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Original Article

Increased erbB3 promotes erbB2/neu-driven mammary tumor proliferation and co-targeting of erbB2/erbB3 receptors exhibits potent inhibitory effects on breast cancer cells

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Abstract: The kinase deficient erbB3 receptor frequently co-expresses and interacts with erbB2 in human breast cancer to activate the oncogenic signaling pathways, and thus promote breast cancer cell survival/proliferation. In the current study, we discovered that the expression of endogenous mouse erbB3 was increased in the mammary tumors-derived from wild type (wt) rat *erbB2/neu*-transgenic mice, and the co-expression of erbB2 and erbB3 significantly promoted mammary tumor proliferation *in vivo*. Co-immunoprecipitation assays detected a heterodimeric complex consisting of the transgene encoded protein rat erbB2 and the endogenous mouse erbB3 in the mammary tumors. Specific knockdown of mouse erbB3 dramatically inhibited proliferation of the mammary tumor cell lines-derived from the transgenic mice. Elevated expression of erbB3 protein, but not mRNA, was observed in human breast cancer cells upon ectopic expression of erbB2. Additional studies revealed that overexpression of erbB2 downregulated three *erbB3*-targeting miRNAs, miR-125a, miR-125b, and miR-205, whereas the erbB2 kinase inhibitor (lapatinib) significantly enhanced expression of the three miRNAs in breast cancer cells, suggesting that erbB2 might regulate erbB3 expression through a miRNA-dependent mechanism. Furthermore, an anti-erbB3 monoclonal IgG1 antibody (Ab) in combination with Herceptin mainly inactivated Akt and significantly inhibited proliferation of erbB2-overexpressing breast cancer cells. Collectively, our data indicate that increased expression of erbB3 plays a pivotal role in activating downstream PI-3K/Akt pathway and promoting erbB2-driven mammary/breast tumorigenesis. Simultaneous targeting of erbB2 and erbB3 with two IgG1 Abs may be an effective strategy to treat breast cancer patients whose tumors overexpress both erbB2 and erbB3.

Keywords: erbB2, erbB3, mammary tumor, miRNA, antibody isotype

Introduction

Gene amplification and/or overexpression of *erbB2* (also known as *HER2/neu*) have been reported in approximately 25-30% of human breast cancer and are significantly associated with aggressive tumor growth and poor clinical outcome [1-3]. The erbB2 receptor severs as an excellent target for breast cancer therapy. Although the anti-erbB2 therapy, such as trastuzumab (or herceptin) and lapatinib, has been successfully used in the treatment of breast cancer patients with erbB2-overexpressing (erbB2+) tumors, drug resistance frequently occurs and compromises the efficacy of the erbB2-targeted therapy. Thus, understanding the molecular features associated with erbB2+ breast cancer that contribute to the resistance is important to develop more effective strategy for breast cancer treatment.

ErbB2 belongs to a family of receptor tyrosine kinases (RTKs), which also includes the epidermal growth factor receptor (EGFR, or erbB1/HER1), erbB3 (HER3), and erbB4 (HER4) [4]. The common molecular structure of erbB receptors consists of an extracellular domain, a transmembrane domain, and an intracellular

domain [5]. The extracellular domain is primarily involved in ligand binding and contributes to protein-protein interaction for dimer formation. Unliganded receptor adopts a conformation that inhibits dimerization. Binding of ligands to the extracellular domain induces formation of homo- or hetero-dimers, resulting in activation of the intracellular tyrosine kinases. The tyrosine residues on the C-terminal tails are then phosphorylated, leading to subsequent activation of the downstream signaling, such as phosphoinositide-3-kinase (PI-3K)/protein kinase B (Akt) pathway, mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein kinase (MAPK) pathway, the Src kinase, and etc. The erbB receptor signaling pathways take part in a variety of biological responses, including cellular proliferation, maturation, survival, apoptosis, and angiogenesis [6-9].

Unlike other family members, erbB2 has no known ligands. However, erbB2 is in a constitutively active conformation with an exposed dimerization arm, making it the most preferred dimerization partner for other erbB receptors [10, 11]. The erbB3 receptor, which was found to be kinase impaired, has only 1/1000th the autophosphorylation activity of EGFR [12, 13]. Thus, the interaction of erbB3 with its binding partners is essential for its biological activity. It is not surprised that among all possible erbB dimers, the erbB2/erbB3 heterodimer is considered to be the most biologically active and potent for activation of the PI-3K/Akt signaling pathway [14, 15]. This is in part because erbB2 has a strong tyrosine kinase activity and erbB3 is the preferred partner of erbB2 [16, 17]. In addition, the intracellular domain of erbB3 contains 6 recognition sites for the SH2 domain of the p85 subunit of PI-3K [18, 19]. Moreover, co-expression of erbB3 and erbB2 is frequently observed in human breast cancers [20, 21] and breast cancer cell lines [22, 23]. The erbB2/3 heterodimer has been proposed to be an "oncogenic unit" and key to the proliferation of human breast cancer cells [24]. It has been shown that erbB3 serves as a critical co-receptor of erbB2 and plays a vital role in the development of erbB2+ breast cancer [25]. Studies on the underlying mechanism indicate that erbB3 functions as a major cause of treatment failure in cancer therapy [26]. Our recent studies reveals that expression/activation of erbB3 confers multiple drug resistance in erbB2+ breast cancer cells [27, 28]. Thus, erbB3 is thought to be an important compensatory target for combinatorial strategy to overcome drug resistance since its powerful, potent capability to activate PI-3K/Akt signaling. In the current study, the mammary tumors-derived from MMTV-*erbB2/neu* transgenic mice were used to investigate the role of endogenous erbB3 in erbB2-driven tumorigenesis. We also evaluated the inhibitory effects of co-targeting of both erbB2 and erbB3 receptors on erbB2+ breast cancer cells.

Materials and methods

Reagents and antibodies

Lapatinib was purchased from LC Labs (L-4804). MM-121 was kindly provided by Merrimack Pharmaceuticals, Inc. (Cambridge, MA). Trastuzumab (Herceptin®, Genentech, South San Francisco, CA) was obtained from University of Colorado Hospital pharmacy. Mouse IgG and protein A-agarose were purchased from Roche Diagnostics Corp. (Indianapolis, IN). The lentiviral vector pLKO.1-puro containing a mouse specific *erbB3* shRNA (pLKO.1-ErbB3shRNA: TRCN0000023429~33, labeled as sh-1~5 in text) was from Functional Genomics Facility at the University of Colorado at Boulder. The lentivirus packaging plasmids pCMV-VSVG and pCMV-ΔA.9 were kindly provided by Dr. Haihua Gu (Department of Pathology, University of Colorado Denver School of Medicine, Aurora, CO).

