

Artemin, a Member of the Glial Cell Line-derived Neurotrophic Factor Family of Ligands, Is HER2-regulated and Mediates Acquired Trastuzumab Resistance by Promoting Cancer Stem Cell-like Behavior in Mammary Carcinoma Cells*

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Background: Artemin is a cancer stem cell (CSC) and metastatic factor in mammary carcinoma.

Results: Artemin promotes trastuzumab resistance by enhancing the cancer stem cell-like population in mammary carcinoma.

Conclusion: Artemin mediates acquired resistance to trastuzumab in mammary carcinoma.

Significance: Functional antagonism of Artemin may enhance trastuzumab sensitivity and reverse acquired resistance to trastuzumab in mammary carcinoma.

Previous studies have demonstrated that Artemin (ARTN) functions as a cancer stem cell (CSC) and metastatic factor in mammary carcinoma. Herein, we report that ARTN mediates acquired resistance to trastuzumab in HER2-positive mammary carcinoma cells. Ligands that increase HER2 activity increased ARTN expression in HER2-positive mammary carcinoma cells, whereas trastuzumab inhibited ARTN expression. Forced expression of ARTN decreased the sensitivity of HER2-positive mammary carcinoma cells to trastuzumab both *in vitro* and *in vivo*. Conversely, siRNA-mediated depletion of ARTN enhanced trastuzumab efficacy. Cells with acquired resistance to trastuzumab exhibited increased ARTN expression, the depletion of which restored trastuzumab sensitivity. Trastuzumab resistance produced an increased CSC population concomitant with enhanced mammospheric growth. ARTN mediated the

enhancement of the CSC population by increased BCL-2 expression, and the CSC population in trastuzumab-resistant cells was abrogated upon inhibition of BCL-2. Hence, we conclude that ARTN is one mediator of acquired resistance to trastuzumab in HER2-positive mammary carcinoma cells.

The ERBB (HER) receptor tyrosine kinase family comprises four receptors: HER1 (also known as epidermal growth factor receptor (EGFR)), HER2 (ERBB2), HER3 (ERBB3), and HER4 (ERBB4) (1). Variable homo- and/or heterodimerization among the members of this receptor family activates tyrosine kinase activity and downstream signaling pathways, leading to cell survival, proliferation, and tumor progression (2). The HER2 protein possesses a number of unique characteristics compared with other members of the HER family. For example, HER2 is capable of ligand-independent dimerization and may remain constitutively activated. HER2 is also the preferred co-receptor for HER1 and HER4 (2).

The *HER2* gene is amplified in ~20–25% of mammary carcinoma, and *HER2* amplification is significantly correlated with an invasive phenotype, often associated with poorer clinical outcome (3). Trastuzumab (Herceptin®) is a humanized monoclonal antibody targeting the extracellular domain of HER2 and used for therapeutic purposes. It was approved by The United States Food and Drug Administration as an adjuvant therapy based on clinical trial data that demonstrated a significant prolongation of disease-free survival when trastuzumab was combined with chemotherapy in HER2-positive mammary carcinoma (4). However, despite some clinical success, a significant fraction of HER2-positive mammary carcinoma demonstrates

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de novo or acquired resistance to trastuzumab (3). Altered functions and/or mutations in PI3K/AKT/mTOR downstream signaling have been proposed to generate trastuzumab insensitivity (5). Alternatively, inhibition of HER2 by trastuzumab may also result in the compensatory activation of alternative signaling pathways, such as the IGF-1R and HER3 signaling pathways, which promote resistance to trastuzumab (2). Investigation of the mechanisms of acquired resistance to trastuzumab has identified a further complex interaction among various molecules including PI3K/AKT, PTEN, IGF-1R, MET, and VEGF among others (3, 6, 7). Hence, it is desirable to further determine the detailed and varied molecular mechanisms of acquired resistance to trastuzumab in mammary carcinoma.

Artemin (ARTN)⁴ is one member of the glial cell line-derived neurotrophic factor (GDNF) family of ligands, which includes three other members, namely GDNF, neurturin, and persephin. ARTN signals through a multicomponent receptor complex by forming a heterodimer with either GFR α 1 or GFR α 3, which subsequently activates RET receptor tyrosine kinase (8). ARTN also mediates signaling through other receptor systems such as syndecan-3 (9, 10). ARTN is expressed in a range of solid tumors and proposed to exert an oncogenic function including tumor growth, metastasis (11), and *de novo* angiogenesis (12). ARTN expression in mammary carcinoma is significantly associated with residual disease after chemotherapy, relapse, and death (13). In addition, ARTN expression in endometrial carcinoma is significantly associated with higher tumor grade and invasiveness (14). ARTN expression decreased sensitivity to paclitaxel in mammary carcinoma (15), both paclitaxel and doxorubicin in endometrial carcinoma (14), and decreased sensitivity to ionizing radiation in mammary carcinoma (15). Furthermore, ARTN also functions to mediate acquired resistance to chemotherapeutics and ionizing radiation in mammary carcinoma by enhancing the cancer stem cell-like (CSC) population (15). In addition, ARTN is an estrogen-inducible gene and mediates acquired antiestrogen (tamoxifen) resistance in mammary carcinoma. Depletion of ARTN partially restores tamoxifen sensitivity in ER⁺ tamoxifen-resistant cells (16). Depletion of ARTN also reverses acquired chemo- and/or radio-resistance via depletion of CSC population in ER-mammary carcinoma (15).

Acquired resistance to trastuzumab has been reported to be associated with increased CSC-like behavior in mammary carcinoma (3) and proposed to be responsible for disease relapse (5, 17, 18). HER2-overexpressing cell lines possess a higher level of BCL-2 expression (19), and increased BCL-2 expression has been reported to contribute to the development of trastuzumab resistance (20). Previously, ARTN has also been demonstrated to regulate BCL-2 in mammary carcinoma (15, 16) and enhance the CSC population in a BCL-2-dependent manner. Independently, CSCs have also been reported to utilize BCL-2 for survival (21). ARTN expression was reported to be significantly correlated with HER2/neu positivity in a cohort of mammary carcinoma patients (13). Hence, ARTN may be a potential intermediary in the link between acquired resistance to trastuzumab and disease relapse in mammary carcinoma.

We herein demonstrate that ARTN is HER2-regulated in HER2-positive mammary carcinoma cells. We show that forced expression of ARTN decreased trastuzumab sensitivity and that ARTN mediated acquired resistance to trastuzumab in HER2-positive mammary carcinoma cells through regulation of CSC-like behavior. Depletion of ARTN restored trastuzumab sensitivity in cells with acquired resistance to trastuzumab. Inhibitors to ARTN may therefore be considered as potential adjuvant therapeutic candidates to enhance trastuzumab efficacy in HER2-positive mammary carcinoma.

EXPERIMENTAL PROCEDURES

Cell Culture—Cell lines used in this study were obtained from the ATCC (American Type Culture Collection) and cultured as recommended. To generate BT474 and SKBR3 cells with forced expression of ARTN, respective cells were stably transfected with pIRESneo3-ARTN plasmid (13). pIRESneo3 plasmid was used to construct respective Vec control cells (13). To generate BT474 and SKBR3 cells with depleted expression of ARTN, the respective cells were stably transfected with pSilencer-ARTN plasmid (13). pSilencer-CK plasmid was used to construct respective siRNA control cells (CK-siRNA) (13).

Generation of BT474 and SKBR3 trastuzumab-resistant cells (HR pools) has been described previously (22). In brief, the cells were treated continuously with trastuzumab (5 μ g/ml in pool 1 and 10 μ g/ml in pool 2) until we observed significant trastuzumab resistance of trastuzumab-treated BT474 and SKBR3 cells by assessing cell viability after trastuzumab treatment for 6 days.

Reagents—Trastuzumab (Herceptin) was a gift from the pharmacy of National University Hospital (NUH), Singapore. Heregulin was purchased from Calbiochem. Epidermal growth factor (EGF), basic FGF, bovine insulin, and B27 were purchased from Invitrogen. The ALDEFLUOR assay kit was purchased from Stem Cell Technologies (Melbourne, Australia).

Quantitative PCR—qPCR was performed as described earlier (11). ARTN primers used for qPCR were: forward, 5'-ATG-AACACTACAGTGGCTGAGG-3' and reverse, 5'-AGCTCC-CATGAGTGTGACTACAGG-3'. BCL-2 primers used for qPCR were: forward, 5'-CGCCCTGTGGATGACTGAGT-3' and reverse, 5'-GGGCCAAACTGAGCAGAGTC-3'.

Immunoblotting—Western blot analysis was performed as described earlier (23). Western blot analysis was performed using the following antibodies: goat ARTN polyclonal antibody (R&D Systems), rabbit HER2 polyclonal antibody (Santa Cruz Biotechnology sc-284), rabbit p-HER2 polyclonal antibody (Santa Cruz Biotechnology, sc-12352-R), mouse BCL-2 monoclonal antibody (Santa Cruz Biotechnology sc-509), and mouse β -actin monoclonal antibody (Sigma).

Cell Function Assays—MTT assay, Soft agar colony formation assay, and three-dimensional cell growth assays were performed as described previously (13). Cell Counting Kit-8 (Dojindo, Japan) was used to quantify the three-dimensional Matrigel growth. The heterotypic cell adhesion assay was performed as described previously (11). The ALDEFLUOR assay

⁴ The abbreviations used are: ARTN, artemin; CSC, cancer stem cell; ER, estrogen receptor; GDNF, glial cell line-derived neurotrophic factor; HRG, heregulin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; qPCR, quantitative real-time PCR.

and mammosphere assays were performed as described previously (15).

Tumor Xenograft in Nude Mice—All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Ethics Committee of The University of Science and Technology of China. A 4×10^6 sample of viable BT474-VEC and BT474-ARTN cells was injected subcutaneously into the flanks of 4-week-old female BALB/c nude mice (Shanghai Slaccas Co, Shanghai, China). Fifteen days after cell inoculation, mice were randomized to receive trastuzumab (10 mg/kg) or vehicle as control. Sizes of the tumors were measured every 4 days. Histological analysis with hematoxylin and eosin (H&E) and terminal transferase deoxyuridine triphosphate nick end labeling (TUNEL) immunostaining were performed as described previously (24). Immunohistochemical analysis of paraffin-embedded specimens of tumors formed in mice was performed by using an UltraSensitive-SP kit (Maxin-Bio, Fuzhou, China) with a mouse polyclonal antibody against Ki67 (Zhongshan Goldenbridge Biotechnology Co., Beijing, China).

RNA Analysis—RNA was extracted by the TRIzol Plus RNA Purification system from 52 mammary carcinoma patients who underwent surgery at the First Affiliated Hospital of Anhui Medical University (Hefei, P. R. China) between 2009 and 2010. The histopathological diagnosis of the specimens was consistent with breast neoplasm and was in accordance with World Health Organization guidelines. The current protocol for use of tissue samples was approved by the Institutional Review Board, and written informed consent was obtained from each patient. SYBR Premix Ex Taq Kit (Takara) was used to determine the expression levels of *ARTN*, *BCL-2*, and *HER2*. The relative amount of gene transcripts was normalized to *GAPDH*.

Statistics—All numerical data are expressed as mean \pm S.D. Data were analyzed using an unpaired two-tailed *t* test or analysis of variance by GraphPad Prism 5. $p < 0.05$ was considered as statistically significant.

