What tumor cells cannot resist

Mechanisms of therapy resistance in esophageal adenocarcinoma

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Esophageal Adenocarcinoma Cells and Xenograft Tumors Exposed to Erb-b2 Receptor Tyrosine Kinase 2 and 3 Inhibitors Activate Transforming Growth Factor Beta Signaling, Which Induces Epithelial to Mesenchymal Transition

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Abstract

Background & Aims: Drugs that inhibit the erb-b2 receptor tyrosine kinase 2 (ERBB2 or HER2) are the standard treatment of patients with different types of cancer, including HER2-overexpressing gastroesophageal tumors. Unfortunately, cancer cells become resistant to these drugs, so overall these drugs provide little benefit to patients with these tumors. We investigated mechanisms that mediate resistance of esophageal adenocarcinoma (EAC) cells and patient-derived xenograft (PDX) tumors to ERBB inhibitors.

Methods: We cultured primary tumor cells, isolated from EAC patient samples, and OE19 and OE33 EAC cell lines with trastuzumab (an inhibitor of HER2), with or without pertuzumab (inhibits dimerization of HER2 with HER3) or a specific antibody against HER3 (anti-HER3). HER2 was knocked down by expression of small hairpin RNAs. In addition, cells were incubated with NRG-1β, a mediator of HER2–HER3 signaling, or A83-01, an inhibitor of transforming growth factor beta (TGF-β) signaling. Cells were analyzed for markers of the epithelial to mesenchymal transition (EMT) using flow cytometry, immunofluorescence, and quantitative reverse transcription PCR. We performed limiting dilution, transwell, and cell viability assays to study the functional effects of HER2 and HER3 inhibition and reactivation. We analyzed publicly available EAC gene expression datasets to correlate expression of ERBB genes with genes encoding epithelial and mesenchymal proteins. NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were given subcutaneous injections of AMC-EAC-007B cells and also given injections of single or combined inhibitors; growth of these PDX tumors was quantified.

Results: EAC cells incubated with trastuzumab decreased expression of epithelial markers (CD24, CD29, and CDH1) and increased expression of mesenchymal markers (CXCR4, VIM, ZEB1, SNAI2, and CDH2), compared to cells not exposed to trastuzumab, indicating induction of EMT. Addition of NRG-1β to these cells restored their epithelial phenotype. Incubation of EAC cells with trastuzumab and pertuzumab accelerated the expression of EMT markers, compared to cells incubated with trastuzumab alone. EAC cells cultured for 2 months with a combination of trastuzumab and pertuzumab became resistant to chemotherapeutic agents (5-fluorouracil, carboplatin, cisplatin, eribulin, and paclitaxel), based on their continued viability, which was accompanied with an enhanced migratory capacity in transwell assays, and clonogenicity in limiting dilution analyses. In comparisons of EAC gene expression patterns, we associated high expression of ERBB3 with an epithelial gene expression signature; expression of transforming growth factor beta (TGFβ1) correlated with expression of EMT-related genes, and we found an inverse correlation between expression of TGFβ1 and ERBB3. EAC cells incubated with ERBB inhibitors began to secrete ligands for the TGF-β receptor and underwent EMT. Incubation of EAC cells with trastuzumab, followed by 10 days incubation with the TGF-β receptor inhibitor in the presence of trastuzumab, caused cells to regain an epithelial phenotype. EAC patient-derived xenograft tumors grew more slowly in mice given the combination of trastuzumab, pertuzumab, and the TGF-β inhibitor than in mice given single agents or a combination of trastuzumab and pertuzumab. Tumors exposed to trastuzumab and pertuzumab expressed EMT markers o and were poorly differentiated, whereas tumors exposed to the combination of trastuzumab, pertuzumab, and the TGF-β inhibitor expressed epithelial markers and were more differentiated.
Conclusions: EAC cells become resistant to trastuzumab and pertuzumab by activating TGF-β signaling, which induces EMT. Agents that block TGF-β signaling can increase the anti-tumor efficacies of trastuzumab and pertuzumab.

