

Human Epidermal Growth Factor Receptor 2 Regulates Angiopoietin-2 Expression in Breast Cancer via AKT and Mitogen-Activated Protein Kinase Pathways

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Abstract

Abnormal activation of human epidermal growth factor receptor 2 (HER2; ErbB-2) in breast tumors results in increased metastasis and angiogenesis, as well as reduced survival. Here, we show that angiopoietin-2 (Ang-2) expression correlates with HER2 activity in human breast cancer cell lines. Inhibiting HER2 activity with anti-HER2 monoclonal antibody trastuzumab (Herceptin) or HER2 short interfering RNA in tumor cells down-regulates Ang-2 expression. Consistent with the important roles of AKT and mitogen-activated protein kinase in the HER2 signaling pathway, AKT and ERK mitogen-activated protein kinase (MAPK) kinase activity is necessary for Ang-2 up-regulation by HER2. Moreover, over-expression of HER2 protein up-regulates Ang-2 expression. Heregulin- β 1-induced Ang-2 up-regulation is abrogated when AKT and ERK kinase activity are blocked. Immunohistochemical analysis of HER2 and Ang-2 proteins in human breast carcinomas shows that Ang-2 expression in breast cancer correlates with HER2 expression. These studies provide evidence that the Ang-2 gene is regulated by HER2 activity in breast cancer, and propose an additional mechanism for HER2 contributing to tumor angiogenesis and metastasis. [Cancer Res 2007;67(4):1487–93]

Introduction

Breast cancer is the most common malignancy of women in the U.S. and is the second leading cause of cancer mortality (1). Approximately 25% to 30% of patients with breast cancer have tumors overexpressing human epidermal growth factor receptor 2 (HER2), a receptor tyrosine kinase in the epidermal growth factor receptor (EGFR) family (2). The EGFR family consists of four members: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4 (3, 4). All EGFR family members are characterized by a modular structure consisting of an extracellular ligand-binding domain, a single hydrophobic transmembrane region, and an intracellular region harboring a highly conserved tyrosine kinase domain. Ligand binding to ErbB receptors induces the formation of receptor homodimers and heterodimers, and the activation of the intrinsic kinase domain, resulting in the phosphorylation of specific tyrosine residues within the cytoplasmic tail. These phosphorylated residues serve as docking sites for a range of proteins, the recruitment of which leads to the activation of intracellular

signaling pathways. None of the ligands bind HER2, but HER2 is the preferred dimerization partner for all of the ErbB receptors. The role of HER2 as an important predictor of patient outcome and response to various therapies in breast cancer has been clearly established (2, 5–7). Patients with HER2-overexpressing breast tumors have an increased incidence of metastasis and a poorer survival rate when compared with patients whose tumors express HER2 at normal levels.

The angiopoietins (Ang) are novel endothelial growth factors, found to be ligands for the endothelium-specific tyrosine receptor Tie-2 (8, 9). Ang-1 plays a role in maintaining and stabilizing mature vessels by promoting the interaction between endothelial cells and the surrounding support cells (10–12). Ang-2 is expressed at sites of vascular remodeling and is thought to antagonize the stabilizing action of Ang-1 (9, 13). Ang-2 expression was elevated in tumor tissue and was associated with angiogenesis in tumor progression (14–18). Ang-2 also acts in concert with vascular endothelial growth factor (VEGF) to regulate vessel growth. In human cancers, increased expression of Ang-2 in tumor cells is closely correlated to the progression, invasiveness, and metastases of lung, gastric, colon, and breast cancers (16, 17, 19–24). Furthermore, gene transduction studies have shown that over-expression of Ang-2 by human tumor cells promotes tumor growth, angiogenesis, and metastases in animals (15, 25, 26). These findings suggest that Ang-2 may be involved in tumor-associated neovascularization and metastasis.

In a preliminary report, we have shown that human breast cancers express Ang-2, and that HER2 signaling seems to up-regulate Ang-2 mRNA expression (27). To begin elucidating the molecular mechanisms underlying HER2 regulation of Ang-2 expression, we investigated the signaling pathways directing Ang-2 production in breast cancer cells. Our results show that Ang-2 expression correlates with HER2 activity in human breast cancer cell lines and tissues. AKT and ERK mitogen-activated protein kinase (MAPK) kinase activity is necessary for Ang-2 up-regulation by HER2.

Materials and Methods

Cell culture and reagents. All human breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM supplemented with 10% fetal bovine serum (FBS). The monoclonal antibody trastuzumab manufactured by Genentech (South San Francisco, CA) was purchased by prescription and used as a HER2 inhibitor. Heregulin- β 1 (HRG- β 1) recombinant protein was purchased from R&D Systems (Minneapolis, MN). LY 294002, PD98059, U0126, and PP2 were purchased from Calbiochem (San Diego, CA). All reagents were prepared and used as recommended by their suppliers. Polyclonal antibodies recognizing EGFR (1005), HER2 (C-18), ErbB-3 (C-17), ErbB-4 (C-18), Ang-2 (C-20), and Akt1/2 (N-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine (clone 4G10) monoclonal antibody was purchased from Upstate (Lake Placid, NY). Phospho-Src family (Tyr⁴¹⁶),

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phospho-Akt (Thr³⁰⁸), and phospho-p44/p42 MAPK (Thr²⁰²/Thr²⁰⁴) antibodies were bought from Cell Signaling Technology (Danvers, MA). ERK (pan-ERK) was from BD Transduction Laboratories (Franklin Lakes, NJ). Monoclonal anti- β -actin antibody was from Sigma (St. Louis, MO).

Transient transfection. pcDNA3-HER2 (wild-type HER2) was a generous gift from Dr. M. Hung of M.D. Anderson Cancer Center (Houston, TX). Transfection of MDA-MB-435s cells with either pcDNA3-HER2 or pcDNA3 was done using LipofectAMINE (Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's protocol. Control short interfering RNA (siRNA) and human HER2/Neu siRNA oligos were obtained from Dharmacon RNA Technologies (Lafayette, CO). Each one contains four pooled siRNAs. Transient transfection of siRNA oligos into SK-BR-3 cells were done at 10, 50, or 100 nmol/L with DharmaFECT 1 reagent.