RESULTS

ARTN Expression Is Increased by Activation of HER2 with EGF and Heregulin—ARTN expression was previously reported to be positively correlated with HER2 expression and indicative of survival outcomes in a cohort of mammary carcinoma patients (13). To further determine a potential association between *ARTN* and *HER2* mRNA expression in mammary carcinoma, we performed qPCR on a cohort of 52 mammary carcinoma patients to compare the relative expression of *ARTN* mRNA and *HER2* mRNA. We divided the cohort into two groups according to the expression of *HER2* (low and high expression). We observed that in the *HER2* high group, *ARTN* expression was also significantly higher (Fig. 1A). Hence, *HER2* mRNA expression in mammary carcinoma is significantly correlated with *ARTN* mRNA expression.

We next examined for regulation of ARTN by EGF or heregulin (HRG); ligands that increase HER2 activity in mammary carcinoma. EGF binds directly to EGFR which recognizes HER2 as co-receptor; all isoforms of HRG bind to HER3 and HER4 and recognize EGFR and HER2 as co-receptors (25, 26). To validate herein that EGF and HRG increase activation of HER2, wild-type HER2⁺ BT474 and SKBR3 cells were exposed

to either EGF or HRG. Western blotting results demonstrated an increased phosphorylation of HER2 in both BT474 and SKBR3 cells upon EGF or HRG treatment (Fig. 1B). Treatment of BT474 or SKBR3 cells with EGF produced a dose-dependent graded increase of *ARTN* mRNA expression (Fig. 1C). EGF at 20 ng/ml produced the highest *ARTN* mRNA expression in both cell lines. Treatment of BT474 and SKBR3 cells with EGF (20 ng/ml) produced a time-dependent graded increase of *ARTN* mRNA expression (Fig. 1D). Seventy-two hours of EGF treatment produced the highest *ARTN* mRNA expression in both cell lines. As above, treatment with HRG in a dose-dependent manner produced a concordant graded increase in *ARTN* expression in both BT474 and SKBR3 cells (Fig. 1E). HRG at 100 ng/ml (BT474) and 50 ng/ml (SKBR3), respectively, resulted in the highest *ARTN* mRNA expression. Treatment of BT474 or SKBR3 cells with HRG (50 ng/ml) produced a time-dependent graded increase of *ARTN* mRNA expression (Fig. 1F). Seventy-two hours of HRG treatment produced the highest *ARTN* mRNA expression in both cell lines. Concordant with the mRNA expression, protein levels of ARTN were also increased upon treatment with either EGF or HRG (Fig. 1G) in BT474 and SKBR3 cells, respectively. Hence, expression of ARTN is increased by ligand-dependent activation of HER2 in mammary carcinoma.

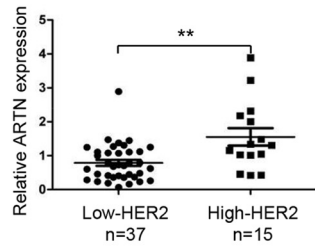
ARTN Expression Is Decreased by Inhibition of HER2 with Trastuzumab—To demonstrate more directly that HER2 regulated ARTN expression, we utilized the humanized monoclonal antibody trastuzumab, which specifically inhibits HER2 (2). Inhibition of HER2 by trastuzumab resulted in decreased *ARTN* mRNA expression in both serum-replete and serum-free media in BT474 cells and in serum-free media in SKBR3 cells (Fig. 2A). Similarly, Western blotting data also demonstrated a significant decrease in ARTN protein expression after trastuzumab treatment in both BT474 and SKBR3 cells (Fig. 2B).

Forced Expression of ARTN Reduced the Sensitivity of Mammary Carcinoma Cells to Trastuzumab in Vitro—Trastuzumab is used clinically to improve disease prognosis in HER2⁺ metastatic mammary carcinoma (27, 28). To determine the functional interactions of ARTN in response to trastuzumab treatment in mammary carcinoma, we first generated stable cell clones with forced expression of ARTN in BT474 and in SKBR3 cells (Fig. 3, A and B). Forced expression of ARTN significantly decreased the relative sensitivity to trastuzumab as evidenced from monolayer proliferation, soft agar colony formation, and three-dimensional Matrigel growth of BT474 and SKBR3 cells (Fig. 3). Treatment with trastuzumab reduced the basal capacity for monolayer proliferation, colony formation in soft agar, and three-dimensional Matrigel growth of BT474-Vec and SKBR3-Vec cells, whereas trastuzumab treatment of BT474 and SKBR3 cells with forced expression of ARTN exerted less significant inhibitory effects on colony formation in soft agar and three-dimensional Matrigel growth (Fig. 3). Hence, ARTN decreased the sensitivity of mammary carcinoma cells to trastuzumab *in vitro*.

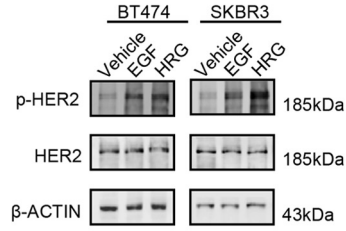
Depletion of Endogenous ARTN Increased the Sensitivity of Mammary Carcinoma Cells to Trastuzumab in Vitro—Next, we depleted endogenous ARTN from BT474 and SKBR3 cells with small interfering RNA (Fig. 3, A and B) and examined the

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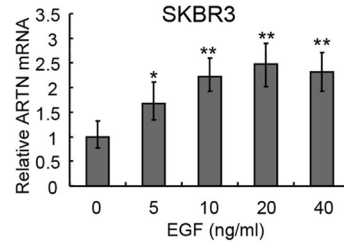
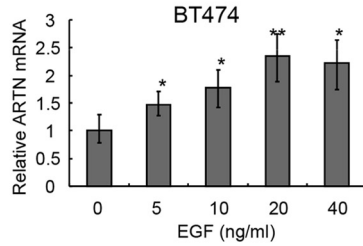
A mRNA correlation



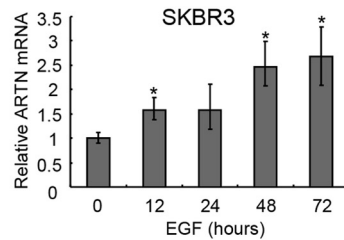
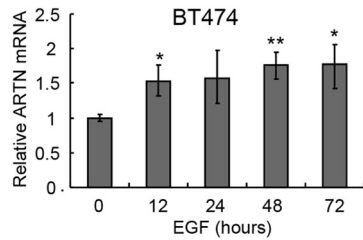
B Western blot



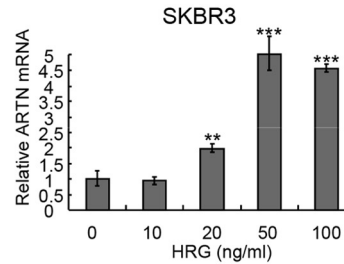
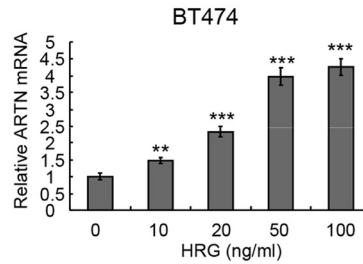
C qPCR



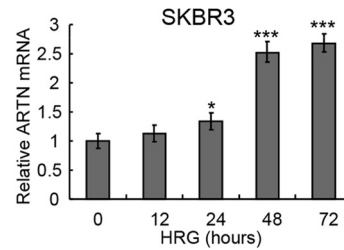
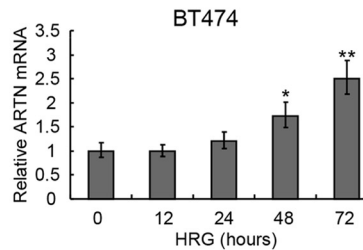
D qPCR



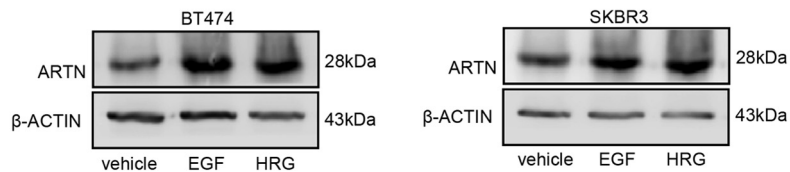
E qPCR



F qPCR



G Western blot



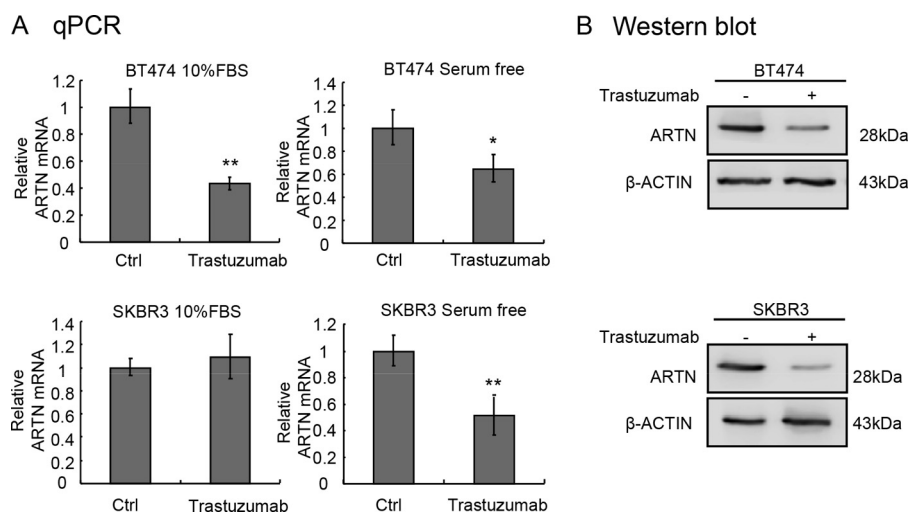


FIGURE 2. Inhibition of HER2 with trastuzumab down-regulates ARTN mRNA and protein expression in mammary carcinoma cells. *A*, qPCR. BT474 and SKBR3 cells were treated with trastuzumab (100 μ g/ml) for 72 h either in serum-free or full serum containing media, and ARTN mRNA expression level was measured. *B*, Western blot analysis. BT474 and SKBR3 cells were treated with trastuzumab (100 μ g/ml) for 72 h in serum-free medium, and ARTN protein expression level was measured. β -Actin was used as input control. *, $p < 0.05$; **, $p < 0.01$. Error bars, S.D.

effects of depletion of ARTN on cell behavior in response to trastuzumab treatment. Depleted expression of ARTN significantly increased sensitivity to trastuzumab as evidenced from monolayer proliferation, soft agar colony formation, and three-dimensional Matrigel growth of BT474 and SKBR3 cells (Fig. 4). Treatment with trastuzumab reduced the basal capacity for monolayer proliferation, colony formation in soft agar, and three-dimensional Matrigel growth of BT474-Vec and SKBR3-Vec cells, whereas trastuzumab treatment of BT474 and SKBR3 cells with depleted expression of ARTN exerted greater relative inhibitory effects on monolayer proliferation, colony formation in soft agar, and three-dimensional Matrigel growth (Fig. 4). Hence, the level of ARTN expression modulates trastuzumab sensitivity in mammary carcinoma cells.