**INTRODUCTION**

Esophageal carcinoma is the eighth most common cancer worldwide, with a rapidly increasing incidence of esophageal adenocarcinoma (EAC). Patients with EAC face a poor 5-year survival of 15-25% despite multimodality treatment, indicating an urgent need for better therapeutic options.

The erb-b2/HER/ERBB-family consists of the receptor tyrosine kinases (RTKs) epidermal growth factor receptor (EGFR or HER1), erb-b2 receptor tyrosine kinase 2 (HER2 or Neu), erb-b2 receptor tyrosine kinase 3 (HER3), and erb-b2 receptor tyrosine kinase 4 (HER4) with well-studied roles in the development and progression of various epithelial tumor types. Receptor activation is initiated by receptor dimerization resulting in phosphorylation of the tyrosine kinase domain leading to enhanced cell viability and proliferation. The most potent HER signaling is mediated through HER2-HER3 heterodimers, which signal via downstream AKT after binding of the beta isoform of neuregulin-1 beta (NRG-1β) to HER3. Overexpression of HER2 in esophageal cancer depends on the histological subtype, with adenocarcinomas being most often HER2 positive (21-31%).

Compared to other cancer types, targeted therapy of EAC is lagging behind. For first-line palliative systemic treatment of HER2-positive esophagogastric adenocarcinomas, trastuzumab, a humanized antibody against HER2 is the only available targeted treatment option. Although a significant survival benefit was shown in the phase III ToGA trial for addition of trastuzumab to standard chemotherapy in advanced-stage esophagogastric adenocarcinoma patients, the improvement was modest. Even after an initial response patients ultimately all showed progressive disease. Therefore, it would be of great clinical value to identify the mechanisms of resistance to HER2 targeting and devise strategies to circumvent them.

The HER3-NRG-1β signaling axis is involved in various drug resistance mechanisms. When NRG-1β is bound to HER3, trastuzumab is unable to inhibit HER2-HER3 dimerization and signaling. Consequently, combined HER2-HER3 targeting is currently studied in clinical trials in gastroesophageal cancer using the compounds trastuzumab and pertuzumab (TRAP; NCT02120911, JACOB; NCT01461057). Pertuzumab inhibits HER2-HER3 signaling regardless of HER3-bound NRG-1β. In addition to the reactivation of signaling pathways through other HER/ERBB family members, epithelial to mesenchymal transition (EMT) may also mediate resistance against HER2-targeted therapy. Although EMT has been identified in breast and gastric cancer, the mechanisms that mediate EMT differ between tumor types. The role of this phenotypic transition in EAC is unknown and EMT-induced drug resistance has not yet been identified in EAC. Also, no clinically feasible treatment strategy to circumvent such resistance against ERBB-targeting has been described. In this study, primary cultures and patient-derived xenograft models were used to identify mechanisms of resistance against ERBB inhibition. We identified EMT as a novel resistance mechanism in EAC, and showed
that the induction of EMT was accelerated by simultaneous inhibition of multiple ERBB-family members. This was accompanied by increased migratory capacity, clonogenicity, and chemoresistance. The EMT process was mediated by TGF-β in a non-cell autonomous manner. Targeting both the ERBB and TGF-β signaling pathways resulted in a significant anti-tumor effect both in vitro and in vivo. Various drugs targeting TGF-β signaling are currently tested in clinical trials. We therefore propose a feasible strategy to circumvent acquired resistance against ERBB-targeting agents in EAC.