Immunoprecipitation and immunoblotting. Whole-cell lysates were prepared in lysis buffer [25 mmol/L Tris-HCl (pH 7.2), 150 mmol/L NaCl, 25 mmol/L NaF, 0.5 mmol/L Na₃VO₄, 1 mmol/L benzamidine, 20 mmol/L 4-NPP, 1 mmol/L DTT, 1% Triton X-100, 100 μ g/mL phenylmethylsulfonyl fluoride, 2 μ g/mL aprotinin, 2 μ g/mL leupeptin, and 1 μ g/mL pepstatin] for 20 min on ice. Lysates were centrifuged for 20 min (4°C) at 12,000 \times g, and the supernatant was collected. Total protein concentration was determined using the Bradford assay. For immunoprecipitation, 0.1 mg of total protein in 0.5 mL of lysis buffer was incubated with rabbit polyclonal anti-HER2/Neu antibody overnight at 4°C. Protein antibody complexes were recovered by protein A/G agarose for 3 h at 4°C. The isolated immunocomplexes were washed thrice with lysis buffer and fractionated on SDS-PAGE. Proteins transferred to polyvinylidene difluoride membrane were probed with antibodies against phosphotyrosine. For Western blot, equal amounts of total cellular protein were dissolved in Laemmli SDS-PAGE sample buffer prior to separation by 10% SDS-PAGE. Protein was transferred to polyvinylidene difluoride membrane, and Western blot analysis was done by standard techniques with enhanced chemiluminescence detection.

Quantitative real-time PCR. Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (2 μ g) was reverse-transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis was carried out on the ABI Prism 7900 Sequence Detection System (Applied Biosystems) using the TaqMan Universal PCR Master Mix as recommended by the manufacturer. The cycling conditions were: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The TaqMan MGB probes (FAM dye-labeled) were purchased from Applied Biosystems. rRNA (18S rRNA) was used as an endogenous control. Each sample was tested in triplicate, and the Ang-2 or HER2 mRNA level was normalized to that of 18S rRNA. Thus, the figures show each value as the mean \pm SE of three independent experiments.

Immunohistochemical study. Immunohistochemical staining was done on formalin-fixed, paraffin-embedded sections (17, 23). Five-micrometer-thick sections were cut, deparaffinized in xylene, and rehydrated in graded alcohol. After antigen retrieval and treatment with 3% H₂O₂ in methanol to inhibit endogenous peroxidase activity, the slides were incubated with normal goat serum to block nonspecific antigens. Slides were then incubated at 4°C overnight with rabbit polyclonal antibodies for either HER2 (diluted, 1:500) or Ang-2 (diluted, 1:500). The primary antibody was replaced with nonimmune IgG as a negative control. Antibody localization was detected by sequential application of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in PBS, and an avidin-biotin complex conjugated to horseradish peroxidase (Vector Laboratories). The substrate used was diaminobenzidine (Vector Laboratories), which forms an insoluble brown precipitate, and nuclei were counterstained blue with Mayer's hemalum solution.

Slides were bar-coded and blinded for automated slide scan imaging and processing. The Ariol SL-50 from Applied Imaging is an automated slide scanner. The system's built-in classifiers include the analysis capability for nuclear, cytoplasmic, and membranous event classification. Detailed images were processed using the Multistain Imaging Module for Ang-2 and HER2 tissue slides.

Statistical analysis. The association between HER2 and Ang-2 was evaluated using nonparametric Spearman's Rank correlation. $P < 0.05$ values were considered statistically significant.

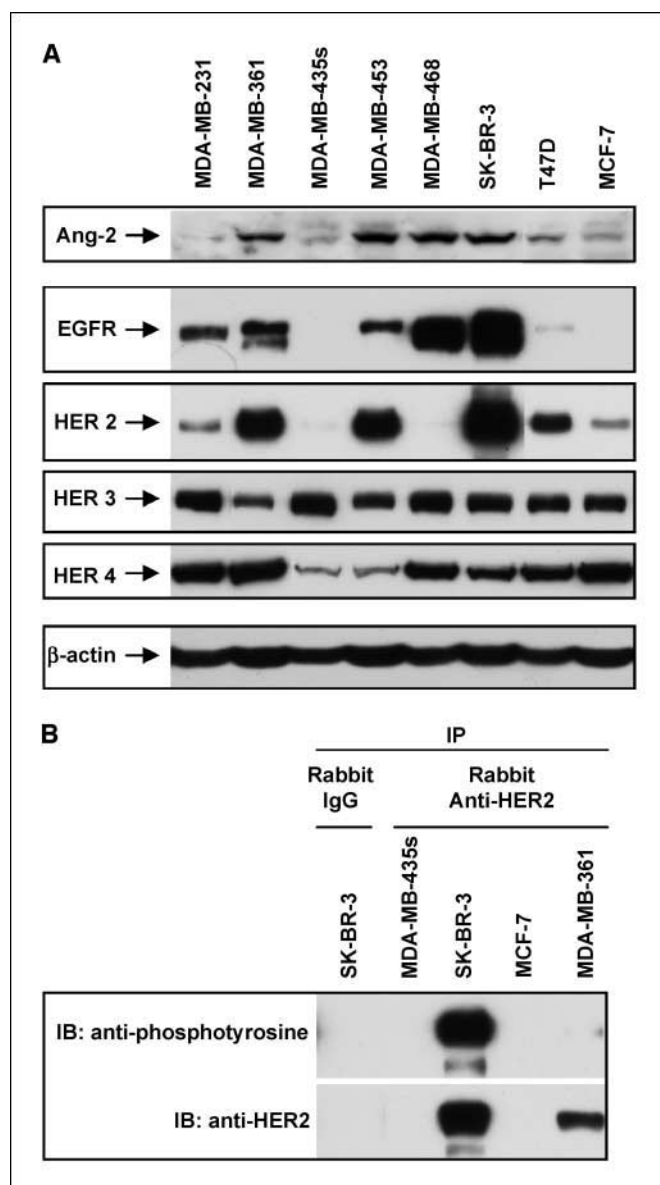


Figure 1. Correlation of Ang-2 expression with HER2 activity in human breast cancer cell lines. *A*, the expression of Ang-2 and EGFR family was determined by Western blot using total cell lysates prepared from human breast cancer cell lines. The relative levels of EGFR family and Ang-2 protein expression were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The correlation coefficient between HER1 and Ang-2 expression was 0.737 ($P < 0.05$), whereas the correlation coefficient between HER2 and Ang-2 expression was 0.849 ($P < 0.05$). There was no correlation in the expression of Ang-2 with HER3 and HER4. *B*, overexpression of HER2 can induce autophosphorylation. Cell lysates were incubated with anti-HER2 antibody to immunoprecipitate HER2 proteins. Immunoprecipitated proteins were probed with an anti-phosphotyrosine antibody.