Forced Expression of ARTN Reduces Trastuzumab Sensitivity of Mammary Carcinoma Cells *in Vivo*—We next injected BT474-Vec or BT474-ARTN cells subcutaneously into the flank of female nude mice. Two weeks after injection, both cell lines formed palpable and measurable tumors (~ 100 mm³) in the respective group of mice and were randomized to receive either trastuzumab or vehicle as control. At 6 weeks, the tumors formed by BT474-ARTN cells were larger than those formed by control BT474-Vec cells (Fig. 5A). Treatment with trastuzumab decreased tumor growth in mice with tumors formed by BT474-Vec cells. In contrast, forced expression of ARTN reduced the relative efficacy of trastuzumab, producing a tumor growth rate comparable with that observed in BT474-Vec vehicle-treated control. Histological examination revealed distinct differences in the appearances of the BT474-Vec- and BT474-

ARTN-derived tumor group, both with and without trastuzumab treatment (Fig. 5B). The BT474-ARTN-derived tumors were predominantly disorganized in structure and infiltrating in nature compared with the BT474-Vec-derived tumor. In addition, trastuzumab treatment had minimal effects on tumor histology in mice with tumors formed by BT474-ARTN compared with the mice with tumors formed by BT549-Vec cells (Fig. 5B). Ki67 and TUNEL assays were performed on the respective tumors sections to quantify proliferation and apoptosis within the tumor at the time of termination of the experiment. As reported similarly for non-HER2 cell lines (13), forced expression of ARTN in BT474 cells generated tumors with increased proliferation and decreased apoptosis compared with the BT474-Vec cells. Trastuzumab reduced Ki67 positivity and increased apoptosis in tumors derived from BT474-Vec cells (Fig. 5, C and D). However, BT474-ARTN-derived tumors treated with trastuzumab maintained cell proliferation and survival rates comparative with vehicle-treated tumors derived from BT474-Vec cells (Fig. 5, C and D). Hence, ARTN reduced the relative sensitivity of mammary carcinoma cells to trastuzumab *in vivo*.

ARTN Mediates Acquired Resistance to Trastuzumab in Mammary Carcinoma Cells—To determine whether ARTN possesses a functional role in acquired resistance to trastuzumab, we developed two pools of trastuzumab-resistant mammary carcinoma cells from both BT474 and SKBR3 cells (pool 1, 5 μ g/ml; and pool 2, 10 μ g/ml) (as described under “Experimental Procedures”). Cell viability assays were performed to confirm the resistance to trastuzumab of these two resistant

FIGURE 1. Activation of HER2 with EGF and HRG up-regulates ARTN mRNA and protein expression in mammary carcinoma cells. *A*, mRNA correlation. HER2 and ARTN mRNA expression was determined by qPCR in a cohort of 52 patients with mammary carcinoma. HER2 expression was divided into two groups (low and high) according to the level of expression. *B*, Western blot analysis. BT474 and SKBR3 cells were treated with either EGF (20 ng/ml) or HRG (50 ng/ml) for 15 min, and HER2, p-HER2 protein expression level was measured. β -Actin was used as input control. *C*, qPCR. BT474 and SKBR3 cells were treated with the indicated concentrations of EGF for 48 h, and ARTN mRNA expression level was measured. *D*, qPCR. BT474 and SKBR3 cells were treated with EGF (20 ng/ml), and ARTN mRNA expression level was measured at the indicated time points. *E*, qPCR. BT474 and SKBR3 cells were treated with the indicated concentrations of HRG for 72 h, and ARTN mRNA expression level was measured. *F*, qPCR. BT474 and SKBR3 cells were treated with HRG (50 ng/ml), and ARTN mRNA expression level was measured at the indicated time points. *G*, Western blot analysis. BT474 and SKBR3 cells were treated with either EGF (20 ng/ml) for 48 h or HRG (50 ng/ml), for 72 h and ARTN protein expression level was measured. β -Actin was used as input control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to zero for each experiment. Error bars, S.D.

ARTN Mediates Acquired Resistance to Trastuzumab

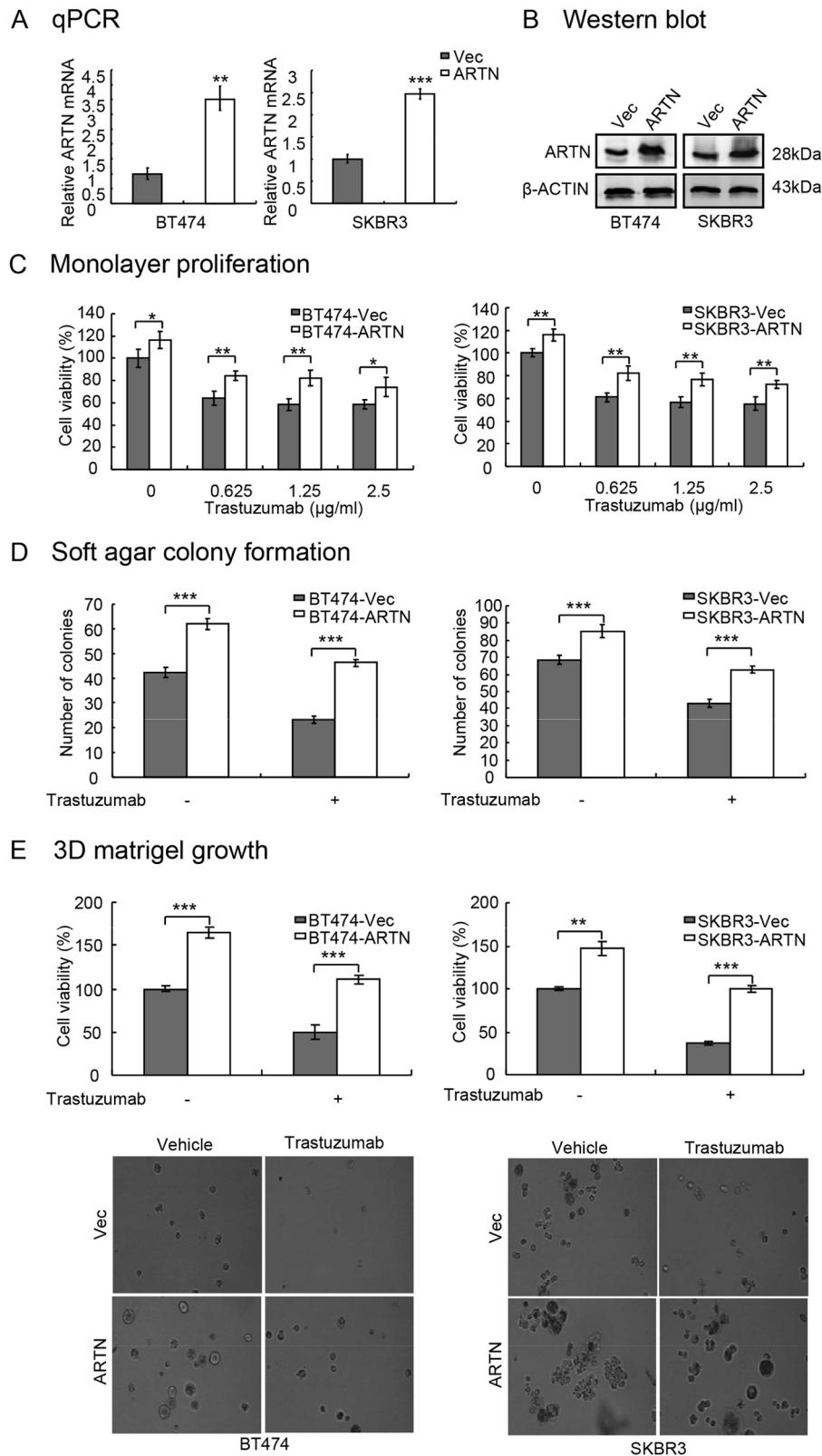


FIGURE 3. Forced expression of ARTN decreases the sensitivity of mammary carcinoma cells to trastuzumab. Generation of stable forced expression of ARTN in BT474 and SKBR3 cells. BT474 and SKBR3 cells were stably transfected with forced expression of ARTN plasmids. Empty vector was used as control. *ARTN* (A) mRNA expression and protein expression (B) were measured. β -Actin was used as input control. C, cell viability assay. BT474 and SKBR3 cells with forced expression of ARTN were treated with the indicated concentrations of trastuzumab for 6 days in the presence of full serum-containing media, and cell viability was calculated by MTT assay. D, soft agar colony formation. Both BT474 and SKBR3 cells with forced expression of ARTN were cultured in 0.35% agar in full media and treated with 1.25 μ g/ml trastuzumab. After 2 weeks colony numbers were counted under a microscope. E, three-dimensional Matrigel growth. Both BT474 and SKBR3 cells with forced expression of ARTN were cultured in full media containing 4% Matrigel and treated with 1.25 μ g/ml trastuzumab. Cell growth was detected using Cell Counting Kit-8. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Error bars, S.D.

