ErbB-2 acts as the preferred heterodimer partner of the other ErbB receptors (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997), and ErbB-2 plays an essential role in the activation of the ErbB signaling by HRG (Lewis *et al.*, 1996). This is particularly true for the kinase-defective ErbB-3 (Guy *et al.*, 1994), which is activated through preferential heterodimer formation with ErbB-2 (Pinkas-Kramarski *et al.*, 1996).

This suggests that the regulation of angiogenesis by the ErbB receptors may be mediated by certain heterodimers containing ErbB-2. Of note, overexpression of ErbB-2 alone in NIH3T3 cells resulted in only modest enhancement of both VEGF expression and angiogenesis, in line with the evidence that homodimers of ErbB2 are weaker, in terms of activation of downstream signaling, than heterodimers containing ErbB-2 (Yarden and Sliwkowski, 2001). Thus, we concluded that overexpression of ErbB-2, particularly in the context of EGFR/ErbB-2 and ErbB-2/ErbB-3 heterodimers, plays a key role in mediating the up-regulation of VEGF and subsequent *in vivo* angiogenesis. This is further supported by our results showing the prevention of VEGF induction in the T47D-5R cell line, whereby it has been previously shown that abolishment of cell surface ErbB-2 expression in these cells results in impaired ligand binding and ErbB signaling (Graus-Porta *et al.*, 1995).

Further indication of the importance of ErbB-2 dimerization was shown using two independent MCF7 breast cancer cell models, whereby ErbB-2 was overexpressed in either a monoclonal or polyclonal population. In both cases, cells overexpressing ErbB-2 transplanted orthotopically into the mammary fat pad resulted in tumors with increased VEGF staining, compared with controls. Immunohistochemistry also revealed a striking increase in vessel density in these same tumors, with larger and more branched vasculature. This VEGF induction seen upon stimulation of MCF7 cells is likely mediated by ErbB-2/ErbB-3 heterodimer signaling, because it is known that elimination of ErbB-2 cell surface

expression completely abolishes heregulin-mediated activation of ErbB-3 (Beerli *et al.*, 1995). We therefore show that ErbB-2-mediated induction of VEGF is a contributor to the angiogenic phenotype observed *in vivo*, which is also supported by our previous *in vitro* studies (Yen *et al.*, 2000).

In contrast to tumors overexpressing EGFR/ErbB-2 and ErbB-2/ErbB-3, VEGF expression was not induced in tumors overexpressing EGFR/ErbB-4, ErbB-2/ErbB-4, and ErbB-3/ErbB-4, despite their increased vascularity (Figure 4). Also, we have found that VEGF mRNA was down-regulated in these same ErbB-4-containing heterodimers (Figure 3). This raises the possibility that signaling from ErbB receptors may involve the differential up-regulation of additional angiogenic factors such as the fibroblast growth factor family or other members of the VEGF family, namely, VEGF-B, VEGF-C, and VEGF-D (Cross and Claesson-Welsh, 2001). Indeed, in head and neck squamous carcinoma cell lines, the ErbB ligands TGF α , BTC, and HRG up-regulated both VEGF and VEGF-C mRNA expression, whereas down-regulating the expression of VEGF-D (O-charoenrat *et al.*, 2000). Additionally, a recent study has shown that the proangiogenic chemokine growth regulated oncogene 1 is induced by EGF stimulation (Loukinova *et al.*, 2001). Further investigations regarding the levels of additional angiogenic factors in response to distinct ErbB receptor overexpression are underway.

An interesting finding in our *in vivo* studies was that overexpression of ErbB-3 alone in NIH3T3 cells was able to induce the formation of small tumors in nude mice. This is in contrast to a previous study showing that NIH3T3-ErbB3 cells were nontumorigenic (Cohen *et al.*, 1996). A number of possible explanations can account for the differences in our findings compared with the Cohen study. For example, their study used single selected NIH3T3 clones, whereas our study used a polyclonal population of cells. Additionally, the Cohen study determined their results at only 4 wk postinjection, which would result in the exclusion of tumors that form at a later stage (such as our ErbB-3-overexpressing tumors, which formed after 2 mo). Although ErbB-3 is considered a kinase-impaired receptor (Guy *et al.*, 1994), we did not discount the idea that it does possess kinase activity, albeit very low. For example, ErbB-3 has been shown to be weakly active when expressed as a homodimer (Pinkas-Kramarski *et al.*, 1996; Cohen *et al.*, 1996). Additionally, stimulation of NIH3T3 cells expressing ErbB-3 alone resulted in robust mitogenesis in response to heregulin stimulation (Zhang *et al.*, 1996). Our results show that the weakly active ErbB-3 in our cells gave rise to the small tumors after a longer latency.