Results

HER2 expression is associated with Ang-2 up-regulation in human breast cancer cell lines. To evaluate if HER2 may contribute to Ang-2 up-regulation in cancer, we first assessed Ang-2 and the EGFR family expression in eight human breast cancer cell lines (Fig. 1A). EGFR was known to contribute to Ang-2 expression in rat cardiac microvascular endothelial cells (28). As shown in Fig. 1A, the expression of Ang-2 correlated with elevated HER2 expression. Several phenomena are responsible for abnormal

activation of HER2 in tumors, including overexpression, amplification, and constitutive activation of mutant receptors or autocrine growth factors loops. Figure 1B shows that overexpression of HER2 is linked with constitutive activation of HER2 receptor.

Blocking HER2 activity or reducing protein levels results in down-regulating Ang-2 levels in HER2-positive breast cancer cells. Trastuzumab is a humanized monoclonal antibody reacting against the extracellular domain of HER2 (29). Trastuzumab binding leads to the suppression of intracellular signaling in HER2 activation. We examined if trastuzumab could down-regulate the Ang-2 levels in HER2-positive breast cancer cells, SK-BR-3. The cells were incubated with varying concentrations of trastuzumab (0, 10, 20, and 50 $\mu\text{g}/\text{mL}$) for 48 h, and the cell lysates were subjected to immunoprecipitation, Western blotting, and real-time PCR. The total protein levels of HER2 did not change in the presence of trastuzumab. However, levels of p-HER2 were decreased in a dose-dependent manner, indicating that trastuzumab was capable of inhibiting HER2 activity (Fig. 2A). The same concentration of trastuzumab treatment resulted in the down-regulation of Ang-2 expression in SK-BR-3 cells, a HER2-overexpressing cell (Fig. 2A), but not in HER2-negative MDA-MB-435s cells (data not shown). To determine whether directly reducing HER2 would suppress Ang-2 expression in cancer cells, we transiently transfected either control siRNA or HER2 siRNA into SK-BR-3 cells. Reducing HER2 expression in these tumor cells led to decreased Ang-2 expression at both the mRNA and protein levels (Fig. 2B).

Ang-2 expression is regulated by ERK and the phosphatidylinositol 3-kinase/AKT pathway of intracellular HER2 signals.

To elucidate the mechanism by which HER2 regulates Ang-2 expression, intracellular signaling molecules associated with HER2 activation were examined. Activated HER2 stimulates many intracellular signaling pathways (4). Three main pathways activated by the HER2 receptor are the MAPK, the phosphatidylinositol 3-kinase (PI3K)-AKT, and the cytoplasmic tyrosine kinase c-Src pathways. Four breast tumor cell lines, which express different levels of HER2, were analyzed for phosphoproteins. Figure 3A showed that in tissue culture with 10% FBS, limited p-AKT was detected in each of the cell lines, whereas p-ERK1/2 was highest in SK-BR-3 cells. Upon serum starvation, MAPK and PI3K/AKT were activated in SK-BR-3 (HER2-overexpressing) cells (Fig. 3B). To further determine the HER2 signaling pathway involved in the regulation of Ang-2 protein levels, we used chemical

inhibitors of these signaling pathways to examine the requirement of activation of each pathway in HER2-mediated Ang-2 up-regulation. PD98059 is known to selectively block the activity of MAPK kinase, an activator of ERK kinases (30). LY294002 is known to selectively block the activity of PI3K, an activator of AKT kinase