Antibodies used for Western blots were from the following sources: erbB2 (c-neu Ab-3, EMD Chemicals, Inc., Gibbstown, NJ); phospho-erbB2 (P-erbB2, clone PN2A), and erbB3 (Ab-7) (Lab Vision/NeoMarkers, Inc., Fremont, CA); phospho-erbB3 (P-erbB3, clone 21D3), phospho-Akt (Ser473), Akt, P-MAPK (Thr202/Tyr204), MAPK (Cell Signaling Technology, Inc., Danvers, MA); and β-actin (clone AC-74, Sigma-Aldrich Co., St. Louis, MO). All other reagents were purchased from Sigma unless otherwise specified.

Cells and cell culture

Murine mammary tumor cell lines 85815 and 85819 were established from mammary tumors derived from the wild type (wt) rat

c-*erbB2/neu* transgenic mice [29, 30]. Human breast cancer cell lines MCF-7, SKBR-3, BT-474, MDA-MB-435, and MDA-MB-453 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM/F-12 medium (1:1, v/v) (Invitrogen Corp., Grand Island, NY) supplemented with 10% FBS (Invitrogen Corp). The *erbB2* transfected MCF-7 cells (MCF-7/erbB2) were obtained from Dr. Christopher C. Benz (Buck Institute for Age Research, Novato, CA). The *erbB2*-transfectant 435.eB1 cell line [31] was kindly provided by Dr. Dihua Yu (MD Anderson Cancer Center). Both MCF-7/erbB2 and 435.eB1 cells were used in our previous studies [32, 33]. All the cell lines were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO_2 and split twice a week.

Mice

All animal care was done in accordance with institutional guidelines in our Association for the Assessment and Accreditation of Laboratory Animal Care-approved mouse facilities. The protocols and experiments were reviewed and approved by our Institutional Animal Care and Use Committee. Virgin female FVB-TgN (MMTV-neu) transgenic mice (wt rat c-*erbB2/neu*) were obtained from Jackson Labs at 4 to 5 weeks of age. Mice were placed on a casein (milk protein based) diet, Purina 5K96 (Ralston Purina Co., St. Louis, MO). Mice were checked twice weekly for tumor formation. Tumor latency was calculated from the date of the first palpable tumor. Tumors were measured twice weekly using calipers to evaluate tumor growth rates as shown in **Figure 1B**. Once tumors reached 1.2 cm in greatest dimension, animals were euthanized to prevent undue stress to the animals. Histologic examination of all palpable or visible tumors was preformed to verify tumorigenesis and evaluate tumor histology. The histological pattern, tumor diagnoses, and heterogeneity of the mammary tumors were confirmed by microscopic analysis and have been reported elsewhere [29, 30, 34].

Production of lentivirus containing specific shRNA and knockdown of erbB3 expression

As we performed previously [33, 35], the lentiviral vector pLKO.1-ConshRNA or pLKO.1-ErbB3shRNA and packaging plasmids pCMV-VSVG and pCMV-ΔA.9 were co-transfected into 293T cells using Fu-GENE-6 (Roche Diagnostics Corp.). After 24 hr, the culture media were replaced with fresh DMEM/F12 medium. The virus in conditioned medium were harvested in 3 consecutive day, and then filtered with low protein binding filters (Millex-hV 0.45 mm polyvinylidene difluoride, EMD Millipore Corp., Billerica, MA) before they were aliquot and stored at -80°C freezer. To knockdown erbB3 expression, the ConshRNA or ErbB3shRNA lentivirus-containing media (5 mL each tube) were thawed completely at room temperature. Another 5-mL fresh DMEM/F12 medium (10% FBS) and polybrene (8 μg/mL) were added into the virus-containing media. Then, the cell culture media were replaced with the ConshRNA or ErbB3shRNA lentivirus-containing media. After 24 hr, the virus-infected cells were selected with puromycin (1 μg/mL) for 48 hr and subjected to required experiments.

Reverse transcription (RT)-PCR

Total RNA was extracted using a modified chloroform/phenol procedure (TRIZOL, Life Technologies Corp., Carlsbad, CA). First-strand cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corp.). The analysis of human *erbB2, erbB3, and β-actin* expression was examined by conventional RT-PCR as we described previously [36].

Analysis of miRNA expression

Total RNA, including small RNA, was extracted and purified using the miRNeasy Mini Kit (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. The expression levels of miRNA were measured as described [36]. In brief, TaqMan MicroRNA Reverse Transcription kit (Life Technologies Corp.) was first used to generate cDNA with the hairpin primers, which were specific to mature miRNAs. The expression levels of miR-125a-5p, miR-125b, and miR-205 were then measured by real-time PCR using TaqMan MicroRNA Assays (assay ID: 002198, 000449, 000509, respectively; Life Technologies Corp.) according to the manufacturer's protocol. RNU6B was used as an internal control to normalize all data using the TaqMan RNU6B Assay (assay ID: 001093; Life Technologies Corp.). The relative miRNA levels were calculated using the comparative Ct method (ΔΔCt).

Immunohistochemistry

Immunohistochemical staining of mammary tumor tissues was performed as previously described [27-29]. Briefly, after deparaffinization and rehydration, tissue sections were steamed in a 10 mmol/L citrate buffer, pH 6.0, for 30 minutes. Non-specific reactivity was blocked with 0.3% H_2O_2 in buffer. Primary antibodies included an anti-erbB2 (reactive with rat c-*neu*/erbB2 rabbit polyclonal; dilution 1:1000; DAKO, Carpinteria, CA, for 2 hr incubation at room temperature), anti-erbB3 (reactive with mouse and human, mouse mAb; dilution 1:50; NeoMarker Inc., overnight incubation at 4°C), anti-phospho-erbB2 (Y1221/Y1222 and Y877) and anti-phospho-erbB3 (Y1289) (rabbit monoclonal; diluted in 5% normal goat serum 1:12.5; Cell Signaling Technology, overnight at 4°C). After multiple washes with buffer, tissue sections were sequentially incubated for 30 minutes at room temperature with diluted biotinylated secondary antibody (1:500, DAKO) and VECTASTAIN Elite ABC reagent (Vector Laboratories, Inc.) diluted in PBS. After reaction with diaminobenzidine (DAKO) and counterstaining with hematoxylin, tumors were individually examined. The cases with a diffuse intense circumferential membrane "chicken-wire" staining of erbB2 were observed. Membrane and/or cytoplasm staining for erbB3 were observed. Each slide was read by two independent scientists. For both erbB2 and erbB3, positive staining in >30% of the mammary tumor cells was considered overexpression.