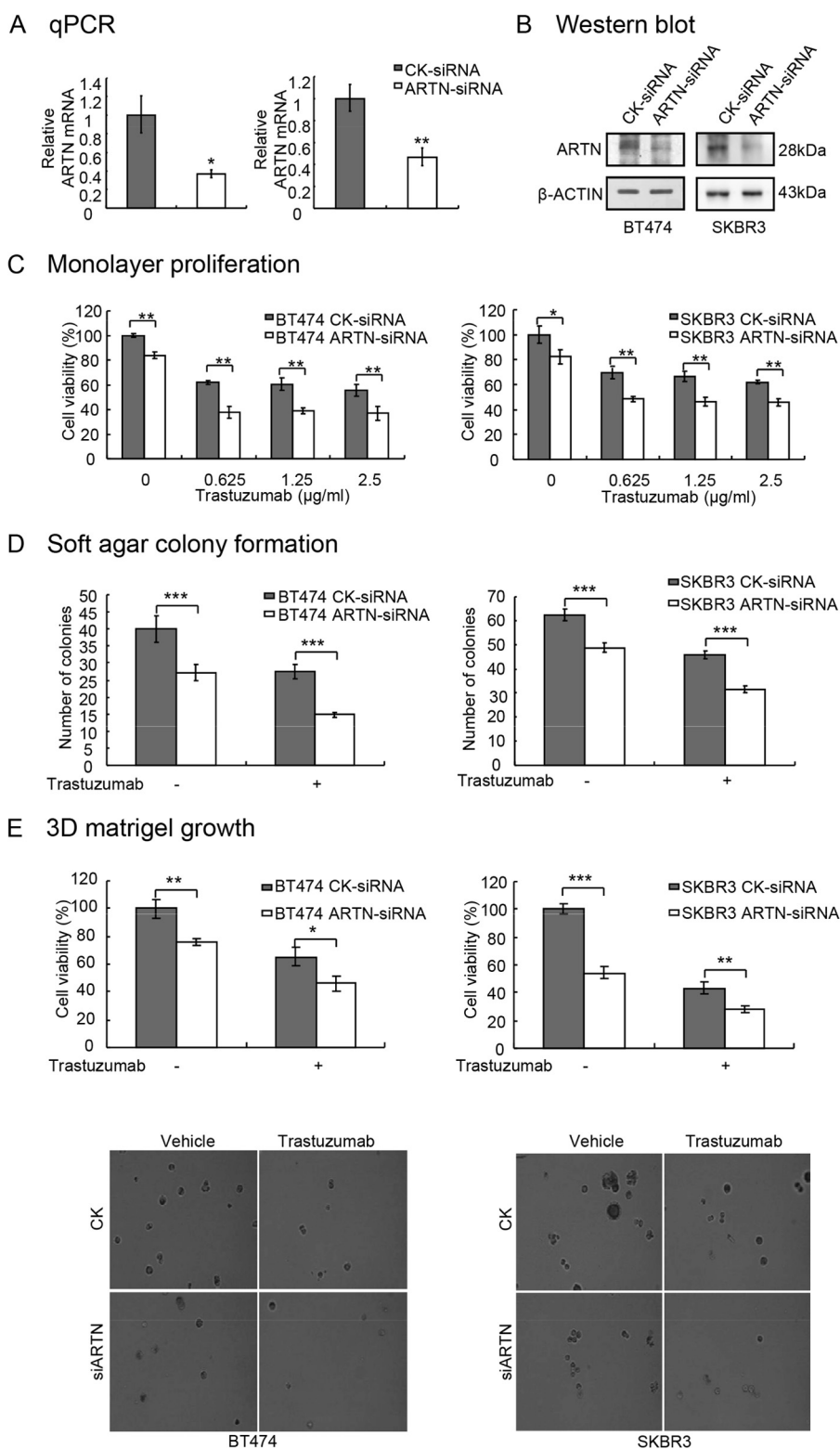


FIGURE 4. Depleted expression of ARTN increases the sensitivity of mammary carcinoma cells to trastuzumab. Generation of stable depleted expression of ARTN in BT474 and SKBR3 cells is shown. BT474 and SKBR3 cells were stably transfected with depleted expression of ARTN plasmids. Empty vector was used as control. *A* and *B*, ARTN mRNA expression (*A*) and protein expression (*B*) were measured. β -Actin was used as input control. *C*, cell viability assay. BT474 and SKBR3 cells with depleted expression of ARTN were treated with the indicated concentrations of trastuzumab for 6 days in the presence of full serum-containing media, and cell viability was calculated by MTT assay. *D*, soft agar colony formation. Both BT474 and SKBR3 cells with depleted expression of ARTN were cultured in 0.35% agar in full media and treated with 1.25 μ g/ml trastuzumab. After 2 weeks colony numbers were counted under the microscope. *E*, three-dimensional Matrigel growth. Both BT474 and SKBR3 cells with depleted expression of ARTN were cultured in full media containing 4% Matrigel and treated with 1.25 μ g/ml trastuzumab. Cell growth was detected using Cell Counting Kit-8. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Error bars, S.D.

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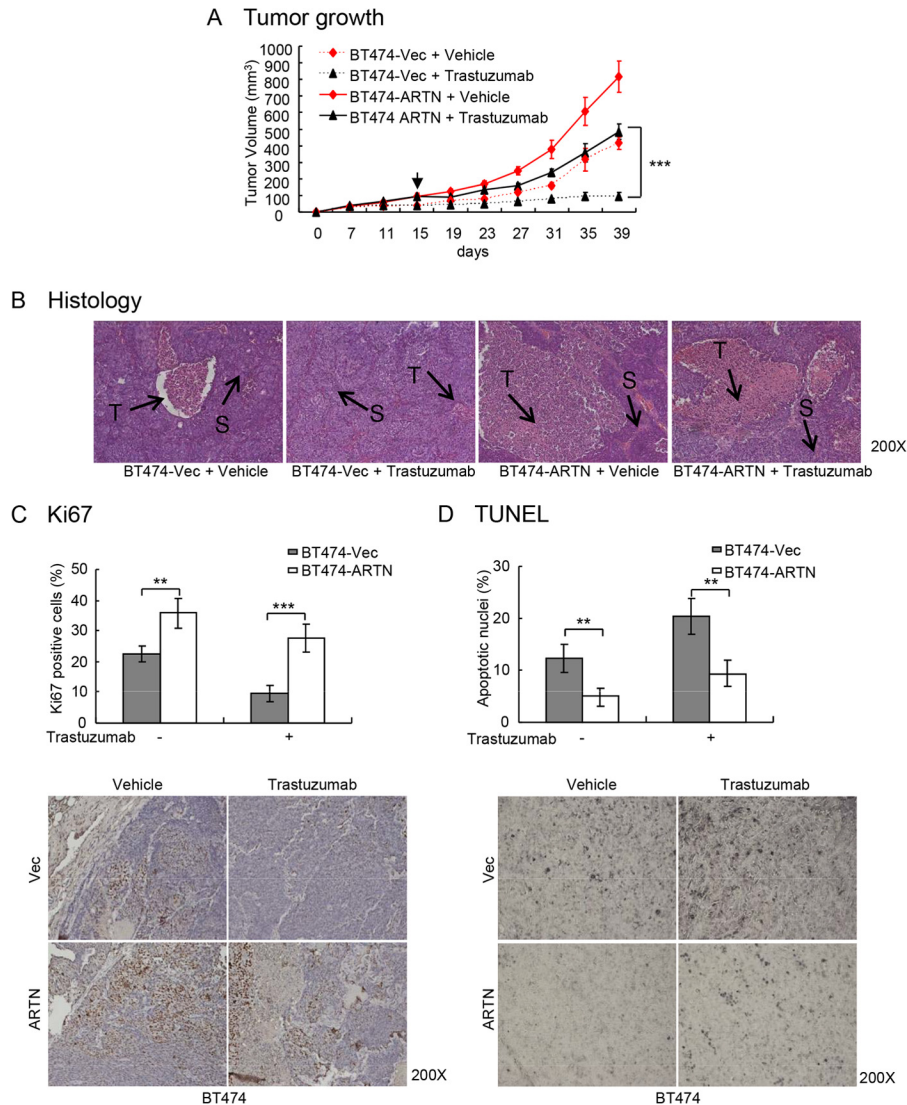


FIGURE 5. Forced expression of ARTN decreases the sensitivity of mammary carcinoma cells to trastuzumab *in vivo*. *A*, xenograft growth of BT474 cells with forced expression of ARTN in immunodeficient mice. BT474-VEC and BT474-ARTN cells were injected subcutaneously into nude mice. After 15 days, the mice were randomized to receive trastuzumab (10 mg/kg) or vehicle as control. At 39 days, tumors were harvested. *B*, H&E staining of representative tumors from mice that received BT474 (VEC or ARTN) cells \pm trastuzumab injected subcutaneously. *T*, tumor; *S*, stroma. Magnification, $\times 200$. *C*, Ki67 staining of tumor sections derived from mice that received BT474 (Vec and ARTN) cells \pm trastuzumab injected subcutaneously. *D*, TUNEL nuclei staining of tumor sections derived from mice that received BT474 (Vec and ARTN) cells \pm trastuzumab injected subcutaneously. **, $p < 0.01$; ***, $p < 0.001$. Error bars, S.D.

pools of BT474 and SKBR3 cells, respectively (Fig. 6A). We subsequently compared ARTN expression in both parental and resistant cell populations. mRNA expression of *ARTN* was increased in both pool 1 and pool 2 of trastuzumab-resistant BT474 cells and in pool 2-derived trastuzumab-resistant SKBR3 cells (Fig. 6B). However, we observed increased ARTN protein expression in both pools of both BT474 and SKBR3 trastuzumab-resistant cells (Fig. 6C). It has been reported previously that the mechanisms contributing to acquired resistance to trastuzumab are heterogeneous (3), and even the trastuzumab doses at which resistance is acquired can produce different cell phenotypes and transcriptome profiles (29). We chose to use pool 2 from each of the cell lines because pool 2 from each of the lines consistently exhibited increased mRNA and protein for ARTN.

We therefore next depleted endogenous ARTN by siRNA in both trastuzumab-resistant (pool 2 only) and -sensitive cells of

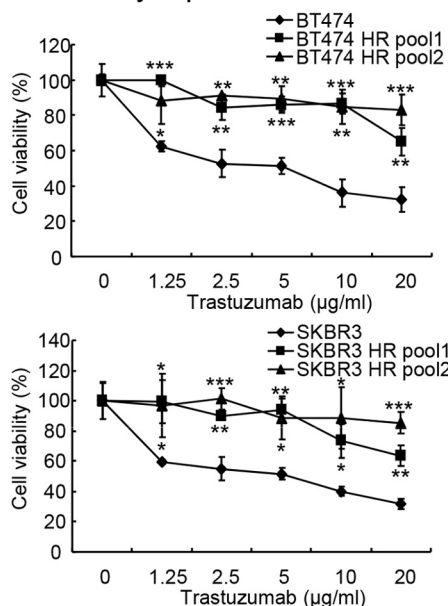
BT474 and SKBR3, respectively (Fig. 6, D and E). Depletion of ARTN by siRNA significantly improved trastuzumab sensitivity assayed by growth in three-dimensional Matrigel growth, in both BT474 and SKBR3 trastuzumab-resistant cells compared with their respective control cells (Fig. 6F). Depletion of ARTN by siRNA completely restored the response to trastuzumab in trastuzumab-resistant cells (Fig. 6F). Hence, ARTN is functionally involved in acquired trastuzumab resistance.

ARTN Modulates the CSC-like Population in Trastuzumab-resistant Mammary Carcinoma Cells—The pivotal role of enhanced CSC-like cell behavior in acquired resistance to trastuzumab has been reported previously (30). Increased expression of HER2 and ARTN has also been observed in the mammospheric cell population of mammary carcinoma (15, 18). To determine the role of ARTN in the CSC-like behavior of trastuzumab-resistant mammary carcinoma cells, ARTN was depleted by siRNA in BT474 and SKBR3 trastuzumab-resistant

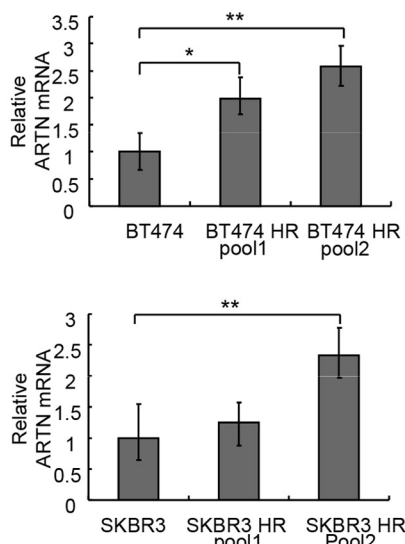
cells and the cells cultured under ultra low attachment conditions. Both BT474 and SKBR3 trastuzumab-resistant cells exhibited increased growth of mammospheres compared with

the respective parental cells (Fig. 7A), consistent with previous literature (29). Conversely, depletion of ARTN by siRNA significantly decreased mammospheric growth in both BT474 and