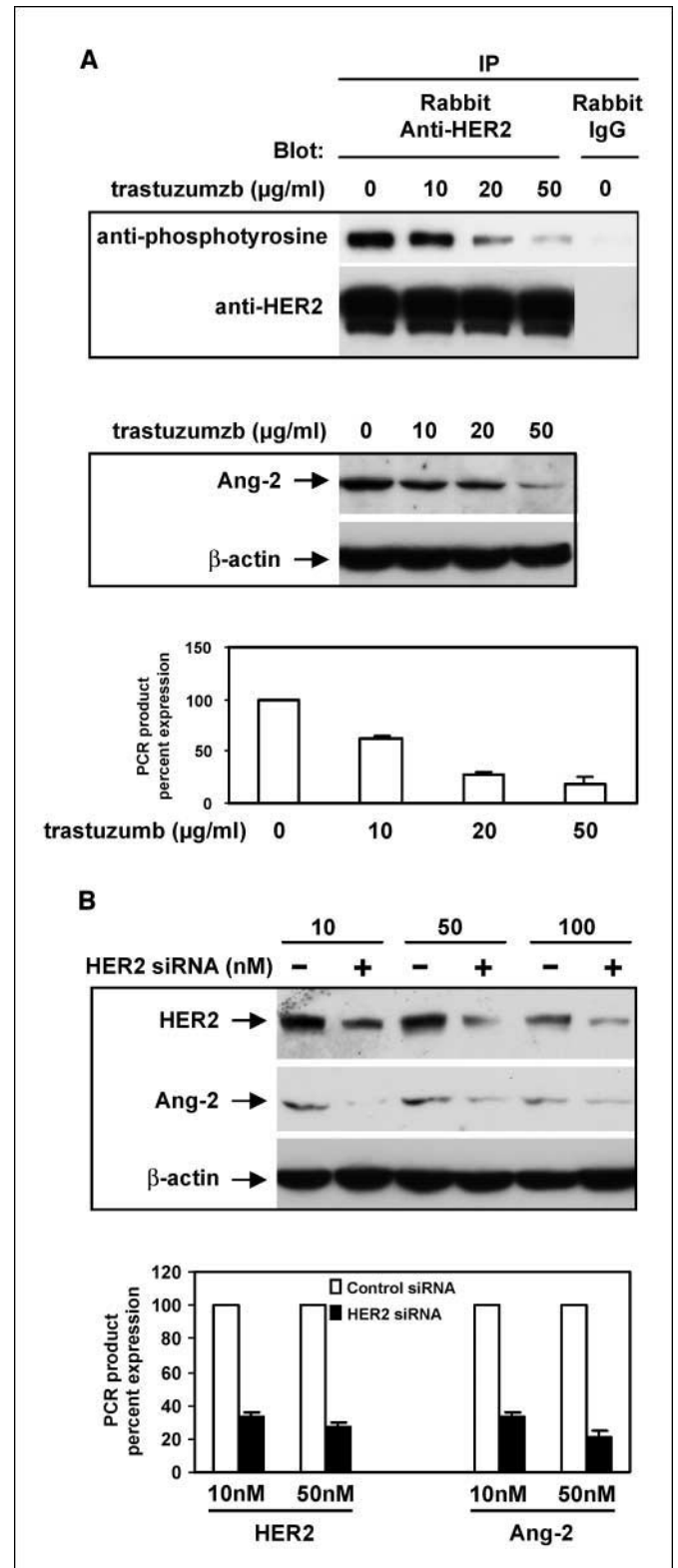


Figure 2. Inhibiting HER2 activity in SK-BR-3 tumor cells reduces the expression of Ang-2. *A*, blocking HER2 activity by anti-HER2 monoclonal antibody trastuzumab reduces the phosphorylation of HER2 and decreases Ang-2 expression. SK-BR-3 was treated for 48 h with 10, 20, or 50 $\mu\text{g}/\text{mL}$ of trastuzumab. *Top*, HER2 was immunoprecipitated from protein lysates and immunoblotted for phosphotyrosine. The phosphorylation of HER2 was reduced in cells treated with trastuzumab. The membranes were stripped and reprobed with anti-HER2 antibody to verify the protein levels. *Middle*, total lysates (100 μg) were immunoblotted for Ang-2 expression. Ang-2 was decreased when reducing the phosphorylation of HER2. *Bottom*, real-time PCR examining Ang-2 mRNA. The amount of Ang-2 mRNA in each sample was normalized against the amount of 18S rRNA in the same sample and presented as a percentage of control against trastuzumab (control being set at 100%). The data were the average of three independent experiments. *B*, inhibiting HER2 expression down-regulates Ang-2 expression. *Top*, Western blot analysis of SK-BR-3 cells transfected with either control or HER2 siRNA. Seventy-two hours after transfection, cells were collected for Western blotting. *Bottom*, real-time PCR assay. Forty-eight hours after transfection, cells were collected for real-time PCR. The amount of HER2 mRNA and Ang-2 mRNA are presented as a percentage of control siRNA against HER2 siRNA (control being set at 100%). The data were the average of three independent experiments.

(31). SK-BR-3 cells were incubated with either LY294002 or PD98059 for the indicated times, and then levels of phosphoproteins and Ang-2 were analyzed by Western blotting. The Ang-2 level was decreased after incubation with the PI3K inhibitor or MEK1

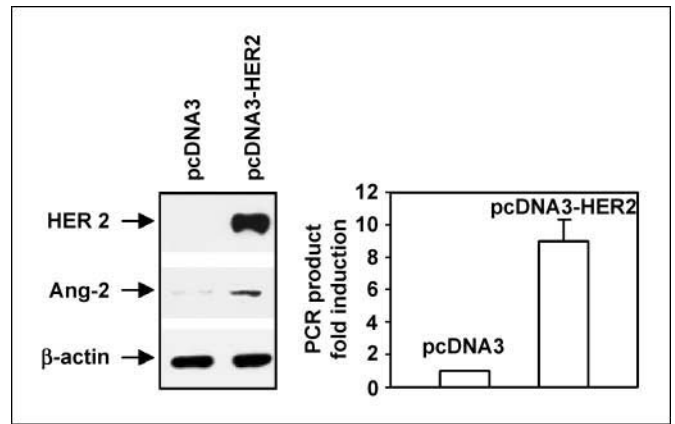
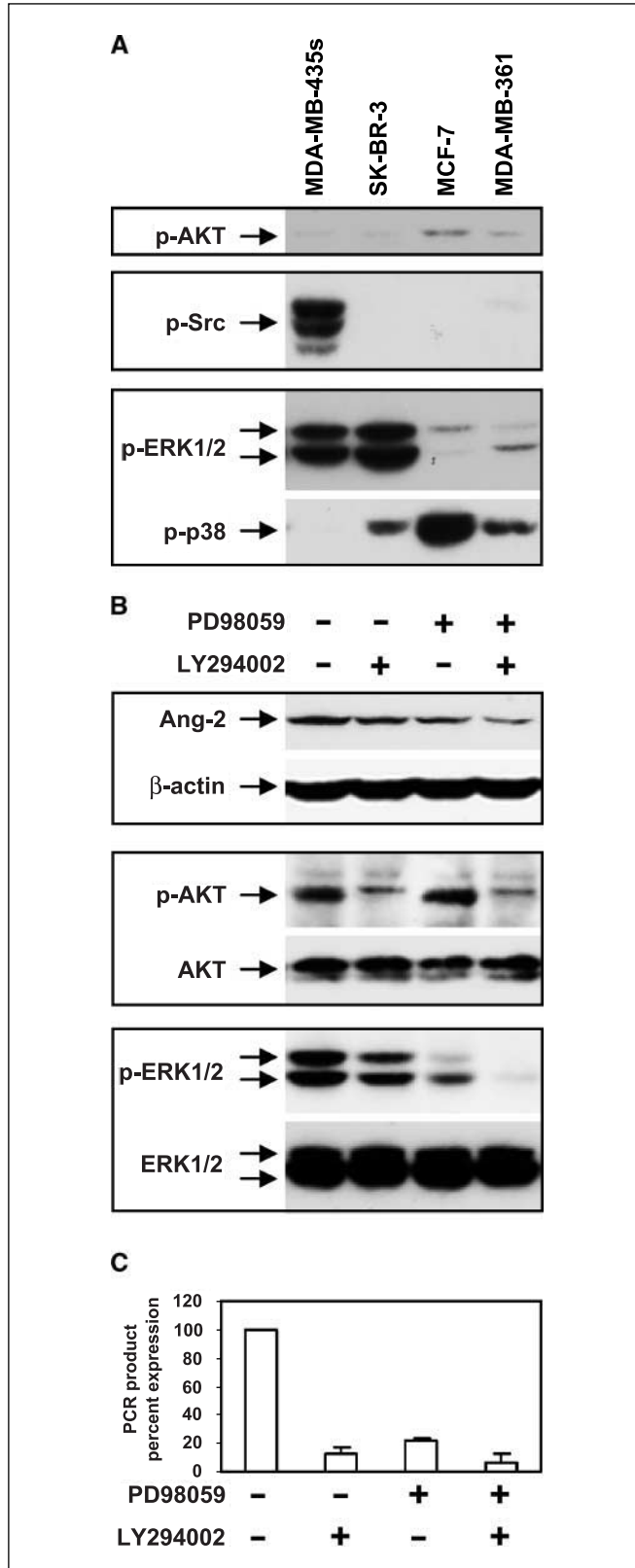