**Materials and Methods**

**Cell culture and chemicals**

OE19 and OE33 cells (ATCC, Manassas, VA) were maintained in RPMI with 8% fetal bovine serum (FBS), L-glutamine (2mM), penicillin (100units/mL), and streptomycin (500µg/mL) (Lonza, Basel, Switzerland) according to standard culture procedures. Trastuzumab incubated cells were continuously cultured with trastuzumab for 6 months, unless noted otherwise (Roche, Grenzach-Wyhlen, Germany). Cells incubated with trastuzumab and pertuzumab were continuously cultured with both components for 2 months (Roche). Both therapeutics were kindly provided by the Academic Medical Center pharmacy and used in vitro at 1µg/ml, in vivo at 1mg/kg. Recombinant NRG-1β was purchased from R&D systems (Oxon, United Kingdom) and used at 1ng/ml. Anti-HER3 antibody (H3.105.5) was purchased from Millipore (Temecula, CA) and used at 0.1µg/ml. 5-Fluoruracil (5-FU), Carboplatin, Cisplatin, Eribulin, Paclitaxel were purchased from the Academic Medical Center pharmacy. A83-01 was purchased from Tocris Bioscience (Bristol, United Kingdom) and used in vitro at 100-500nM, in vivo at 1mg/kg. TGF-β neutralizing antibody was purchased from R&D (clone 1D11) and used at 1µg/ml. Recombinant TGF-β was purchased from Peprotech (Rocky Hill, United States) and used at 1-5ng/ml. Trastuzumab or trastuzumab and pertuzumab was maintained in all experiments involving a combination with cytotoxic agents or recombinant proteins.

**Immunofluorescence**

Cells were plated on 1% gelatin coated glass slides in 12-well plates using either control- or trastuzumab-complemented medium. Reaching 60-80% confluency, cells were washed with PBS, fixed using 4% paraformaldehyde 20 min on ice, washed twice with PBS prior to 1h blocking in blocking solution (PBS + 0.1% triton X-100 + 5% normal goat serum (ab7481; Abcam), followed by overnight incubation at 4°C using the antibodies listed in Supplementary Table 1. Cells were washed with blocking solution, and incubated with the appropriate secondary antibodies. Samples were washed with blocking solution, incubated 10 minutes with DAPI at 1µg/ml (D9542; Sigma-Aldrich, St. Louis, MO), and washed with PBS prior to mounting using ProLong Gold (P36930, Invitrogen, Carlsbad, CA). Images were obtained using a fluorescence microscope (Axiovert 200M; Zeiss, Jena, Germany).

**Flow cytometry**

Flow cytometric stainings were performed as previously described. Antibodies and isotype controls are listed in Supplementary Table 1. Data were analyzed with FlowJo 10 (Tree
Intracellular epitopes were revealed using permeabilization buffer (BD Biosciences, San Jose, CA), in these condition no propidium iodide was used. The geometric mean fluorescence (gMFI) intensity in the relevant channel was calculated by correcting the staining gMFI from the samples for the isotype control, yielding the Δ gMFI.

**Quantitative RT-PCR mRNA**

Cells were lysed using Trizol (Invitrogen), RNA was isolated and cDNA was synthesized using Superscript III (Invitrogen). Quantitative Reverse Transcription-PCR (qRT-PCR) was performed using SYBR green (Roche) on a Lightcycler LC480II (Roche), according to manufacturer’s protocols. Values were normalized to GAPDH according to the comparative threshold cycle (Cp) method. Primer sequences: GAPDH-forward: 5’-AATCCCATCACCATCTTCCA, reverse: 5’-TGGACTCCACGACTTCA; VIM-forward: 5’-CCCTCACCTGTGAAGTGGAT, reverse: 5’-TCCAGCAGCTTCTGTAGGTG-; ZEB1-forward: 5’-GCACAAGAAGGCCACAAGTA, reverse: 5’-GCAAGACAAGTTCAAGGGTTC; CDH2-forward: 5’-ACAGTGCCCCACTCAAAGG, reverse: 5’-CCGAGATGGGCTTGATAG; SNAI2-forward: 5’-GGTCAAGAAGCATTTCAACG, reverse: 5’-CACAGTGATGGGCGTATAG.

**Quantitative RT-PCR miRNA**

Total RNA was extracted using the NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany). Reverse transcription PCR was performed using the HiFlex buffer of the miScript II RT Kit (cat. no. 218160; Qiagen, Hilden, Germany) and the miScript SYBR Green PCR Kit (cat. no. 218073; Qiagen) was used for qRT-PCR. miScript Primer assays: Hs_RNU6-2_1, Hs_SNORD44_1, Hs_miR-200b_3, Hs_miR-200a_1, Hs_miR-429_1, Hs_miR-200c_1, and Hs_miR-141_1. Data were normalized to the expression of SNORD44. Normalization to RNU6-2 yielded comparable results.