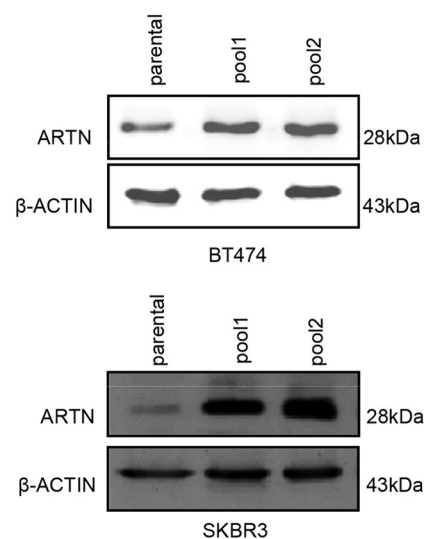
A Monolayer proliferation



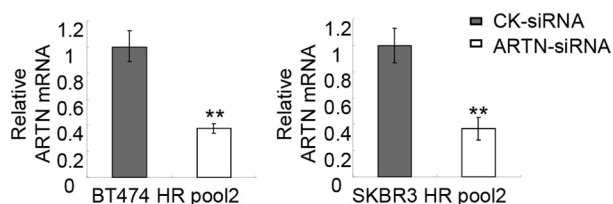
B qPCR



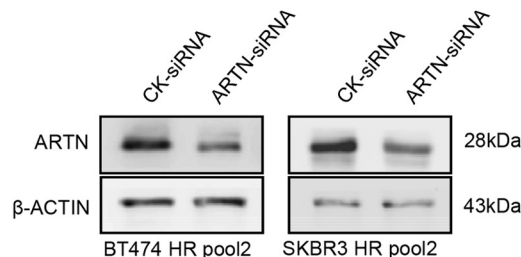
C Western blot



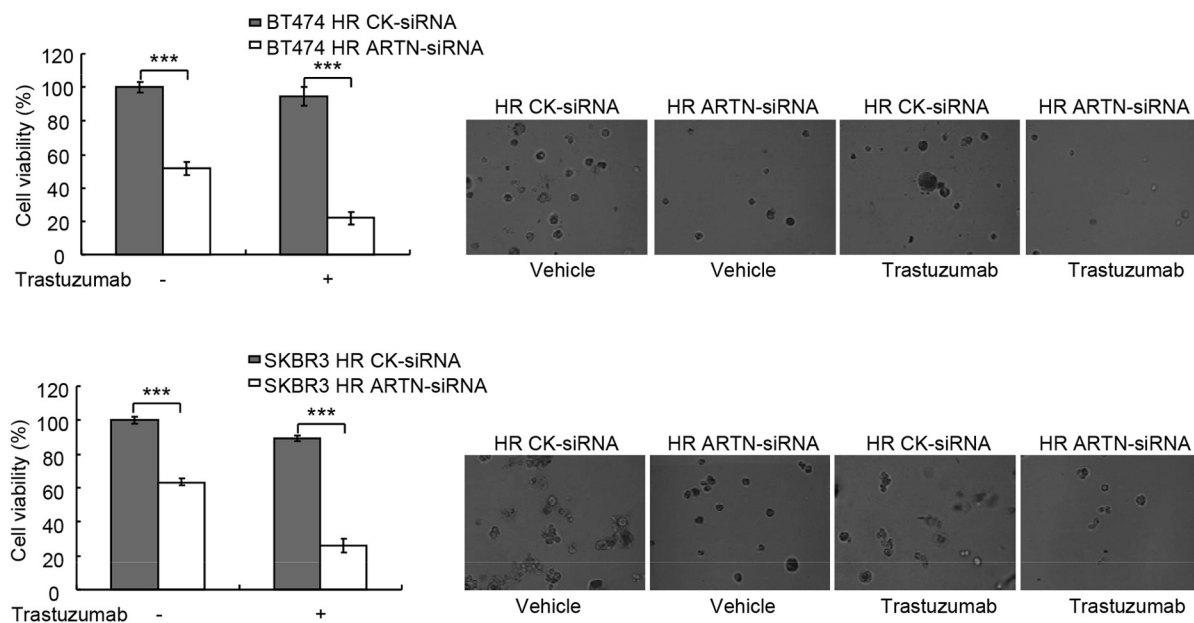
D qPCR



E Western blot



F 3D matrigel growth



ARTN Mediates Acquired Resistance to Trastuzumab

SKBR3 parental cells (Fig. 7A). Furthermore, after depletion of ARTN by siRNA, trastuzumab-resistant cells also exhibited decreased mammospheric growth, indicative that ARTN may potentially be involved in promoting CSC-like behavior in trastuzumab-resistant cells.

ALDH1 positivity in mammary carcinoma cells has been reported to identify CSC-like cells (31). Furthermore, increased expression of HER2 or ARTN has been reported to increase the ALDH1⁺ cell population in mammary carcinoma cells (15, 18). To determine whether ARTN mediated the increase in the ALDH1⁺ cell population in trastuzumab-resistant BT474 and SKBR3 cells, we performed the ALDEFUOR assay in both BT474 and SKBR3 Hereceptin-resistant cells \pm siARTN. In accordance with the previous report (18), trastuzumab-resistant BT474 and SKBR3 cells resulted an increased ALDH1⁺ cell population compared with their respective parental cells (Fig. 7B). Depletion of ARTN by siRNA decreased the basal ALDH1⁺ cell population in both BT474 and SKBR3 parental cells. Furthermore, depletion of ARTN by siRNA in BT474 and SKBR3 trastuzumab-resistant cells completely abrogated the increased ALDH1⁺ cell population due to trastuzumab resistance (Fig. 7B). Hence, ARTN mediates the increase in the ALDH1⁺ cell population conferred by trastuzumab resistance.

The ARTN-dependent Increase in the CSC-like Population in Trastuzumab-resistant Mammary Carcinoma Cells Requires BCL-2—Previous reports have identified a functional role of BCL-2 in acquired resistance to trastuzumab in mammary carcinoma (20). BCL-2 is also a direct target for ARTN and mediates ARTN reduction of tamoxifen sensitivity in ER⁺ mammary carcinoma cells (16). Furthermore, BCL-2 has been demonstrated to possess a functional role in the CSC-like population in mammary carcinoma (15, 21) and including ARTN promotion of CSC-like behavior. To determine a potential association between BCL-2 and ARTN mRNA expression in both BT474 parental and trastuzumab-resistant cells, we performed qPCR analysis. Trastuzumab-resistant cells exhibited increased BCL-2 mRNA expression (Fig. 8A). siRNA-mediated depletion of ARTN in both parental and trastuzumab-resistant BT474 cells significantly decreased BCL-2 mRNA expression (Fig. 8A). Furthermore, BT474 parental and trastuzumab-resistant cells were grown in mammosphere culture in ultra low attachment plates. Mammospheric cells are enriched in mammary stem-like cells, capable of self-renewal and dedifferentiation (32). Mammospheres were isolated, and mRNA expression of ARTN and BCL-2 was compared between BT474 parental and the trastuzumab-resistant cells grown in either monolayer or mammospheric culture. Consistent with the previously published data (15), both ARTN and BCL-2 mRNA expression was significantly increased in the respective CSC-rich mammo-

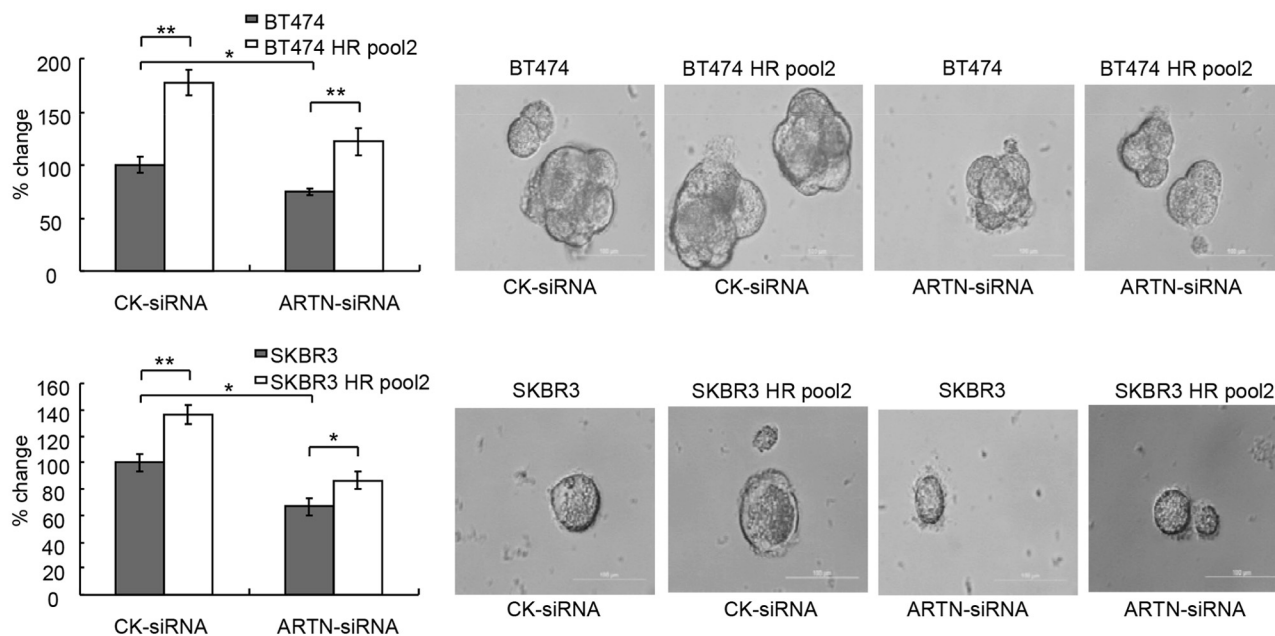
spheric cell population compared with their respective cells grown in monolayer. Trastuzumab-resistant BT474 cells grown in mammospheric culture exhibited the highest ARTN and BCL-2 mRNA expression (Fig. 8, B and C). Similarly, BT474-ARTN cells grown in mammospheric culture also exhibited the highest BCL-2 mRNA expression compared with the BT474-Vec-derived mammosphere and/or monolayer grown cells (Fig. 8D). Consistent with the qPCR analysis, trastuzumab-resistant BT474 cells demonstrated increased BCL-2 protein expression, and siRNA-mediated depletion of ARTN significantly decreased BCL-2 protein expression (Fig. 8E).

To determine the functional role of BCL-2 in the ARTN-mediated increased CSC-like population in trastuzumab-resistant mammary carcinoma cells, we employed the BCL-2 inhibitor YC137 to functionally inhibit BCL-2 in both parental and trastuzumab-resistant BT474 cells. Functional inhibition of BCL-2 by YC137 significantly inhibited three-dimensional Matrigel growth in the parental cells and the enhanced growth observed in the trastuzumab-resistant BT474 cells (Fig. 8F). In addition, the BCL-2 inhibitor also significantly reduced the ALDH1⁺ cell population in parental cells and completely abrogated the enhanced ALDH1⁺ cell population observed in trastuzumab-resistant BT474 cells (Fig. 8G). Hence, BCL-2 is required for the ARTN-stimulated increase in the CSC-like population in trastuzumab-resistant mammary carcinoma cells.

ARTN Modulates Trastuzumab-resistant Mammary Carcinoma-derived CSC-like Cell Growth in Three-dimensional Matrigel—Previous reports have indicated that the CSC-like cell population is responsible for trastuzumab resistance and is related to disease progression (33). To determine whether ARTN mediates the increased tumorigenicity of BT474 and SKBR3 trastuzumab-resistant cells, we examined the three-dimensional Matrigel growth of mammosphere-derived CSC-like cells from both BT474 and SKBR3 mammospheric cells (which were derived from respective trastuzumab-resistant cells) \pm siARTN. As observed in Fig. 9, BT474 and SKBR3 trastuzumab-resistant CSC like-cells produced significantly more three-dimensional Matrigel colonies compared with the respective parental CSC-like population. Depletion of ARTN by siRNA not only resulted in less growth of three-dimensional Matrigel colonies of BT474 and SKBR3 parental CSC-like population, but also specifically decreased the growth of three-dimensional Matrigel colonies of BT474 and SKBR3 trastuzumab-resistant cells (Fig. 9A). Hence, we conclude that ARTN partially mediates the enhanced three-dimensional Matrigel cell growth of the trastuzumab-resistant cell-derived CSC-like cells.