Figure 4. Overexpression of HER2 protein up-regulates Ang-2 expression at both the mRNA and the protein levels. MDA-MB-435s cells, which lack HER2 and Ang-2 expression, were transiently transfected with a HER2 expression vector. Cells were lysed 48 h after transfection. *Left*, Western blot analyses. *Right*, real-time PCR for Ang-2 mRNA.

inhibitor, whereas each inhibitor was able to reduce the specific downstream phosphoprotein levels, p-AKT and p-ERK, respectively (Fig. 3B and C). A synergistic effect was seen in combination with both inhibitors. Collectively, it seems that the Ang-2 protein level could be regulated by both PI3K/AKT and MAPK pathways of intracellular HER2 signals in breast cancer cells.

Overexpression of HER2 up-regulates Ang-2 at both the mRNA and protein levels in breast cancer cells. To directly test the hypothesis that activated HER2 has a causal role in Ang-2 induction in tumor cells, we determined whether overexpression of HER2 by gene transfer could up-regulate Ang-2 expression. MDA-MB-435s and MCF-7 were transfected with pcDNA3-HER2 expression plasmid. Forty-eight hours after transfection, cell lysates were collected and examined for HER2 and Ang-2 protein levels by Western blotting, and for mRNA level by real-time PCR (Fig. 4). MDA-MB-435s cells displayed neither HER2 nor Ang-2 expression (Fig. 1A). When HER2 was overexpressed in MDA-MB-435s cells, the Ang-2 level was markedly increased. A similar result was obtained from the transfection study using MCF-7 cells, which had low endogenous HER2 expression (data not shown). These data imply that the Ang-2 level could be up-regulated by the expression of HER2.

HRG-β1 up-regulates Ang-2 in human breast cancer cells. HRG-β1, a member of the epidermal growth factor-like family, acts as a combinatorial ligand for the HER3 and HER4 receptors in

Figure 3. The AKT and ERK pathway is required for Ang-2 up-regulation by HER2. *A*, a Western blot of total protein showed comparable p-AKT, p-Src, and p-ERK in MDA-MB-435s, SK-BR-3, MCF-7, and MDA-MB-361 cells grown in tissue culture media with 10% FBS. All cell lines exhibited limited p-AKT, whereas p-ERK1/2 was highest in SK-BR-3 cells. *B*, inhibiting AKT and ERK signaling inhibits Ang-2 protein levels. SK-BR-3, starved by an overnight exposure to serum-free medium, was exposed to 50 μmol/L of PD98059 or 50 μmol/L of LY294002 alone or in combination for 48 h. The phosphorylated (p) proteins were revealed by incubating with p-AKT antibody (*middle*) or p-ERK (*bottom*) following enhanced chemiluminescence detection. The membranes were stripped and reprobed with the respective proteins antibodies to verify the protein levels. *C*, real-time PCR for Ang-2 mRNA. SK-BR-3 cells, starved by an overnight exposure to serum-free medium, was exposed to 50 μmol/L of PD98059 or 50 μmol/L of LY294002 alone or in combination for 24 h. The amount of Ang-2 mRNA was presented as a percentage of control (DMSO) against specific inhibitors (control being set at 100%). The data are the average of three independent experiments.

breast cancer cells (4, 32). The binding of HRG- β 1 to its receptors induces either HER3 or HER4 to form homodimers, or to form heterodimers with HER2, thus triggering diverse signaling cascades. A panel of breast cancer cell lines were examined for their response to HRG- β 1 treatment to produce Ang-2. The cell lines included MDA-MB-435s (no HER2 expression), MCF-7 (low-level HER2 expression), and MDA-MB-361 (intermediate-level HER2 expression). HRG- β 1 treatment significantly increased Ang-2 expression in MCF-7 and MDA-MB-361 cell lines (Fig. 5A), but not in MDA-MB-435s cell lines (data not shown). The induction of Ang-2 protein was time-dependent and could be readily detected when MCF-7 and MDA-MB-361 cells were treated with 50 ng/mL of HRG- β 1 for 3 h, and was sustained for up to 24 h. As shown in Fig. 5A (bottom), the Ang-2 mRNA level was elevated with a peak at 3 h after 50 ng/mL of HRG- β 1 treatment. This finding suggests that HRG- β 1 induces Ang-2 up-regulation at the protein and RNA levels. Figure 5B showed that HRG- β 1 could also activate ERK and AKT pathways in MCF-7 and MDA-MB-361 cells. To address if

these signaling pathways might be involved in the up-regulation of Ang-2 by HRG- β 1, MCF-7 and MDA-MB-361 cells were pretreated with LY294002 or PD98059, or its combination for 30 min and followed by treatment with HRG- β 1 for 3 or 6 h. Total protein and RNA were used to determine Ang-2 expression. Figure 5C reveals that LY 294002 or PD 98059 could partially reduce the increased level of Ang-2 protein (top) and mRNA (bottom) induced by HRG- β 1, whereas the combination of the two kinases completely blocked the up-regulation of Ang-2 induced by HRG- β 1. Figure 5D shows that these two signaling pathways were effectively blocked in MCF-7 and MDA-MB-361 cells when treated with pharmacologic inhibitors. These data suggest that those pharmacologic inhibitors were functionally active in inhibiting the specific signaling pathways.