Transcription of VEGF mRNA is up-regulated by a variety of cytokine and growth factors, including interleukin-1 α and - β , TGF β 1, interleukin-6, TGF α , TGF β , and PDGF (Ferrara and Davis-Smyth, 1997; Ferrara, 1999). We have previously reported that HRG induces up-regulation of VEGF expression (Yen *et al.*, 2000). Herein, we performed promoter studies by using a series of VEGF promoter constructs. On overexpression of ErbB-2 in MCF7 cells, the basal level of VEGF promoter transcription was increased. Transcription of the VEGF promoter was further enhanced upon stimulation of the cells with HRG. Furthermore, abolishment of ErbB-2 signaling results in a reduction in VEGF transcription and decreased responsiveness to HRG, as seen in our T47D-5R

breast cancer cells. Thus, overexpression of ErbB-2 in breast cancer cells results in increased VEGF transcription, with enhanced responsiveness to stimulation by HRG. Using deletion analysis and mutants of the VEGF promoter region, we have mapped the region of the VEGF promoter that mediates the responsiveness to HRG. This region, between -88 and -66 base pairs upstream of the transcriptional start site, encompasses a GC-rich region that was found to bind the AP-2 and Sp-1 transcription factors constitutively. This binding was enhanced upon stimulation with HRG. Thus, regulation of VEGF by HRG occurs through transcriptional activation of the VEGF gene promoter and this activation is mediated by Sp-1 and AP-2 transcription factors binding within the -88 to -66 region of the promoter. ErbB receptors are coupled to several signaling pathways, including ERK1/2 (p44/p42) MAPKs, phospholipase C γ , phosphatidylinositol 3-kinase, and c-Jun NH $_2$ -terminal kinases (Yarden and Slivkowski, 2001). We have shown that inhibition of the ERK1/2 pathway resulted in inhibition of VEGF promoter responsiveness to HRG. Indeed, HRG has been shown to stimulate the activation of ERK1/2 (Marte *et al.*, 1995), and ERK1/2 is known to play an important role in the transcriptional regulation of VEGF (Milanini *et al.*, 1998). Additionally, the overexpression of ErbB-2 can lead to a prolonged activation of the MAPK pathway by EGF or heregulin (Karunagaran *et al.*, 1996) and blocking the expression of ErbB-2 at the cell surface results in a dramatic decrease in heregulin-mediated activation of ERK2 (Beerli *et al.*, 1995). Furthermore, our findings showing Sp-1 involvement in VEGF transcription are consistent with a previous study showing that Sp-1 seems to act downstream of all members of the ErbB family and is essential for ErbB signaling after stimulation by HRG (Alroy *et al.*, 1999). Of note, it is possible that additional regions of HRG responsiveness may reside within the VEGF promoter. For example, deletion of the region between -136 and -88 was found to abolish HRG-induced luciferase activity in the control MCF-7-neo cells (Figure 8B), thus suggesting a presence of additional HRG-mediated responsive regions further upstream of the -88 to -66 region. Future studies will address the possible presence of additional HRG regulatory regions.

Transcriptional activation of VEGF gene expression in response to growth factors or hypoxia is mediated, in part, by hypoxia-inducible factor 1 (HIF1) (Forsythe *et al.*, 1996; Carmeliet *et al.*, 1998; Ryan *et al.*, 1998; Zetter, 1998; Zhong *et al.*, 2000) binding to a HIF1 binding site within the VEGF promoter (Guillemin and Krasnow, 1997). Laughner *et al.* (2001) recently showed that ErbB-2 signaling can up-regulate synthesis of the HIF1 α subunit of HIF1 in MCF7 and NIH3T3 cells, thus providing a possible mechanism for erbB-mediated up-regulation of VEGF expression (Laughner *et al.*, 2001). However, in our VEGF promoter studies, transcriptional activation of the promoter in response to HRG occurred even upon deletion of the HIF1-binding site within the HRE (Figure 8). In agreement with Laughner *et al.* (2001) our results show that deletion of the HIF1 binding site does result in a moderate decrease in HRG responsiveness, thus confirming an additional contribution of HIF1 signaling in HRG-mediated VEGF induction. Taken together, our results indicate that HRG can transactivate the VEGF promoter in a mechanism independent of HIF1.

Our study has also shown that this proximal AP-2/Sp-1 region of the promoter is a common site that is important for the induction of VEGF transcription in response to other ErbB ligands besides HRG. Induction of VEGF promoter activity was seen upon stimulation with two EGFR-specific ligands, namely, EGF and AR. Additionally, studies in human mammary cells have shown that EGF preferentially activates heterodimers of EGFR/ErbB-2, whereas HRG preferentially activates ErbB-2/ErbB-3 heterodimers (Chen *et al.*, 1996). Therefore, it is likely that the up-regulation in VEGF expression seen in our MCF7 tumor model is mediated by EGFR/ErbB-2 and ErbB-2/ErbB-3 heterodimers, which is consistent with the data obtained with the NIH3T3-ErbB model.

Taken together, our findings add new evidence on biological diversity of ErbB signaling in the regulation of angiogenesis. Our results also highlight the importance of studying the ErbB receptors in the context of heterodimer activation, with the recognition that signaling through specific ErbB heterodimers is an important regulator of the tumor microenvironment and angiogenesis.

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