FIGURE 6. ARTN possesses a functional role in acquired resistance of mammary carcinoma cells to trastuzumab. A, cell viability assay. BT474 and SKBR3 parental cells and two pools of trastuzumab-resistant cells (pool 1, resistant to 5 μ g/ml; and pool 2, resistant to 10 μ g/ml) were treated with the indicated concentrations of trastuzumab for 6 days in the presence of full serum-containing media, and cell viability was calculated by MTT assay. B, qPCR. BT474 and SKBR3 parental cells and two pools of trastuzumab-resistant cells (pool 1 and pool 2) were cultured in full media without trastuzumab for 48 h, and ARTN mRNA expression level was measured. C, Western blot analysis. BT474 and SKBR3 parental cells and two pools of trastuzumab-resistant cells (pool 1 and pool 2) were cultured in full media without trastuzumab for 48 h, and ARTN protein expression level was measured. β -Actin was used as input control. D, qPCR. BT474 HR pool 2 and SKBR3 HR pool 2 cells were transfected with siRNA to ARTN. Empty vector was used as control. ARTN mRNA expression is shown. E, Western blot. Protein expression was measured. β -Actin was used as input control. F, three-dimensional Matrigel growth. Trastuzumab-resistant BT474 and SKBR3 cells (pool 2) \pm siARTN were cultured in full media containing 4% Matrigel and treated with trastuzumab (10 μ g/ml). After 10 days, cell growth was detected using Cell Counting Kit-8. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Error bars, S.D.

A Mammosphere growth



B Aldefluor assay

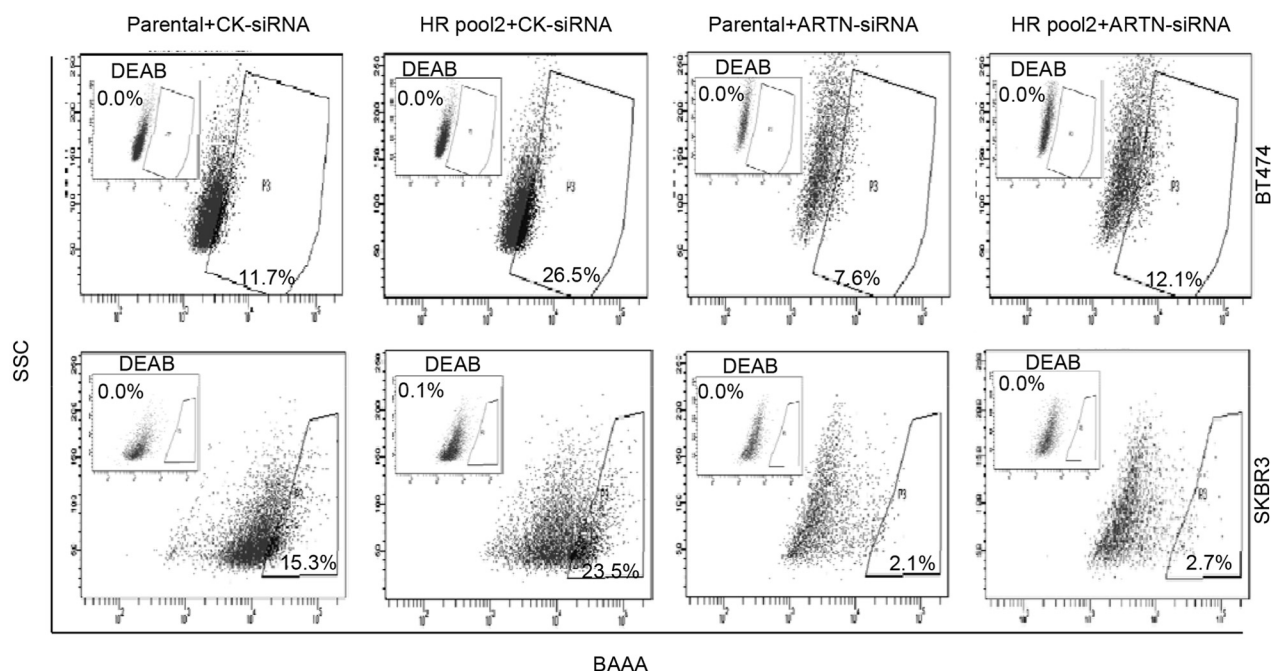


FIGURE 7. Increased ARTN expression mediates the enhanced CSC-like population in trastuzumab-resistant mammary carcinoma cells. A, BT474 and SKBR3 parental and trastuzumab-resistant (pool 2) cells \pm siARTN were seeded in ultra low attachment plates in mammospheric growth media. After 10 days, growth was measured by alamarBlue. Representative images of mammospheres generated by either respective parental or trastuzumab-resistant cells \pm siARTN of BT474 and SKBR3 cells. B, ARTN modulates the ALDH1⁺ cell population in trastuzumab-resistant BT474 and SKBR3 cells. BT474 and SKBR3 parental (wild-type) and trastuzumab-resistant BT474 (pool 2) and SKBR3 (pool 2) cells \pm siARTN were incubated with ALDEFLUOR substrate (BAAA, BODIPY[®]-aminoacetaldehyde) to define the ALDEFLUOR-positive, and a specific inhibitor of ALDH1, diethylaminobenzaldehyde (DEAB), was used to establish the baseline fluorescence. Flow cytometry plots indicate side scatter (SSC) versus fluorescence intensity. Depletion of ARTN by siARTN significantly decreased the ALDH1⁺ cell population in trastuzumab-resistant and WT cells of BT474 and SKBR3 cells, respectively. *, $p < 0.05$; **, $p < 0.01$. Error bars, S.D.

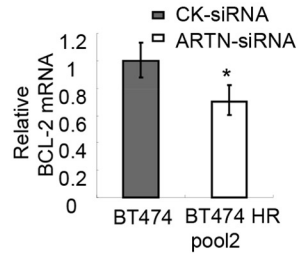
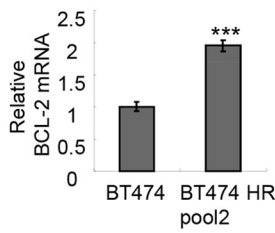
DISCUSSION

Herein, we have demonstrated that increased ARTN expression functionally contributed to acquired resistance to trastuzumab by promotion of the CSC-like cell population and CSC-like behavior in mammary carcinoma cells. Similarly, we have

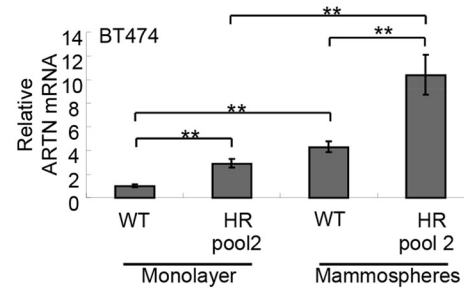
demonstrated previously that increased expression of ARTN is observed in tamoxifen-resistant ER⁺ mammary carcinoma cells and depletion of ARTN by siRNA partially restored tamoxifen sensitivity (16). Furthermore, increased ARTN expression has also been reported to be functionally associated

ARTN Mediates Acquired Resistance to Trastuzumab

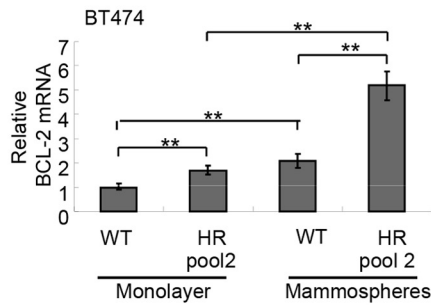
A qPCR



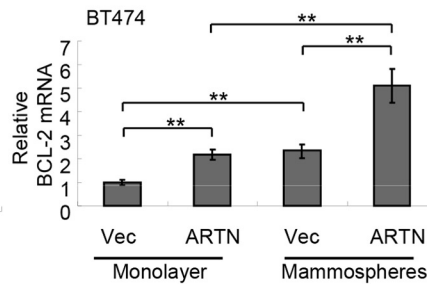
B qPCR



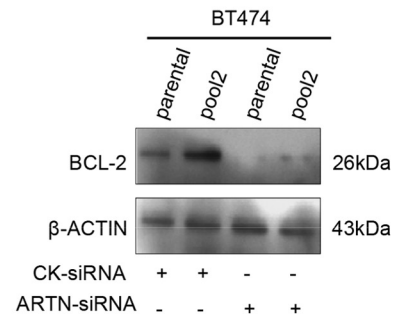
C qPCR



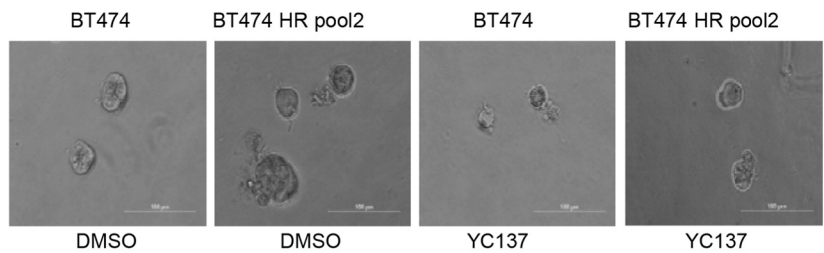
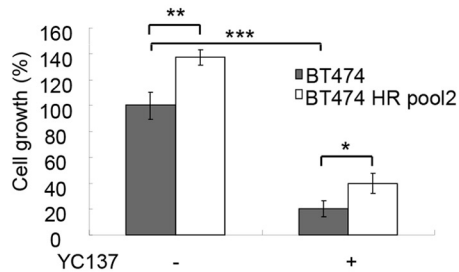
D qPCR



E Western blot



F 3D matrigel growth



G Aldefluor assay

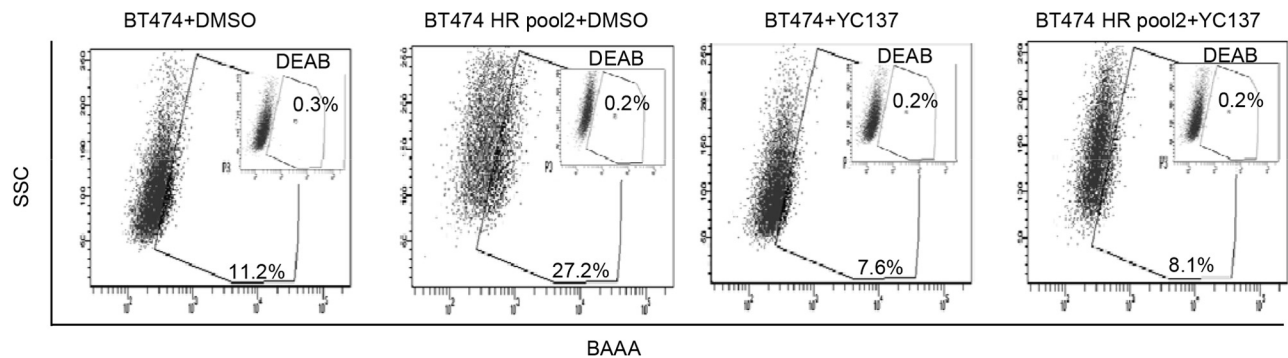


FIGURE 8. ARTN activates BCL-2 to promote a CSC-like phenotype in trastuzumab-resistant BT474 mammary carcinoma cells. *A*, qPCR. Trastuzumab-resistant BT474 (pool 2) cells exhibit increased *BCL-2* mRNA expression compared with the BT474 parental cells. siRNA-mediated depletion of ARTN significantly decreased *BCL-2* mRNA expression in trastuzumab-resistant BT474 cells. *B* and *C*, qPCR analysis for *ARTN* (*B*) and *BCL-2* (*C*) mRNA expression in BT474 parental (wild-type, WT) and trastuzumab-resistant (pool 2) cells grown under monolayer and mammospheric conditions, respectively. *D*, qPCR analysis for *BCL-2* mRNA expression in BT474-Vec and BT474-ARTN cells grown in monolayer and mammospheric conditions, respectively. *E*, Western blot analysis. BT474 parental and trastuzumab-resistant cells (pool 2) \pm siARTN were cultured in full media, and BCL-2 protein expression level was measured. β -Actin was used as input control. *F*, three-dimensional Matrigel growth. Trastuzumab-resistant BT474 cells \pm YC137 (10 μ M) were cultured in full media containing 4% Matrigel. After 10 days, cell growth was detected using alamarBlue. *G*, trastuzumab-resistant BT474 (pool 2) cells \pm YC137 (10 μ M) were incubated with ALDEFLUOR substrate to define the ALDEFLUOR-positive and a specific inhibitor of ALDH1, diethylaminobenzaldehyde (DEAB), was used to establish the baseline fluorescence. Flow cytometry plots indicate side scatter (SSC) versus fluorescence intensity. Functional inhibition of BCL-2 significantly decreased the ALDH1⁺ cell population in trastuzumab-resistant and WT cells of BT474, respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Error bars, S.D.