Immunohistochemical detection of Ang-2 and HER2 in breast cancer tissues. To determine whether HER2-up-regulated Ang-2 expression could also be observed in primary tumor tissues, formalin-fixed, paraffin-embedded breast cancer specimens were

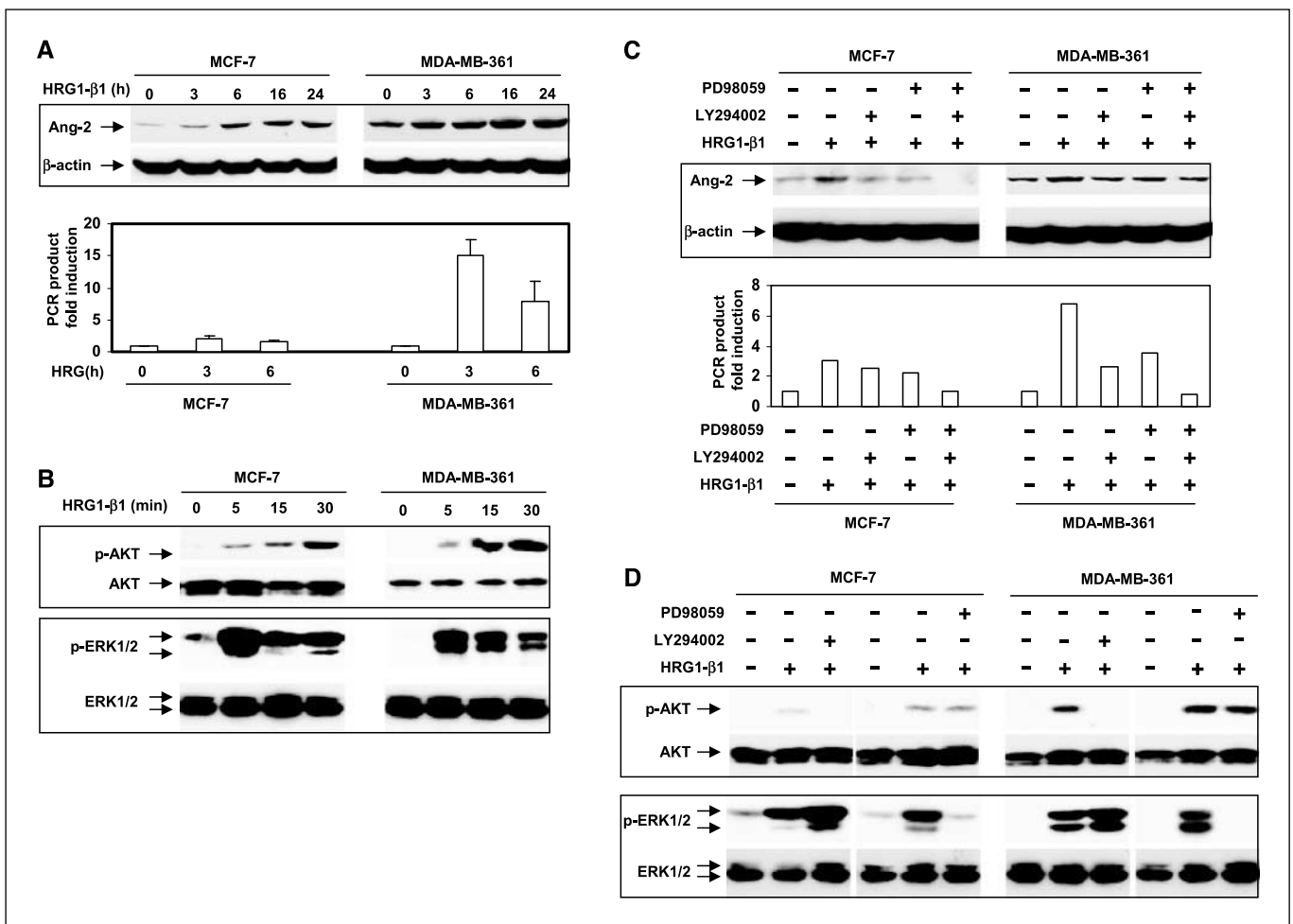


Figure 5. HRG- β 1 up-regulates Ang-2 in HER2 expression breast cancer cell lines and requirement of the AKT and ERK pathway for Ang-2 up-regulation by HRG- β 1. **A**, HRG- β 1-mediated up-regulation of Ang-2 mRNA and protein expression in MCF-7 and MDA-MB-361 cells is time-dependent. Total RNA and protein were collected from serum-starved MCF-7 and MDA-MB-361 breast cancer cells treated with 50 ng/mL of HRG- β 1 for various times as indicated. *Top*, Western blot analysis; *bottom*, real-time PCR for Ang-2 mRNA. **B**, HRG- β 1 activated multiple signal pathways. MCF-7 and MDA-MB-361 cells were treated by HRG- β 1 for 5, 15, or 30 min. Cell lysates were prepared, and Western blots were done. **C**, MCF-7 and MDA-MB-361 cells, starved overnight in serum-free medium, were pretreated with 50 μ mol/L of LY294002 or 50 μ mol/L of PD98059 or its combination for 30 min, then treated with 50 ng/mL of HRG- β 1 for 6 h (for Western blotting) or 3 h (for real-time PCR). Ang-2 mRNA or protein was detected by using Western blotting (*top*) and real-time PCR (*bottom*). **D**, to test the efficacy of the inhibitors, MCF-7 cells, starved overnight, were pretreated with the inhibitors at the concentrations as in Fig. 6A for 30 min, and stimulated by 50 ng/mL of HRG- β 1 for 30 min (Western blot analysis for p-AKT) or 5 min (Western blot analysis for p-ERK1/2). Cell lysates were prepared and subjected to Western blot analysis using antibodies against p-ERK1/2 and p-AKT. Then the membranes were stripped and re probed with the respective protein antibodies to verify the protein levels.

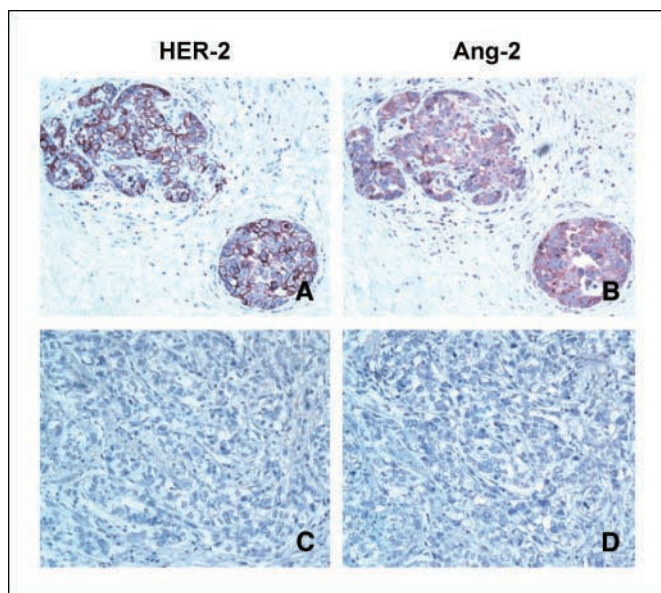


Figure 6. Immunohistochemical analysis of HER2 and Ang-2 proteins in human breast carcinomas (17, 23). A and C, HER2 staining. B and D, Ang-2 staining.