**Establishment of primary cell cultures**

Primary cultures were established from tumor material of patients diagnosed with EAC in the Academic Medical Center (Amsterdam, The Netherlands) as described earlier and was approved by the institute’s ethical committee (MEC 01/288#08.17.1042). For patient material expansion by xenograft, NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ (NSG) immunodeficient animals were bred and maintained at the local animal facility according to local legislation and ethical approval was obtained (LEX100780 and LEX102774). Patient-derived xenograft (PDX) tumors were processed as previously shown. The primary cultures used are: AMC-EAC-007B (007B) and AMC-EAC-031M (031M).

**Cell viability assay**

Cell viability was determined using the Cell Titer-Blue Cell Viability Assay kit (G8081; Promega, Madison, WI). Before the assay, cells were either cultured under normal conditions or as outlined for the trastuzumab and pertuzumab incubated condition; cells were cultured in trastuzumab and pertuzumab supplemented medium and were refreshed/passaged twice a week. Cells were seeded into 96-well plates at a density of 1000 cells per well in triplicates. After cell adhesion overnight, baseline cell viability was measured, drugs were added (see below), and 20 µl of Cell Titer-Blue reagent was added to each well after the indicated incubation period followed by 3h incubation. Plates were read in a cytofluorometer (BioTek Instruments, Winooski, VT).
viability was calculated by comparing the values obtained from cells incubated with inhibitors and or chemotherapeutics versus control cells, minus the baseline cell viability. The following therapeutic agents were administered at the indicated concentrations and incubated for the indicated time; 5-Fluoruracil (5-FU), Carboplatin, Cisplatin, Eribulin, and Paclitaxel. The presence of trastuzumab and pertuzumab was continued during the assay.

Limiting dilution assay and cell sorting
Control or cells incubated with trastuzumab and pertuzumab were harvested, and sorted on a BD FACSAria III. For the limiting dilution assay, cells were sorted into 96 wells, per plate accordingly; 16x1, 16x2, 16x4, 8x8, 8x16, 8x32, 8x64, 8x128, and 8x254 cells/well. The clonogenic capacity was calculated using L-Calc™ Software (STEMCELL Technologies, Vancouver, Canada). (http://www.stemcell.com/en/Products/All-Products/LCalc-Software.aspx). Trastuzumab and pertuzumab incubation was continued during the assay. Antibodies and isotype controls used are listed in Supplementary Table 1.

Migration assay
Migration was performed using Corning FluoroBlok cell culture inserts with 8.0µm pore filters (351152; Corning, Corning, NY)). Cells were washed with PBS, incubated for 1h with 10µM cell tracker Green (C2925; Invitrogen) in serum-free RPMI, washed, and incubated 1h in serum-free RPMI. 600µl RPMI with/without 1% serum (as attractant) was placed in the lower compartment of the Corning FluoroBlok plate. Cells were collected, suspended in 5.0×10^4 / 100µl serum-free RPMI, and seeded into the upper compartment. Migration was measured every 2 minutes for 3h at 37ºC using a cytofluorometer (BioTek Instruments). The migration was controlled for the no-attractant control with serum-free RPMI in the lower compartment.

Gene set enrichment analysis and gene correlation
Gene set enrichment analysis (GSEA) was performed using Broad Institute tool (http://www.broadinstitute.org/gsea/index.jsp). Data analyzed were derived from a TCGA (The Cancer Genome Atlas) study (Study ID: TCGA-ESCA; https://gdc-portal.nci.nih.gov/projects/TCGA-ESCA)25, and were selected to contain EAC tumors only. Samples were dichotomized by median ERBB3 expression. P-values indicating the significance of enrichment were determined by 100 permutations. The EMT gene set was assembled from two published EMT signatures.26, 27 Gene expression levels of ERBB3 and TGFB1 were extracted from the same TCGA-ESCA study as used for the GSEA, and correlations were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, Ca).