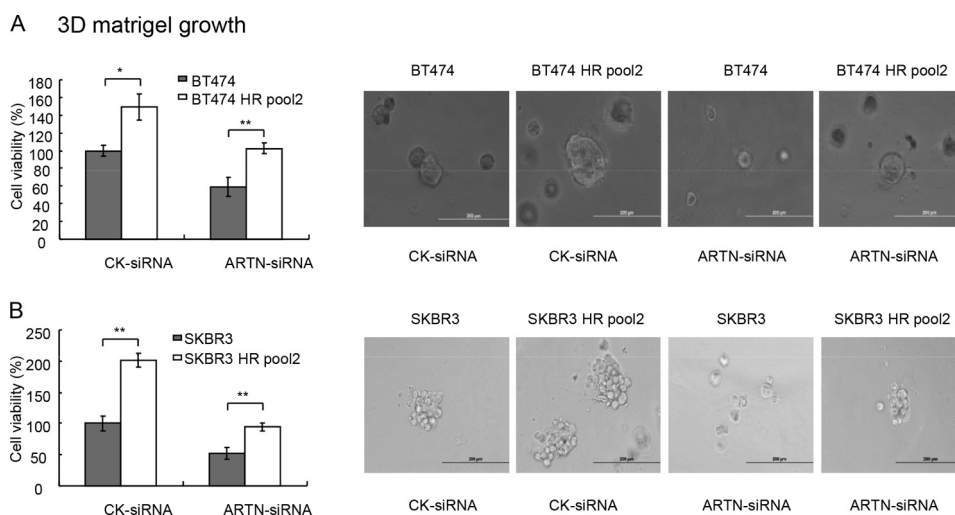


FIGURE 9. **ARTN modulates trastuzumab-resistant CSC-like cell growth in three-dimensional Matrigel.** A, trastuzumab-resistant BT474- (pool 2) and (B) SKBR3- (pool 2) derived mammospheric cells promoted three-dimensional Matrigel growth. Both parental and trastuzumab-resistant (pool 2) of BT474 and SKBR3 cells \pm siARTN- (transient transfection) derived mammospheric cells were cultured in full media containing 4% Matrigel. After 10 days, growth was measured by alamarBlue. *, $p < 0.05$; **, $p < 0.01$. Error bars, S.D.

with acquired resistance of ER⁻ mammary carcinoma cells to paclitaxel and ionizing radiation and associated with ARTN promotion of the CSC phenotype (15). Indeed, various reports have postulated that *de novo* or acquired resistance of mammary carcinoma to therapeutic interventions may be related to the capacity to enhance a CSC-like cell phenotype (2, 17). HER2 expression has been similarly positively correlated with increased chemo- and radiotherapeutic acquired resistance in different carcinoma cell types and associated with an increased CSC-like cell phenotype (34, 35). Both ARTN and HER2 have been previously demonstrated to increase the ALDH1⁺ cell population (15, 18). ALDH1 positivity in mammary carcinoma is considered to be a marker of mammary CSC-like cells (31). In another recent report, HER2 has been observed to promote luminal-type mammary CSC in absence of *HER2* amplification (36). Furthermore, epithelial to mesenchymal transition of mammary carcinoma cells is associated with a CSC-like phenotype and is also associated with primary resistance to trastuzumab in mammary carcinoma (37). HER2 activation has been reported to promote epithelial to mesenchymal through the Wnt/ β -catenin pathway activation in mammary carcinoma cells (38). Furthermore, in the same study, Wnt activation was associated with increased TWIST1 expression and decreased E-cadherin expression. Interestingly, ARTN has also been demonstrated to promote epithelial to mesenchymal transition in ER⁻ mammary carcinoma cells via increased expression of TWIST1 and decreased expression of E-cadherin (11). Concordantly, high expressions of both ARTN and HER2 have been reported to be significantly associated with disease relapse and poor survival outcomes in subtypes of mammary carcinoma (2, 11). Hence, ARTN may be one mediator of a common adaptive mechanism promoting resistance of mammary carcinoma cells to hostile environments given that ARTN has been demonstrated to promote acquired resistance to various therapeutic approaches by promotion of CSC-like cell population. ARTN may, in addition, also be a pivotal mediator of trastuzumab resistance in HER2⁺ mammary carcinoma.

The mechanistic involvement of PI3K/AKT pathway activation in both acquired trastuzumab resistance by promotion of CSC-like population and in ARTN-mediated promotion of CSC-like population in mammary carcinoma has been previously demonstrated (12, 36). ARTN utilized an AKT-TWIST1-BCL-2 signaling to increase the CSC-like cell population in ER⁻ mammary carcinoma cells (15). Similarly, HER2-stimulated increased expression of both TWIST1 and BCL-2 has been reported in mammary carcinoma cells (3, 19). Consistently, trastuzumab has been reported to down-regulate BCL-2 in HER2-amplified mammary carcinoma cells (39). Hence, ARTN could utilize TWIST1-dependent expression of BCL-2 to mediate acquired trastuzumab resistance by promotion of a CSC-like cell population in mammary carcinoma. Given herein that we have demonstrated the requirement of BCL-2 in the ARTN-mediated increase in the CSC-like population in trastuzumab-resistant cells, we postulate that BCL-2 may act as an ARTN-dependent potential downstream target in a wide a range of mammary carcinoma subtypes including HER2-dependent mammary carcinoma (20). In this regard it is important to note that regardless of the prognostic value of BCL-2 expression (40–42), it is the interactions among the various BCL-2 family member proteins (*e.g.* BAK, BAX, BAD) that exert significant effects on BCL-2 function (43). Furthermore, compensatory activation of parallel signaling pathways (*e.g.* EGFR, IGF-1R, MET, VEGF (3, 6, 7)) have been reported to circumvent trastuzumab sensitivity (6, 44, 45). In this regard it is interesting that members of the GDNF family of ligands have been reported to bind to and/or activate MET and other receptors (46) in addition to the GFR α family. Furthermore, IGF-1 and GDNF have been demonstrated to possess synergistic activities in models of neurologic development and disease (47, 48). Indeed, preliminary evidence from the laboratory suggests that ARTN is indeed IGF-1 regulated.⁵ Hence, despite the independent iden-

⁵ A. Yap, P. E. Lobie, and J. K. Perry, unpublished data.

tification of multiple and different factors and signaling pathways involved in acquired HER-2 resistance (2), it is likely and plausible that they are functionally interrelated and/or synergistic. Given the regulation of ARTN by estrogen (16) EGF, HRG, and IGF-1, ARTN regulation of VEGF (12) and ER activity (16), and as yet to be defined potential interactions with other molecules involved in trastuzumab resistance, it is possible that ARTN may be one common upstream mediator of resistance to HER-2 inhibition. That ARTN expression is increased in other examples of resistant cancer cell phenotypes (e.g. tamoxifen (16), ionizing radiation (15), paclitaxel (15)), and the increase in ARTN expression is functionally relevant, lends credence to this notion.

The HER2-enriched ER⁺/HER2⁺ luminal B subtype of mammary carcinoma responds to both ER and HER2 inhibition by tamoxifen and trastuzumab, respectively. However it has been demonstrated that ER⁺/HER2⁺ mammary carcinoma may acquire cross-resistance to either antiestrogens and/or HER2 inhibitors (49). HER2 overexpression in ER⁺ mammary carcinoma cells results in down-regulation of ER α and subsequent resistance to tamoxifen (50–52). Conversely, inhibition of HER2 results in increased activation of ER α -mediated transcription followed by acquired resistance against HER2-directed agents (49). Hence, in ER⁺/HER2⁺ mammary carcinoma cells, combined inhibition of both ER and HER2 signaling is considered to be a more effective approach (49). It is noted that ARTN is both E2- (16) and HER2-regulated, and increased expression of ARTN is functionally relevant in acquired resistance to both antiestrogens (16) and HER2 inhibition. Hence, inhibition of ARTN may obviate the development of acquired resistance to both antiestrogens and HER2 inhibitors in the luminal B subtype of mammary carcinoma.

REFERENCES

1. Stern, D. F. (2000) Tyrosine kinase signalling in breast cancer: ErbB family receptor tyrosine kinases. *Breast Cancer Res.* **2**, 176–183
2. Wong, A. L., and Lee, S. C. (2012) Mechanisms of resistance to trastuzumab and novel therapeutic strategies in HER2-positive breast cancer. *Int. J. Breast Cancer* **2012**, 415170
3. Korkaya, H., Kim, G. I., Davis, A., Malik, F., Henry, N. L., Ithimakin, S., Quraishi, A. A., Tawakkol, N., D'Angelo, R., Paulson, A. K., Chung, S., Luther, T., Paholak, H. J., Liu, S., Hassan, K. A., Zen, Q., Clouthier, S. G., and Wicha, M. S. (2012) Activation of an IL6 inflammatory loop mediates trastuzumab resistance in HER2⁺ breast cancer by expanding the cancer stem cell population. *Mol. Cell* **47**, 570–584
4. Gradishar, W. J. (2013) Emerging approaches for treating HER2-positive metastatic breast cancer beyond trastuzumab. *Ann. Oncol.* **24**, 2492–2500
5. Huang, Y., Fu, P., and Fan, W. (2013) Novel targeted therapies to overcome trastuzumab resistance in HER2-overexpressing metastatic breast cancer. *Curr. Drug Targets* **14**, 889–898
6. Nahta, R., Yuan, L. X., Zhang, B., Kobayashi, R., and Esteva, F. J. (2005) Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. *Cancer Res.* **65**, 11118–11128
7. Shattuck, D. L., Miller, J. K., Carraway, K. L., 3rd, and Sweeney, C. (2008) Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. *Cancer Res.* **68**, 1471–1477
8. Airaksinen, M. S., and Saarma, M. (2002) The GDNF family: signalling, biological functions and therapeutic value. *Nat. Rev. Neurosci.* **3**, 383–394
9. Beshpalov, M. M., Sidorova, Y. A., Tumova, S., Ahonen-Bishopp, A., Magalhães, A. C., Kuleskiy, E., Paveliev, M., Rivera, C., Rauvala, H., and Saarma, M. (2011) Heparan sulfate proteoglycan syndecan-3 is a novel receptor for