analyzed for the expression of Ang-2 and HER2 by immunohistochemical staining (Fig. 6). Immunohistochemical scoring was determined by automated slide scan imaging and processing. Of the 66 breast cancer specimens, Ang-2 staining was positive in 93.7% (15 of 16) of HER2-positive cases, whereas it was negative in 72% (36 of 50) of HER2-negative cases (Table 1). The correlation between HER2 and Ang-2 was statistically significant (correlation coefficient, $r = 0.38182$; $P < 0.005$), supporting the conclusion that overexpression of HER2 up-regulates Ang-2 expression *in vivo*.

Discussion

Although Ang-2 has been shown to be highly expressed in a vast array of malignant tissues (16, 17, 19–24), the factors which control its up-regulation in cancer are not fully understood. Hypoxia results in increases in both Ang-1 and Ang-2 levels, but Ang-2 is elevated to a far greater extent (33–37). A few investigations have shown that VEGF could induce Ang-2 gene expression in human endothelial cells (34, 36, 38). Other factors, such as steroid hormones and thrombin, influence Ang-2 expression levels (39, 40). Our study indicates, for the first time, that activation of ERK and PI3K/AKT pathways are required for HER2 overexpression or HRG-

β 1-mediated up-regulation of angiogenic factor Ang-2 in human breast cancer cells. Our current report on the induction of Ang-2 protein synthesis contributes to a better understanding of the mechanism of HER2-induced angiogenesis, tumor growth, and metastasis.

It is generally accepted that VEGF, Ang-1, and Ang-2 are necessary for efficient blood vessel growth and development. It has been proposed that Ang-2, a natural antagonist of Ang-1, may be an important proangiogenic factor in that it may counteract Ang-1-mediated blood vessel stability, thus maintaining the endothelium in a more plastic state and promoting the response of endothelial cells to angiogenic growth factors. Experimental studies have shown the close relationship between VEGF and Ang-2 functions in angiogenesis (41, 42). Ang-2 was found to promote a vessel sprouting in the presence of abundant VEGF, whereas Ang-2 contributed to vessel regression in the absence of VEGF. VEGF induced both a time- and concentration-dependent increase of Ang-2 expression in bovine microvascular endothelial cells. On the other hand, ectopically administered Ang-2 was shown to promote endothelial cell proliferation and sprouting of new blood vessels in the presence of endogenous VEGF. The Ellis group reported that overexpression of Ang-2 by gene transfection in human colon cancer cells lead to enhanced tumor angiogenesis and growth in nude mice (25). Up-regulation of both VEGF and Ang-2 by HER2 strongly indicates that angiogenesis was facilitated by HER2.

The role of Ang-2 in tumor metastasis has been more clearly defined by several investigators (15, 26). Cheng et al. reported that Ang-2 induces human glioma cell invasion (15). In invasive areas of primary human glioma specimens, up-regulated expression of Ang-2 was detected in tumor cells. Correspondingly higher levels of matrix metalloproteinase (MMP)-2 expression was present in Ang-2-expressing tumor cells in these gliomas. Ectopic overexpression of Ang-2 induced glioma invasion in intracranial xenografts in mouse brains. In these invasive tumors, there was an increased expression of MMP-2 at the invasive front. *In vitro* invasion analyses showed that Ang-2 promoted glioma cell invasion and stimulated activation of MMP-2. The Mori group showed that high Ang-2 expression cases showed more frequent vascular involvement and more advanced stages of disease compared with low Ang-2 expression cases in human gastric cancers (26). The survival time for patients in the high-Ang-2 mRNA group was significantly shorter. Ang-2-transfected human MKN-7 gastric cancer cells were implanted *in vivo* into the gastric walls of nude mice. Ang-2-transfectant mice developed highly metastatic tumors with hypervascularity as compared with MKN-7 or control

Table 1. Summary HER2 and Ang-2 immunohistochemical staining in tumor tissue sample from 66 cases of primary breast cancer

	HER2+	HER2–	Total
Ang-2+	15 ($R = 0.38182$, $P < 0.005$)	14 ($R = -0.38182$, $P < 0.005$)	29
Ang-2–	1 ($R = -0.38182$, $P < 0.005$)	36 ($R = 0.38182$, $P < 0.005$)	37
Total	16	50	66

NOTE: The nonparametric Spearman's rank correlation was used to analyze for significance of the relationship between HER2 expression and Ang-2 expression ($P < 0.005$).

vector-transfected tumors. There was a significant correlation between Ang-2 mRNA expression and lower grade of vessel maturation. MMP-1, MMP-9, and urokinase-type plasminogen activator in endothelial cells were strongly up-regulated by Ang-2 in the presence of VEGF *in vitro*. These findings suggest that Ang-2 was involved in tumor metastasis in addition to its primary role in vascular and tissue development.

Clinical and experimental data indicate that increased HER2 activity is an important step in breast cancer progression that negatively affects patient survival. In this study, we have shown that HER2 signaling up-regulates Ang-2 in HER2-expressing cells and that Ang-2 expression correlates with HER2 via breast cancer

expression. Ang-2 up-regulation may contribute to the metastatic phenotype in HER2 overexpressing breast cancer. Blocking Ang-2 may offer an additional therapeutic target.