Immunoprecipitation and western blot analysis

Immunoprecipitation (IP) and western blot assays were performed as previously described [33, 35]. Briefly, cells were lysed and the supernatants were cleared by centrifugation. Protein concentrations were measured using the Coomassie plus protein assay reagent (Pierce Chemical Co., Rockford, IL). Total cell lysates containing 500 μg of protein were subjected to IP in the presence of 1 μg anti-erbB2 Ab (Ab-4, EMD Chemicals, Inc.) for 2 hr at 4°C, followed by incubation with immobilized protein A-agarose (Roche Diagnostics Corp.) at 4°C overnight with rotation. For western blot analyses, the immunoprecipitates or equal amounts of crude extracts were boiled in Laemmli SDS-sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose (Bio-Rad Laboratories, Hercules, CA), and probed with different primary antibodies. After the blots were incubated for another 1 hr at room temperature with horseradish peroxidase-labeled secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG, Jackson ImmunoResearch Laboratories, West Grove, PA), the signals were detected using the enhanced chemiluminescence reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) according to the manufacturer's instructions.

Cell proliferation assay

The IncuCyte™ system (Essen BioScience, Inc., Ann Arbor, MI) was used to kinetically monitor cell growth. It is an automated imaging platform providing real-time images and quantitative data generated throughout cell culture process. Proliferation is successfully measured using an IncuCyte phase-only processing module. Briefly, Cells were plated onto 96-well plates, 2000 cells/well for each cell line. The plate was placed into the IncuCyte™ at 37°C for 2-3 days. During this time, each well was repeatedly imaged at fixed time intervals (every 4 hr). The data were analyzed by the IncuCyte software. Data reflects the means of three independent experiments.

The CellTiter96 AQ nonradioactive cell proliferation kit (Thermo Fisher Scientific Inc., Waltham, MA) was used to determine cell viability as previously described [27-29]. Briefly, cells were plated onto 96-well plates for 24 hr, and then grown in either DMEM/F12 medium with 0.5% FBS as control, or the same medium containing herceptin in the presence or absence of Ab105, and then incubated for another 72 hr. After reading all wells at 490 nm with a microplate reader, the percentages of surviving cells from each group relative to controls, defined as 100% survival, were determined by reduction of MTS.

Statistical analysis

Comparisons of the tumor proliferation rate in mice were calculated using the two-sided Student's *t* test with unequal variance. The first palpable tumor was used to calculate the tumor latency for animals that had multiple tumors. Analysis on tumor latency or *in vitro* cell growth

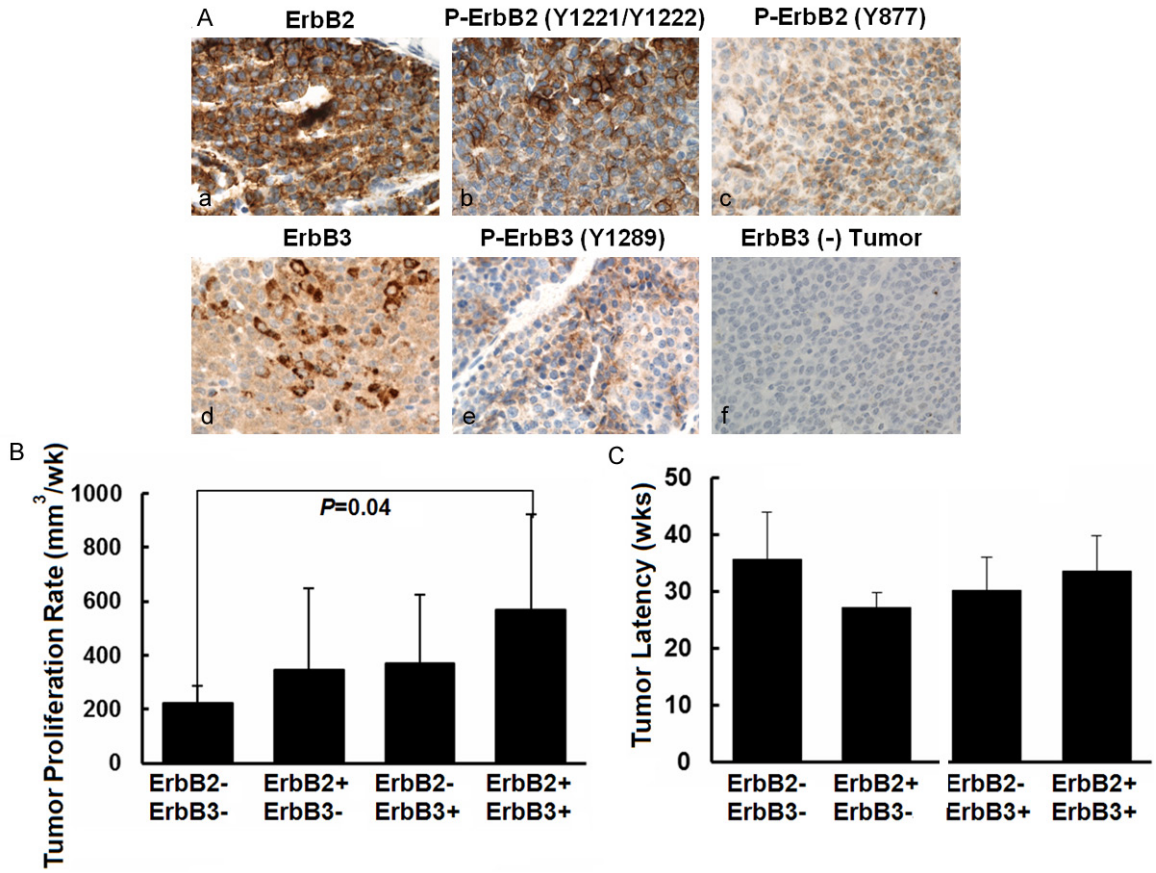

Figure 1. Increased mouse erbB3 significantly promoted wt rat c-*erbB2/neu*-driven mammary tumor growth *in vivo*. (A) Mammary tumors obtained from the transgenic mice were evaluated by IHC analysis with specific Abs against erbB2, P-erbB2, erbB3, and P-erbB3. Representative photomicrographs were taken from 78617 R3 mammary tumor sections (a-e). The positive staining was found mainly on cell membrane. (f) Negative control-78423 R1 mammary tumor section showed no erbB3 expression. 40X. (B) Mice were visually checked for tumors twice weekly until 60 wk. Once palpable, tumors were measured by caliper twice weekly. The interval of growth from the first date palpable to the time the tumor reached 1.2 cm in greatest dimension was recorded as the tumor growth rate. The mammary tumors examined were separated into four groups (erbB2-erbB3- (n=3), erbB2+erbB3- (n=6), erbB2-erbB3+ (n=7), and erbB2+erbB3+ (n=7)) according to their erbB2 and erbB3 status. In each subgroup, the average of time it took to reach 1. 2 cm was calculated and shown. The tumors in erbB2+/erbB3+ group showed significantly higher proliferation rate than those in erbB2-/erbB3- group, *P*=0.04. (C) Tumor latency was calculated from the date of the first palpable tumor. There was no significant difference among four different groups.

assays was performed using the two-sided Student's *t* test with equal or unequal variance. Statistical calculations were carried out by Stat View 5.01 software (SAS Institute Inc., Cary, NC).