- GDNF, neurturin, and artemin. *J. Cell Biol.* **192**, 153–169
10. Wu, Z. S., Pandey, V., Wu, W. Y., Ye, S., Zhu, T., and Lobie, P. E. (2013) Prognostic significance of the expression of GFR α 1, GFR α 3, and syndecan-3, proteins binding Artemin, in mammary carcinoma. *BMC Cancer* **13**, 34
11. Banerjee, A., Wu, Z. S., Qian, P., Kang, J., Pandey, V., Liu, D. X., Zhu, T., and Lobie, P. E. (2011) Artemin synergizes with TWIST1 to promote metastasis and poor survival outcome in patients with ER-negative mammary carcinoma. *Breast Cancer Res.* **13**, R112
12. Banerjee, A., Wu, Z. S., Qian, P. X., Kang, J., Liu, D. X., Zhu, T., and Lobie, P. E. (2012) Artemin promotes *de novo* angiogenesis in ER-negative mammary carcinoma through activation of TWIST1-VEGF-A signalling. *PLoS One* **7**, e50098
13. Kang, J., Perry, J. K., Pandey, V., Fielder, G. C., Mei, B., Qian, P. X., Wu, Z. S., Zhu, T., Liu, D. X., and Lobie, P. E. (2009) Artemin is oncogenic for human mammary carcinoma cells. *Oncogene* **28**, 2034–2045
14. Pandey, V., Jung, Y., Kang, J., Steiner, M., Qian, P. X., Banerjee, A., Mitchell, M. D., Wu, Z. S., Zhu, T., Liu, D. X., and Lobie, P. E. (2010) Artemin reduces sensitivity to doxorubicin and paclitaxel in endometrial carcinoma cells through specific regulation of CD24. *Transl. Oncol.* **3**, 218–229
15. Banerjee, A., Qian, P., Wu, Z. S., Ren, X., Steiner, M., Bougen, N. M., Liu, S., Liu, D. X., Zhu, T., and Lobie, P. E. (2012) Artemin stimulates radio- and chemo-resistance by promoting TWIST1-BCL-2-dependent cancer stem cell-like behavior in mammary carcinoma cells. *J. Biol. Chem.* **287**, 42502–42515
16. Kang, J., Qian, P. X., Pandey, V., Perry, J. K., Miller, L. D., Liu, E. T., Zhu, T., Liu, D. X., and Lobie, P. E. (2010) Artemin is estrogen-regulated and mediates antiestrogen resistance in mammary carcinoma. *Oncogene* **29**, 3228–3240
17. Dean, M., Fojo, T., and Bates, S. (2005) Tumour stem cells and drug resistance. *Nat. Rev. Cancer* **5**, 275–284
18. Korkaya, H., Paulson, A., Iovino, F., and Wicha, M. S. (2008) HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. *Oncogene* **27**, 6120–6130
19. Crawford, A., and Nahta, R. (2011) Targeting Bcl-2 in herceptin-resistant breast cancer cell lines. *Curr. Pharmacogenomics Person. Med.* **9**, 184–190
20. Nahta, R. (2012) New developments in the treatment of HER2-positive breast cancer. *Breast Cancer* **4**, 53–64
21. Lang, J. Y., Hsu, J. L., Meric-Bernstam, F., Chang, C. J., Wang, Q., Bao, Y., Yamaguchi, H., Xie, X., Woodward, W. A., Yu, D., Hortobagyi, G. N., and Hung, M. C. (2011) BikDD eliminates breast cancer initiating cells and synergizes with lapatinib for breast cancer treatment. *Cancer Cell* **20**, 341–356
22. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987) Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. *Science* **235**, 177–182
23. Liu, D. X., and Lobie, P. E. (2007) Transcriptional activation of p53 by Pitx1. *Cell Death Differ.* **14**, 1893–1907
24. Pandey, V., Perry, J. K., Mohankumar, K. M., Kong, X. J., Liu, S. M., Wu, Z. S., Mitchell, M. D., Zhu, T., and Lobie, P. E. (2008) Autocrine human growth hormone stimulates oncogenicity of endometrial carcinoma cells. *Endocrinology* **149**, 3909–3919
25. Klapper, L. N., Glathe, S., Vaisman, N., Hynes, N. E., Andrews, G. C., Sela, M., and Yarden, Y. (1999) The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 4995–5000
26. Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunakaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996) A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol. Cell. Biol.* **16**, 5276–5287
27. Park, B. H., and Davidson, N. E. (2007) PI3 kinase activation and response to trastuzumab therapy: what's neu with herceptin resistance? *Cancer Cell* **12**, 297–299
28. Tseng, P. H., Wang, Y. C., Weng, S. C., Weng, J. R., Chen, C. S., Bruegge-meier, R. W., Shapiro, C. L., Chen, C. Y., Dunn, S. E., Pollak, M., and Chen, C. S. (2006) Overcoming trastuzumab resistance in HER2-overexpressing

- breast cancer cells by using a novel celecoxib-derived phosphoinositide-dependent kinase-1 inhibitor. *Mol. Pharmacol.* **70**, 1534–1541
29. Chakrabarty, A., Bhola, N. E., Sutton, C., Ghosh, R., Kuba, M. G., Dave, B., Chang, J. C., and Arteaga, C. L. (2013) Trastuzumab-resistant cells rely on a HER2-PI3K-FoxO-survivin axis and are sensitive to PI3K inhibitors. *Cancer Res.* **73**, 1190–1200
 30. Bedard, P. L., Cardoso, F., and Piccart-Gebhart, M. J. (2009) Stemming resistance to HER-2 targeted therapy. *J. Mammary Gland Biol. Neoplasia* **14**, 55–66
 31. Ginestier, C., Hur, M. H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., Jacquemier, J., Viens, P., Kleer, C. G., Liu, S., Schott, A., Hayes, D., Birnbaum, D., Wicha, M. S., and Dontu, G. (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* **1**, 555–567
 32. Liu, S., Dontu, G., and Wicha, M. S. (2005) Mammary stem cells, self-renewal pathways, and carcinogenesis. *Breast Cancer Res.* **7**, 86–95
 33. Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001) Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105–111
 34. Duru, N., Fan, M., Candas, D., Menaa, C., Liu, H. C., Nantajit, D., Wen, Y., Xiao, K., Eldridge, A., Chromy, B. A., Li, S., Spitz, D. R., Lam, K. S., Wicha, M. S., and Li, J. J. (2012) HER2-associated radioresistance of breast cancer stem cells isolated from HER2-negative breast cancer cells. *Clin. Cancer Res.* **18**, 6634–6647
 35. Minami, T., Kijima, T., Otani, Y., Kohmo, S., Takahashi, R., Nagatomo, I., Hirata, H., Suzuki, M., Inoue, K., Takeda, Y., Kida, H., Tachibana, I., and Kumanogoh, A. (2012) HER2 as therapeutic target for overcoming ATP-binding cassette transporter-mediated chemoresistance in small cell lung cancer. *Mol. Cancer Ther.* **11**, 830–841
 36. Ithimakin, S., Day, K. C., Malik, F., Zen, Q., Dawsey, S. J., Bersano-Begey, T. F., Quraishi, A. A., Ignatoski, K. W., Daignault, S., Davis, A., Hall, C. L., Palanisamy, N., Heath, A. N., Tawakkol, N., Luther, T. K., Clouthier, S. G., Chadwick, W. A., Day, M. L., Kleer, C. G., Thomas, D. G., Hayes, D. F., Korkaya, H., and Wicha, M. S. (2013) HER2 drives luminal breast cancer stem cells in the absence of HER2 amplification: implications for efficacy of adjuvant trastuzumab. *Cancer Res.* **73**, 1635–1646
 37. Oliveras-Ferraros, C., Corominas-Faja, B., Cuffi, S., Vazquez-Martin, A., Martin-Castillo, B., Iglesias, J. M., López-Bonet, E., Martin, Á. G., and Menendez, J. A. (2012) Epithelial-to-mesenchymal transition (EMT) confers primary resistance to trastuzumab (Herceptin). *Cell Cycle* **11**, 4020–4032
 38. Wu, Y., Ginther, C., Kim, J., Mosher, N., Chung, S., Slamon, D., and Vadgama, J. V. (2012) Expression of Wnt3 activates Wnt/ β -catenin pathway and promotes EMT-like phenotype in trastuzumab-resistant HER2-overexpressing breast cancer cells. *Mol. Cancer Res.* **10**, 1597–1606
 39. Milella, M., Trisciuglio, D., Bruno, T., Ciuffreda, L., Mottolise, M., Cianciulli, A., Cognetti, F., Zangemeister-Wittke, U., Del Bufalo, D., and Zupi, G. (2004) Trastuzumab down-regulates Bcl-2 expression and potentiates apoptosis induction by Bcl-2/Bcl-XL bispecific antisense oligonucleotides in HER-2 gene-amplified breast cancer cells. *Clin. Cancer Res.* **10**, 7747–7756
 40. Dawson, S. J., Makretsov, N., Blows, F. M., Driver, K. E., Provenzano, E., Le Quesne, J., Baglietto, L., Severi, G., Giles, G. G., McLean, C. A., Callagy, G., Green, A. R., Ellis, I., Gelmon, K., Turashvili, G., Leung, S., Aparicio, S., Huntsman, D., Caldas, C., and Pharoah, P. (2010) BCL2 in breast cancer: a favourable prognostic marker across molecular subtypes and independent of adjuvant therapy received. *Br. J. Cancer* **103**, 668–675
 41. Tawfik, K., Kimler, B. F., Davis, M. K., Fan, F., and Tawfik, O. (2012) Prognostic significance of Bcl-2 in invasive mammary carcinomas: a comparative clinicopathologic study between “triple-negative” and non-“triple-negative” tumors. *Hum. Pathol.* **43**, 23–30
 42. Yang, Q., Moran, M. S., and Haffty, B. G. (2009) Bcl-2 expression predicts local relapse for early-stage breast cancer receiving conserving surgery and radiotherapy. *Breast Cancer Res. Treat.* **115**, 343–348
 43. Youle, R. J., and Strasser, A. (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nat. Rev. Mol. Cell Biol.* **9**, 47–59
 44. Lu, Y., Zi, X., and Pollak, M. (2004) Molecular mechanisms underlying IGF-I-induced attenuation of the growth-inhibitory activity of trastuzumab (Herceptin) on SKBR3 breast cancer cells. *Int. J. Cancer* **108**, 334–341
 45. Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D., and Pollak, M. (2001) Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J. Natl. Cancer Inst.* **93**, 1852–1857
 46. Sariola, H., and Saarma, M. (2003) Novel functions and signalling pathways for GDNF. *J. Cell Sci.* **116**, 3855–3862
 47. Bilak, M. M., and Kuncel, R. W. (2001) Delayed application of IGF-I and GDNF can rescue already injured postnatal motor neurons. *Neuroreport* **12**, 2531–2535
 48. D’Costa, A. P., Prevette, D. M., Houenou, L. J., Wang, S., Zackenfels, K., Rohrer, H., Zapf, J., Caroni, P., and Oppenheim, R. W. (1998) Mechanisms of insulin-like growth factor regulation of programmed cell death of developing avian motoneurons. *J. Neurobiol.* **36**, 379–394
 49. Nahta, R., and O’Regan, R. M. (2012) Therapeutic implications of estrogen receptor signaling in HER2-positive breast cancers. *Breast Cancer Res. Treat.* **135**, 39–48
 50. Benz, C. C., Scott, G. K., Sarup, J. C., Johnson, R. M., Tripathy, D., Coronado, E., Shepard, H. M., and Osborne, C. K. (1992) Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res. Treat.* **24**, 85–95
 51. Kurokawa, H., Lenferink, A. E., Simpson, J. F., Pisacane, P. I., Sliwkowski, M. X., Forbes, J. T., and Arteaga, C. L. (2000) Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. *Cancer Res.* **60**, 5887–5894
 52. Pietras, R. J., Arboleda, J., Reese, D. M., Wongvipat, N., Pegram, M. D., Ramos, L., Gorman, C. M., Parker, M. G., Sliwkowski, M. X., and Slamon, D. J. (1995) HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. *Oncogene* **10**, 2435–2446