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References

- Herbst RS, Bajorin DF, Bleiberg H, et al. Clinical Cancer Advances 2005: major research advances in cancer treatment, prevention, and screening—a report from the American Society of Clinical Oncology. *J Clin Oncol* 2006;24:190–205.
- Carlsson J, Nordgren H, Sjöström J, et al. HER2 expression in breast cancer primary tumours and corresponding metastases. Original data and literature review. *Br J Cancer* 2004;90:2344–8.
- Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005; 5:341–54.
- Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr Relat Cancer* 2001;8:11–31.
- DiGiovanna MP, Stern DF, Edgerton SM, et al. Relationship of epidermal growth factor receptor expression to ErbB-2 signaling activity and prognosis in breast cancer patients. *J Clin Oncol* 2005;23:1152–60.
- Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987;235:177–82.
- Tsutsui S, Ohno S, Murakami S, Hachitanda Y, Oda S. Prognostic value of c-erbB2 expression in breast cancer. *J Surg Oncol* 2002;79:216–23.
- Davis S, Aldrich TH, Jones PF, et al. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* 1996;87:1161–9.
- Maisonpierre PC, Suri C, Jones PF, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. *Science* 1997;277:55–60.
- Gamble JR, Drew J, Trezise L, et al. Angiopoietin-1 is an antipermeability and anti-inflammatory agent *in vitro* and targets cell junctions. *Circ Res* 2000;87:603–7.
- Suri C, Jones PF, Patan S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 1996;87:1171–80.
- Thurston G, Suri C, Smith K, et al. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* 1999;286:2511–4.
- Parikh SM, Mammoto T, Schultz A, et al. Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans. *PLoS Med* 2006;3:e46.
- Ding H, Roncarì L, Wu X, et al. Expression and hypoxic regulation of angiopoietins in human astrocytomas. *Neurooncol* 2001;3:1–10.
- Hu B, Guo P, Fang Q, et al. Angiopoietin-2 induces human glioma invasion through the activation of matrix metalloproteinase-2. *Proc Natl Acad Sci U S A* 2003;100: 8904–9.
- Koga K, Todaka T, Morioka M, et al. Expression of angiopoietin-2 in human glioma cells and its role for angiogenesis. *Cancer Res* 2001;61:6248–54.
- Sfiligoi C, de Luca A, Cascone I, et al. Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. *Int J Cancer* 2003;103:466–74.
- Stratmann A, Risau W, Plate KH. Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am J Pathol* 1998;153:1459–66.
- Mitsuhashi N, Shimizu H, Ohtsuka M, et al. Angiopoietins and Tie-2 expression in angiogenesis and proliferation of human hepatocellular carcinoma. *Hepatology* 2003;37:1105–13.
- Ochiuni T, Tanaka S, Oka S, et al. Clinical significance of angiopoietin-2 expression at the deepest invasive tumor site of advanced colorectal carcinoma. *Int J Oncol* 2004;24:539–47.
- Ogawa M, Yamamoto H, Nagano H, et al. Hepatic expression of ANG2 RNA in metastatic colorectal cancer. *Hepatology* 2004;39:528–39.
- Takanami I. Overexpression of Ang-2 mRNA in non-small cell lung cancer: association with angiogenesis and poor prognosis. *Oncol Rep* 2004;12:849–53.
- Tanaka F, Ishikawa S, Yanagihara K, et al. Expression of angiopoietins and its clinical significance in non-small cell lung cancer. *Cancer Res* 2002;62:7124–9.
- Tanaka S, Mori M, Sakamoto Y, et al. Biologic significance of angiopoietin-2 expression in human hepatocellular carcinoma. *J Clin Invest* 1999;103:341–5.
- Ahmad SA, Liu W, Jung YD, et al. The effects of angiopoietin-1 and -2 on tumor growth and angiogenesis in human colon cancer. *Cancer Res* 2001;61:1255–9.
- Etoh T, Inoue H, Tanaka S, et al. Angiopoietin-2 is related to tumor angiogenesis in gastric carcinoma: possible *in vivo* regulation via induction of proteases. *Cancer Res* 2001;61:2145–53.
- Carter WB. HER2 signaling-induced microvessel dismantling. *Surgery* 2001;130:382–7.
- Fujiyama S, Matsubara H, Nozawa Y, et al. Angiotensin AT(1) and AT(2) receptors differentially regulate angiopoietin-2 and vascular endothelial growth factor expression and angiogenesis by modulating heparin binding-epidermal growth factor (EGF)-mediated EGF receptor transactivation. *Circ Res* 2001;88:22–9.
- Fendly BM, Winget M, Hudziak RM, et al. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. *Cancer Res* 1990;50:1550–8.
- Lester RD, Jo M, Campana WM, Gonias SL. Erythropoietin promotes MCF-7 breast cancer cell migration by an ERK/mitogen-activated protein kinase-dependent pathway and is primarily responsible for the increase in migration observed in hypoxia. *J Biol Chem* 2005;280:39273–7.
- Mottet D, Dumont V, Deccache Y, et al. Regulation of hypoxia-inducible factor-1 α protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3 β pathway in HepG2 cells. *J Biol Chem* 2003;278:31277–85.
- Crovello CS, Lai C, Cantley LC, Carraway KL III. Differential signaling by the epidermal growth factor-like growth factors neuregulin-1 and neuregulin-2. *J Biol Chem* 1998;273:26954–61.
- Krikun G, Schatz F, Finlay T, et al. Expression of angiopoietin-2 by human endothelial cells: regulation by hypoxia and inflammation. *Biochem Biophys Res Commun* 2000;275:159–63.
- Mandriota SJ, Pepper MS. Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ Res* 1998;83:852–9.
- Mandriota SJ, Pyke C, Di Sanza C, et al. Hypoxia-inducible angiopoietin-2 expression is mimicked by iodine compounds and occurs in the rat brain and skin in response to systemic hypoxia and tissue ischemia. *Am J Pathol* 2000;156:2077–89.
- Oh H, Takagi H, Suzuma K, et al. Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. *J Biol Chem* 1999;274:15732–9.
- Pichiule P, Chavez JC, LaManna JC. Hypoxic regulation of angiopoietin-2 expression in endothelial cells. *J Biol Chem* 2004;279:12171–80.
- Park YS, Kim NH, Jo I. Hypoxia and vascular endothelial growth factor acutely up-regulate angiopoietin-1 and Tie2 mRNA in bovine retinal pericytes. *Microvasc Res* 2003;65:125–31.
- Huang YQ, Li JJ, Hu L, Lee M, Karpatkin S. Thrombin induces increased expression and secretion of angiopoietin-2 from human umbilical vein endothelial cells. *Blood* 2002;99:1646–50.
- Ye F, Florian M, Magder SA, Hussain SN. Regulation of angiopoietin and Tie-2 receptor expression in non-reproductive tissues by estrogen. *Steroids* 2002;67:305–10.
- Lobov IB, Brooks PC, Lang RA. Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival *in vivo*. *Proc Natl Acad Sci U S A* 2002;99:11205–10.
- Visconti RP, Richardson CD, Sato TN. Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). *Proc Natl Acad Sci U S A* 2002;99:8219–24.

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