Results

Co-expression of the endogenous mouse erbB3 and the transgene-encoded protein rat erbB2 promotes mammary tumor proliferation in vivo

Transgenic mice bearing the wt-rat c-*erbB2/neu*, under control of the mouse mammary tumor virus promoter (MMTV-LTR), typically develop unifocal, well-circumscribed, low-grade mammary tumors after a long latency [37]. While we showed that expression of the endogenous mouse erbB3 was elevated and the increased erbB3 formed physical and functional interactions with the transgene-encoded protein erbB2 in tumor-derived cell lines [29], we wondered if this phenomenon might be observed in the primary tumors. The expression of erbB2 and erbB3 in mammary tumors-derived from the wt rat c-*erbB2/neu* transgenic mice was studied by immunohistochemistry (IHC). Elevated expression of erbB3 was detect-

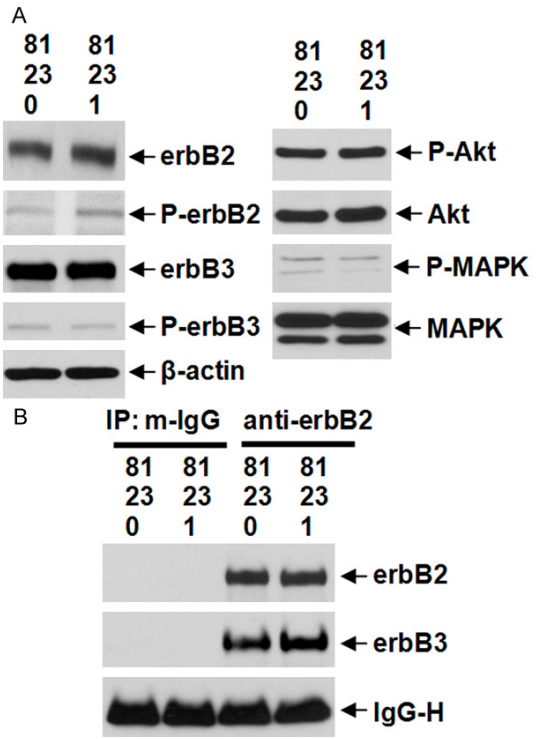

Figure 2. Interactions between mouse erbB3 and rat erbB2 were discovered in the mammary tumors-derived from the wt rat c-*erbB2/neu*-transgenic mice. A. Two representative mammary tumors 81230 and 81231 were homogenized in the presence of Western blot lysis buffer. Same amount of total lysates was then subjected to Western blot analysis with the specific antibodies directed against erbB2, P-erbB2, erbB3, P-erbB3, P-Akt, Akt, P-MAPK, MAPK, or b-actin. B. The same bath of total lysates (500 μg proteins) were used for IP analysis with 1 μg of anti-erbB2 Ab (mouse monoclonal Ab, Ab-4) at 4°C for 2 hr. The same isotype of mouse IgG was used as a negative control. The immobilized protein A-agarose was added into the reaction and incubated at 4°C overnight with rotation. The immunoprecipitates were then subjected to Western blot analysis of erbB2 and erbB3. IgG-H indicates the heavy chain of the IgG Abs used for IP analysis.

ed in 56.3% (18/32) erbB2-positive mammary tumors. Co-expression of erbB2 and erbB3 was found in about 40% (14/45) tumors. In addition, the tumors with co-expression of both receptors had higher levels of phosphorylated erbB2 (P-erbB2) and phosphorylated erbB3 (P-erbB3). Two phosphorylation sites of erbB2, Y1221/1222 and Y877, were examined. The representative data for each antibody staining showed in **Figure 1A**. To investigate the role of the endogenous mouse erbB3 on erbB2-mediated mammary tumor growth *in vivo*, we compared tumor proliferation rate in four different groups (erbB2+erbB3+, erbB2+erbB3-, erbB2-erbB3+, and erbB2-erbB3-) according to IHC results. The mammary tumors with expression of both erbB2 and erbB3 (erbB2+erbB3+) showed a significantly higher growth rate than that with expression of neither receptor (erbB2-erbB3-) (P=0.04) (**Figure 1B**). The expression status of erbB2 and erbB3 appeared to have no significant effect on tumor latency (**Figure 1C**). These data indicated that increased expression of the endogenous mouse erbB3 promoted mammary tumor proliferation in the wt rat c-*erbB2/neu*-transgenic mice.

Increased mouse erbB3 interacts with rat erbB2 in the mammary tumors, and is essential for maintaining proliferation/survival of the tumor-derived cell lines

Consistent with our IHC results, studies with Western Blot analysis confirmed the co-expression and activation of erbB2 and erbB3 in tumor tissues as evidenced by the detection of the protein levels and phosphorylation of erbB2 (P-erbB2) and erbB3 (P-erbB3) in the representative tumor 81230 and 81231 (**Figure 2A**). While the downstream signaling kinases Akt and MAPK were activated, it appeared that Akt was a major one since much higher levels of phosphorylated Akt (P-Akt) than that of phosphorylated MAPK (P-MAPK) were observed (**Figure 2A**). Moreover, we performed co-immunoprecipitation (IP) assays and discovered that the endogenous mouse erbB3 interacted with the protein encoded by the transgene rat *erbB2* in the mammary tumors (**Figure 2B**). These data indicate that mouse erbB3 and wt rat erbB2 protein form physical and functional interactions in the mammary tumors expressing both erbB2 and erbB3.

Our previous studies demonstrated that down-regulation of erbB3 by siRNA inhibited cell proliferation and colony formation in both murine and human mammary/breast cancer cells [32]. Here we used specific shRNAs to knockdown erbB3 expression to further elucidate the role of erbB3 in maintaining proliferation/survival of the mammary tumor-derived cell lines. Five mouse *erbB3*-specific shRNA sequences (sh-1~5) in the lentiviral vector pLKO.1 were tested for their efficiency to silence mouse *erbB3* (data not shown). We found a partial and complete knockdown of erbB3 expression with sh-3

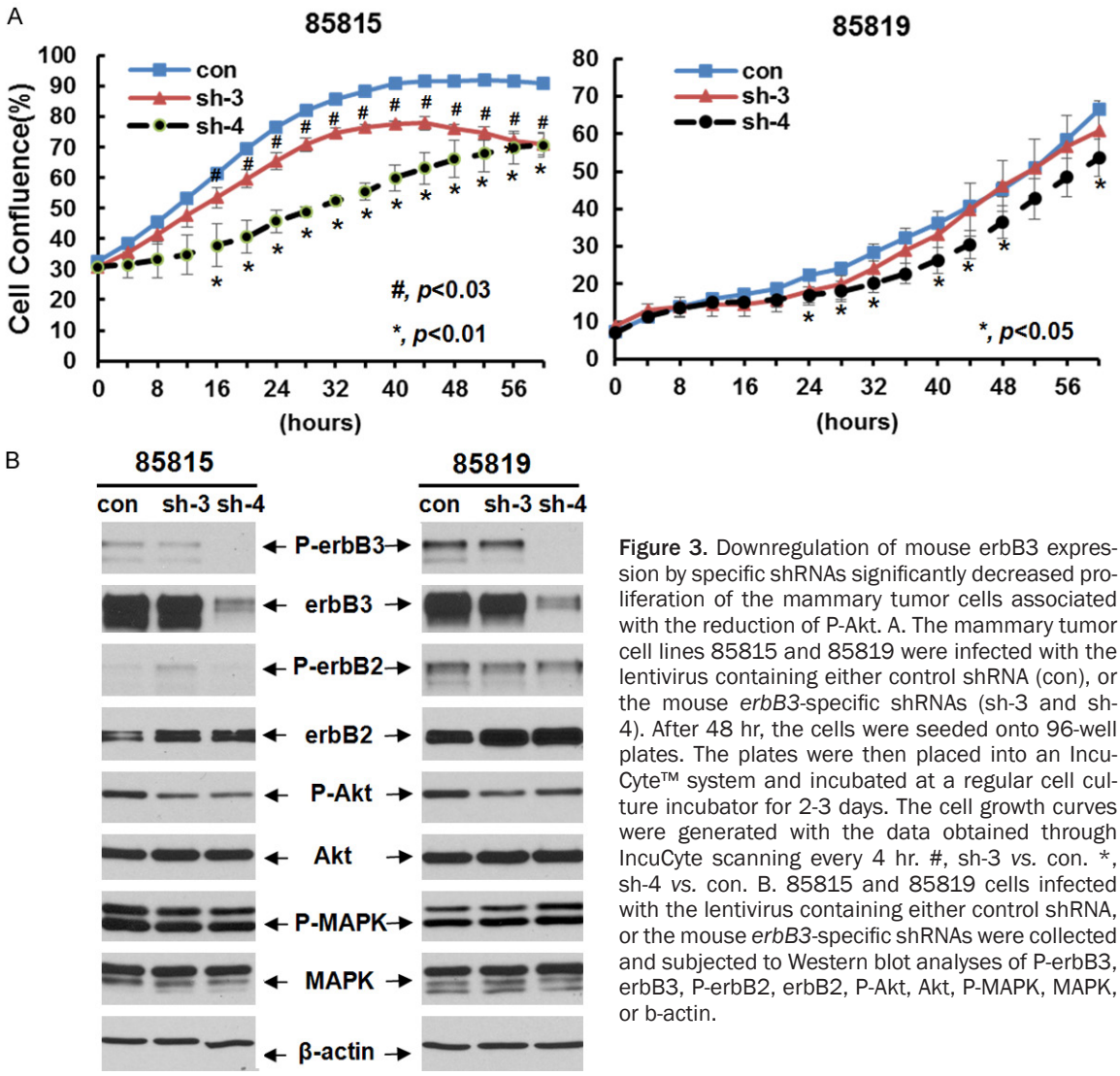

Figure 3. Downregulation of mouse erbB3 expression by specific shRNAs significantly decreased proliferation of the mammary tumor cells associated with the reduction of P-Akt. A. The mammary tumor cell lines 85815 and 85819 were infected with the lentivirus containing either control shRNA (con), or the mouse *erbB3*-specific shRNAs (sh-3 and sh-4). After 48 hr, the cells were seeded onto 96-well plates. The plates were then placed into an IncuCyte™ system and incubated at a regular cell culture incubator for 2-3 days. The cell growth curves were generated with the data obtained through IncuCyte scanning every 4 hr. #, sh-3 *vs.* con. *, sh-4 *vs.* con. B. 85815 and 85819 cells infected with the lentivirus containing either control shRNA, or the mouse *erbB3*-specific shRNAs were collected and subjected to Western blot analyses of P-erbB3, erbB3, P-erbB2, erbB2, P-Akt, Akt, P-MAPK, MAPK, or b-actin.

and sh-4, respectively, which were then used in the following studies. Cell proliferation was monitored by the state-of-art IncuCyte System (Essen BioScience, Inc.), which provided an effective read-out for long-term, live-cell imaging and allowed automated analysis of kinetic quantification of cell growth *in vitro*. The kinetic cell growth curves showed that downregulation of erbB3 with either sh-3 or sh-4 significantly decreased proliferation in 85815 mammary tumor cells, whereas complete knockdown of erbB3 expression with sh-4 markedly inhibited proliferation of both 85815 and 85819 mammary tumor cell lines (**Figure 3A**). Furthermore, simultaneous reduction of P-erbB2 and P-erbB3 levels clearly decreased the levels of P-Akt, but had no effect on P-MAPK (**Figure 3B**). The protein levels of erbB2, Akt, and MAPK remained unchanged. Collectively, our data verified that erbB3 mainly activates the PI-3K/Akt signaling to maintain erbB2-driven mammary tumor cell proliferation/survival, which is consistent with our previous findings in both murine and human mammary/breast cancer cells [32].

Overexpression of erbB2 appears to upregulate erbB3 via a miRNA-dependent mechanism in human breast cancer cells

Co-expression of erbB3 with erbB2 is frequently observed in breast cancers, both in human and mouse. However, the underlying mechanism remains elusive. To investigate the potential role of erbB2 in upregulating erbB3 expression, we took advantage of the *erbB2*-tarns-

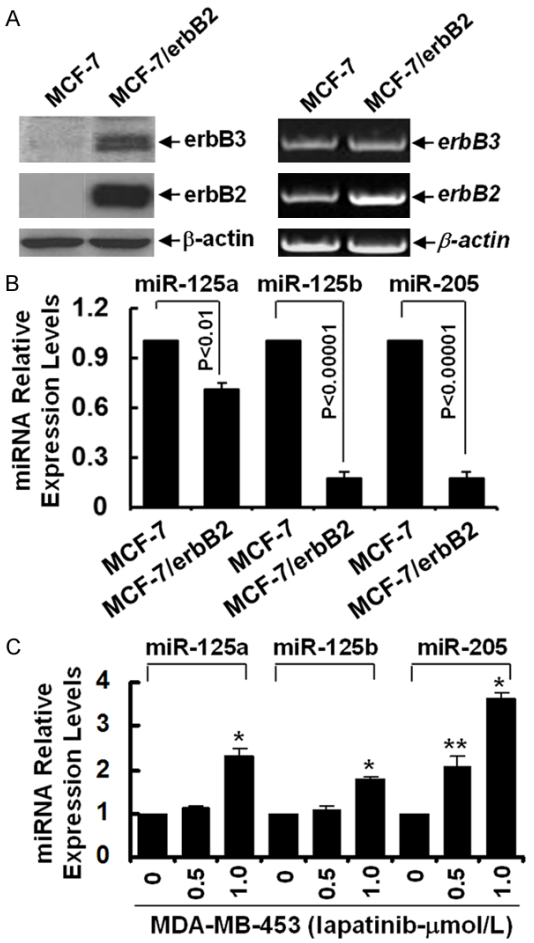

Figure 4. Overexpression of erbB2 increased the levels of erbB3 protein, but not mRNA, in human breast cancer cells through a mechanism involving in miR-125a, miR-125b, and miR-205. A. Same amount of total lysates from MCF-7 and MCF-7/erbB2 cells were analyzed by Western blot analyses of erbB3, erbB2, or b-actin (left). Total RNA was extracted from MCF-7 and MCF-7/erbB2 cells and followed by reverse transcription-PCR analysis with specific primers for *erbB3*, *erbB2*, or b-*actin*. B. MCF-7 and MCF-7/erbB2 cells at normal culture condition; and C, MDA-MB-453 cells untreated or treated with indicated concentrations of lapatinib for 24 hr were subjected to total RNA extraction, inclusive of the small RNA fraction. The expression levels of miR-125a, miR-125b, and miR-205 were measured by qRT-PCR using Taqman miRNA assays. Data were normalized with internal control (RNU6B). **P*<0.001, ***P*<0.005 vs. untreated control.

fected MCF-7 (MCF-7/erbB2) human breast cancer cell line [32], and discovered that ectopic expression of erbB2 increased the protein, but not mRNA levels of *erbB3* (**Figure 4A**). These data suggest that erbB2 possibly regulate erbB3 expression via a mechanism independent of transcription. Since microRNAs (miRNAs), the short noncoding RNAs of ~22 nucleotides, negatively regulate gene expression at the post-transcriptional level, and usually bind to the 3'-UTR of its target mRNA via sequence-guided recognition to trigger mRNA degradation or translational repression [38], we hypothesized that erbB2 may modulate erbB3 expression via a miRNA-dependent mechanism in erbB2+ breast cancer cells. Quantitative real-time (qRT)-PCR analysis revealed that the expression levels of miR-125a, miR-125b, and miR-205, which have been shown to target *erbB3* mRNA [39-42], were significantly lower in MCF-7/erbB2 cells than that in MCF-7 cells (**Figure 4B**). Conversely, the tyrosine kinase inhibitor of erbB2 (lapatinib) dramatically induced expression of miR-125a, miR-125b, and miR-205 in MDA-MB-453, a well-known erbB2+ breast cancer cell line. These data suggest that elevated expression/activation of erbB2 may increase erbB3 potentially through downregulation of miR-125a, miR-125b, and/or miR-205 in breast cancer cells.

Targeting of both erbB2 and erbB3, but not either one of them, exerts a significant inhibitory activity in human breast cancer cells with co-expression of erbB2 and erbB3

Since elevated expression of erbB3 plays a vital role in the development of erbB2+ breast cancer [25], several blocking Abs against erbB3 with therapeutic potential have been developed [43]. MM-121 is a fully human anti-erbB3 monoclonal IgG2 Ab currently under preclinical studies [44, 45] and clinical trials in cancer patients (https://www.clinicaltrials.gov/ct2/results?term=MM-121&Search=Search). We recently showed that MM-121 was able to overcome resistance and significantly enhanced Herceptin-mediated growth inhibition and/or apoptosis in erbB2+ breast cancer cells [27]. To investigate whether the anti-erbB3 Ab with a different isotype may also be able to enhance the efficacy of Herceptin, we next studied the inhibitory effects of Ab105 (clone H3.105.5, a mouse anti-erbB3 monoclonal IgG1 Ab from Oncogene Research Products, Boston, MA) in combination with Herceptin on erbB2+ breast cancer cells. Our purpose was to identify more effective erbB3-targeted therapy that could significantly potentiate Herceptin-mediated anti-proliferative/anti-survival effects on erbB2-driven breast cancer

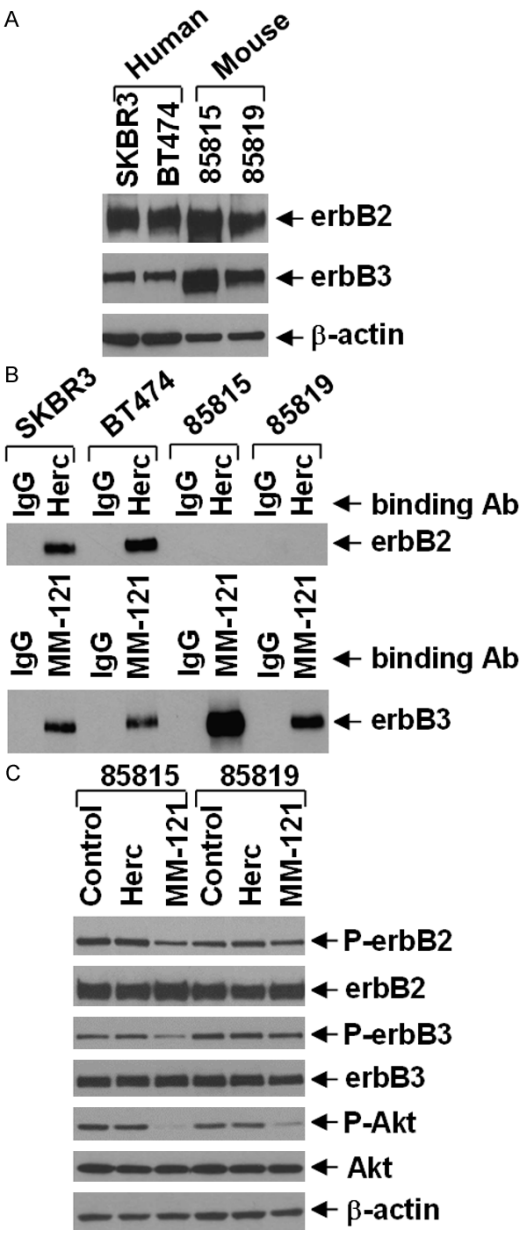

Figure 5. Herceptin did not recognize rat erbB2, whereas MM-121 bound to both human and mouse erbB3 and reduced the levels of P-erbB2, P-erbB3, and P-Akt in mouse mammary tumor cells. A. Human (SKBR3 and BT474) and mouse (85815 and 85819) breast/mammary tumor cell lines were examined by Western blot analyses of erbB2, erbB3, or b-actin. B. The indicated Abs (immunoglobin, IgG; Herceptin, Herc; MM-121) was added into the cell culture media. After 2 hr incubation, the cells were collected and lysed. Same amounts of total cell lysates from each sample were subjected to binding assays with protein A-agarose. The protein A-bound immunocomplexes were then analyzed by Western blot analyses. C. Mouse mammary tumor cells untreated or treated with either Herceptin (20 μg/ml) or MM-121 (10 μg/ml) for 24 hr were examined by Western blot analyses of P-erbB2, erbB2, P-erbB3, erbB3, P-Akt, Akt, or b-actin.

cells. Herceptin is a humanized IgG1 Ab targeting human erbB2, its binding efficiency with rat erbB2, however, is unclear. Thus, we first tested whether Herceptin could bind to rat erbB2 protein. Co-expression of erbB2 and erbB3 was observed in both human breast cancer SKBR3 and BT474 cell lines and the 85815 and 85819 mouse mammary tumor cell lines-derived from the wt rat c-*neu*/erbB2-transgenic mice (**Figure 5A**). Same amount of Herceptin or MM-121 (a mouse IgG was used as a negative) was added into the culture of SKBR3, BT474, 85815, and 85819 cells. After 4 hr, the cells were collected and subjected to IP analysis followed by Western blot assays. Our data showed that MM-121 recognized both human and mouse erbB3, whereas Herceptin only interacted with human erbB2 (**Figure 5B**). Consistent with this observation, MM-121, but not Herceptin, was able to reduce the levels of P-erbB2, P-erbB3, and P-Akt in 85815 and 85819 cells (**Figure 5C**). These data suggest that the mouse mammary tumor cell lines may not be a good model to test Herceptin's activity. Thus, we decided to examine the combinatorial activity of Ab105 and Herceptin in human breast cancer cell lines.

MDA-MB-453 and 435.eB1 (The *erbB2*-transfectant of MDA-MB-435 cells expresses higher levels of erbB3 due to ectopic expression of erbB2 (data not shown)) cells were treated with either vehicle control, or Herceptin or Ab105 alone, or both Abs for 24 hr. Western blot analysis showed that while treatment with single Ab had no significant effect on P-Akt, the combinations of Herceptin and Ab105 clearly reduced the levels of P-Akt (**Figure 6A**). The levels of P-MAPK remained unchanged with any treatment. These data were consistent with our previous findings that MM-121 in combination with Herceptin inhibited Akt, but not MAPK in both Herceptin-sensitive and -resistant breast cancer cell lines [27]. Furthermore, cell proliferation (MTS) assays revealed that while Ab105 alone had no effect, Herceptin alone exhibited some inhibitory effects on SKBR3, BT474, and MDA-MB-453 cells. However, the combinations of Ab105 and Herceptin significantly, as compared to Herceptin alone, inhibited proliferation of 435.eB1, SKBR3, BT474, and MDA-MB-453 cells (**Figure 6B**). The parental cell line MDA-MB-435 does not express erbB2, thus showed no response to any treatment (**Figure 6B**). It appeared that Ab105 in combination with Herceptin mainly induced growth inhibition *in*

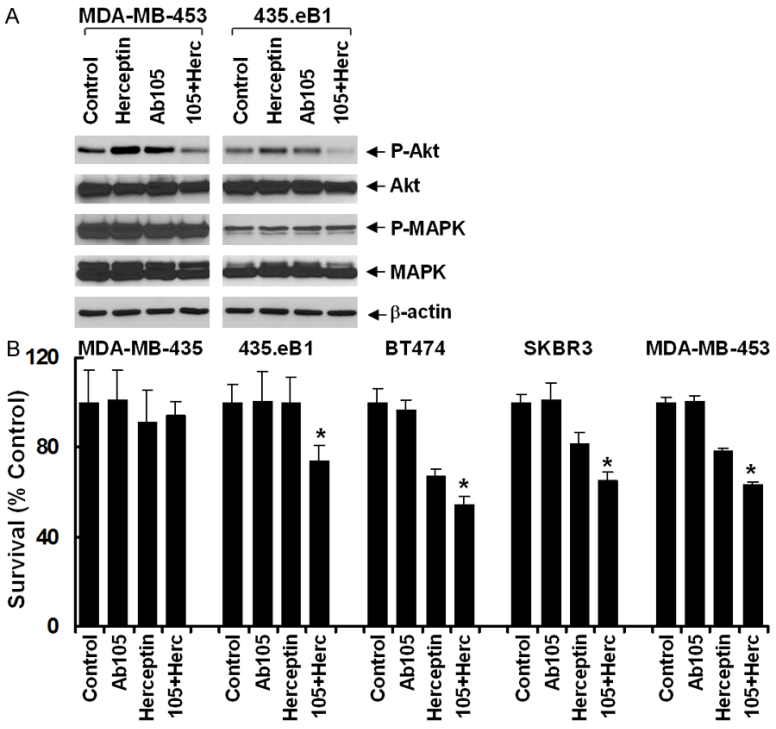

Figure 6. Combinations of Ab105 and Herceptin decreased P-Akt levels and significantly induced growth inhibition in erbB2+ breast cancer cells. A. MDA-MB-453 and 435.eB1 breast cancer cells were cultured in DMEM/F12 medium containing 0.5% FBS or the same medium containing either Herceptin (20 μg/ml), or Ab105 (10 μg/ml) alone, or both Abs (Ab105+Herc) for 24 hr. Cells were collected and subjected to Western blot analyses of P-Akt, Akt, P-MAPK, MAPK, or β-actin. B. MDA-MB-435, 435.eB1, BT474, SKBR3, and MDA-MB-453 cells were plated onto 96-well plates and incubated at 37°C with 5% CO_2. After 24 hr, the culture medium was replaced with 0.1 ml fresh medium containing 0.5% FBS or the same medium containing Ab105 (10 μg/ml), or Herceptin (20 μg/ml) alone, or both Abs (Ab105+Herc) for another 72 hr. The percentages of surviving cells from each cell line relative to controls, defined as 100% survival, were determined by reduction of MTS. *Bars*, SD. Data show a representative of three independent experiments. *P<0.05 vs. Herceptin alone.

vitro, as no significant alterations in apoptosis-related markers were observed (data not shown). Taken together, these data suggest that targeting of erbB3 with the IgG1 Ab (Ab105) will improve the efficacy of erbB2-targeted therapy (Herceptin) against erbB2+ breast cancer.

Discussion

A number of studies indicate that transgenic mice bearing the wt rat c-*erbB2/neu* gene, under control of the MMTV promoter, develop mammary tumors with a long latency, suggesting that additional genetic and/or epigenetic alterations may contribute to the mammary tumorigenesis. We and others reported an elevated expression of endogenous mouse erbB3 in the mammary tumors-derived from the wt or activated rat c-*erbB2/ne* transgenic mice [29, 46]. Our data showed that the increased mouse erbB3 interacted with the transgene *erbB2-encoded protein in the mam*mary tumor-derived cell lines [29], supporting previous findings that heterodimers of erbB2 and erbB3 functioned as an oncogenic unit to maintain breast cancer cell proliferation/survival [47]. However, it was unknown if overexpression of the mouse erbB3 could also interact with rat erbB2 in the mammary tumors and promote tumor growth *in vivo*. In the current study, both physical and functional interactions of erbB2 and erbB3 were identified in the mammary tumors-derived from the wt rat c-*erbB2/neu* transgenic mice. We believe we are the first detecting a direct association of two receptors from different species (rat erbB2/mouse erbB3) *in vivo*. Furthermore, the mammary tumors with co-expression of erbB2 and erbB3 grew significantly faster than those with expression of neither erbB2 nor erbB3. Although the mammary tumors expressing both erbB2 and erbB3 had a trend to proliferate faster than those expressing either receptor alone, the difference did not reach significance, which might be due to limited numbers of tumor samples.

Co-expression of erbB2 and erbB3 receptors is common in human breast cancers [48] and breast cancer cell lines [49]. This observation has been confirmed with the studies of mammary tumorigenesis in *erbB2/neu*-transgenic mice [29, 46]. However, the underlying mechanisms why erbB2/erbB3 frequently co-express in breast cancer are not well understood. Here, we found that the erbB3 protein, not mRNA,

levels were increased upon ectopic expression of erbB2 in human breast cancer MCF-7 cells (**Figure 4A**) as well as in mouse mammary tumors [46], indicating that upregulation of erbB3 may occur at post-transcriptional level. Our studies showed that the expression levels of three *erbB3*-targeting miRNAs, miR-125a, miR-125b, and miR-205 were markedly decreased when erbB2 was overexpressed. In contrast, the miRNAs' expression was significantly increased when erbB2+ breast cancer cells were treated with the erbB2 tyrosine kinase inhibitor, lapatinib (**Figure 4B**). These data suggest that overexpression/activation of erbB2 promotes erbB3 expression via a mechanism involving in miR-125a, miR-125b, and/or miR-205. This conclusion seems to be supported by recent studies. It has been shown that elevated expression of erbB2 downregulates miR-205 in breast cancer [50], and an inverse correlation between erbB2 status and the expression of miR-125a/miR-125b is observed in gastric and esophageal carcinomas [51]. Nonetheless, more detailed analyses of the mouse mammary tumors and clinical samples of erbB2+ breast cancer patients are needed to strengthen our hypothesis.

It is widely recognized that one of the major mechanisms of action of Herceptin is through its IgG1 Fc portion to activate the antibody-dependent cell-mediated cytotoxicity (ADCC) effect via host's innate immune system [52]. Novel strategies which can enhance ADCC effectors, such as natural killer (NK) cells, are sought to improve the therapeutic efficacy of Herceptin. This has been supported by a recent study indicating that stimulation of NK cells with a CD137-specific Ab significantly enhances Herceptin-mediated cell killing *in vitro* and *in vivo* [53]. We showed that combinations of MM-121 and Herceptin not only induced growth inhibition of the Herceptin-resistant breast cancer BT474-HR20 cells, but also promoted the cells undergoing apoptosis *in vivo* [27]. This cell death-inducing effect may be attributed to the enhanced ADCC mainly mediated by Herceptin (not MM-121), because MM-121 is an IgG2 Ab that cannot trigger ADCC [54]. Thus, it is conceivable to hypothesize that an IgG1 isotype of anti-erbB3 Ab in combination with Herceptin will exert superior antitumor activity against breast cancers with co-expression of both erbB2 and erbB3 receptors. Ab105 is a mouse monoclonal IgG1 Ab against human erbB3. It shows no cross reactivity with EGFR, erbB2, or erbB4; and is particularly suited for inhibiting the binding of heregulin to erbB3 [55]. We found that Ab105 in combination with Herceptin mainly inactivated Akt, evidenced by the dramatic reduction of P-Akt in human erbB2+ breast cancer cells. Moreover, the combinations of Ab105 and Herceptin, as compared to single Ab, significantly inhibited cell proliferation in all erbB2+ breast cancer cell lines tested (**Figure 6**). These *in vitro* data not only confirm our findings with MM-121 [27], they also warrant further evaluations to test the antitumor activity of Ab105 in combination with Herceptin against erbB2+ breast cancer *in vivo*.

In summary, we demonstrate that elevated expression of the endogenous mouse erbB3 promotes mammary tumor proliferation in the wt rat c-*erbB2/neu* transgenic mice via interaction with the transgene rat *erbB2/neu*-encoded protein. The novel data showing that overexpression and/or activation of erbB2 enhance erbB3 expression potentially through a miRNA-dependent mechanism may explain a long-standing mystery - why erbB2 and erbB3 (but not EGFR) are frequently co-expressed in a subset of breast cancer. Our studies with Ab105 suggest that co-targeting of erbB2 and erbB3 with two IgG1 Abs may exhibit a more effective antitumor activity against the breast cancers with expression of both erbB2 and erbB3 receptors.

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Disclosure of conflict of interest

None.

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