Western blot
Western blot analyses were performed as previously described.18 Primary antibodies (listed in Supplementary Table 1) were incubated overnight at 4ºC. Proteins were imaged using Lumilight plus Western blot substrate (12015196001; ROCHE) on a FujiFilm LAS 4000 imager.

Lentiviral transduction
ployG-V2 constructs containing Venus (#27340; Addgene, Cambridge, MA) or mCherry (#27339; Addgene)28, were used for transduction. Virus production was performed as previously described.18 After overnight transduction, cells were cultured for 72 hours prior to sorting.
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Cell death and proliferation assay
Cells were harvested, plated in 6 well plates (1-2x10^4 /well), and incubated overnight. The next day, 10-day incubation was started; A83-01, trastuzumab, pertuzumab, trastuzumab and pertuzumab, trastuzumab and pertuzumab+A83-01 (TGF-β pathway inhibitor) (500nM). The inhibitors were refreshed twice a week, floating/dead cells were kept in the assayed population by centrifugation of supernatant. After incubation, cells were harvested and resuspended in FACS buffer containing 100ng/ml propidium iodide (Sigma -Aldrich). Events were acquired in a FACS Canto II (BD Biosciences, Franklin Lakes, NJ). Data were analyzed using FlowJo 10 (Tree Star, Ashland, OR). Cell proliferation was determined by calculating the amount of cells in the life fraction using CountBright™ Absolute Counting Beads (C36950; Thermo Fisher, Waltham, MA).

In vivo experiments
Early passage 007B cells (1x10^6) were subcutaneously injected into the flank of NSG mice. Tumor bearing mice (50-100 mm^3) were randomly divided into six groups and injected with either 1% DMSO-PBS, 1mg/kg trastuzumab, 1mg/kg pertuzumab, 1mg/kg A83-01 as single inhibition. Double inhibition consisted of trastuzumab and pertuzumab at the indicated concentrations and trastuzumab and pertuzumab-A83-01 as triple combination. Mice were injected once a week, tumor growth and the weight of the animal were measured twice a week. The injections were continued until the tumors reached the maximum allowed size of 1000mm^3, after which mice were euthanized and the tumors were harvested and measured. In the serial passage experiments, a tumor piece of 4mm^3, obtained from PDX tumors of the previous experiment, was subcutaneously grafted. Tumors were grown until the first tumor reached a maximum allowed size of 1000mm^3. Animals in these experiments were bred and maintained at the local animal facility according to local legislation and ethical approval was obtained (LEX103096).

Statistical Analysis
For the in vitro experiments, statistical analyses were performed using two-sided unpaired t tests, unless noted otherwise. P-values and the R-values of gene expression correlations were determined by linear regression analysis. For the in vivo survival analysis, statistical significance was determined using Log-rank (Mantel-Cox) test. All statistical analyses were performed using GraphPad Prism 6. Error bars show the mean ± SEM. A P-value of <.05 was considered statistically significant.
Chapter 6

Results

HER2 inhibition induces a mesenchymal phenotype that can be mitigated by reactivation of growth factor receptor signaling.

To investigate the mechanisms mediating resistance to HER2 inhibition, HER2 positive OE19 and OE33 EAC cell lines were cultured for 6 months in the presence of 1µg/ml trastuzumab, which resulted in a shift towards a mesenchymal morphology (Figure 1A). To characterize this observation in more detail, EMT markers were measured using immunofluorescence and we found increased expression of vimentin (VIM) and ZEB1, whereas cadherin 1 (CDH1), an epithelial marker, was decreased (Figure 1B). Further analysis by flow cytometry and qRT-PCR showed a decreased expression of epithelial markers CD24, CD29, and CDH1, and an increased expression of EMT markers CXCR4, VIM, ZEB1, SNAI2, and CDH2. A concomitant induction of cancer stem cell (CSC) markers CD44, CD133, and LGR5 was observed (Figure 1C, D and Supplementary Figure 1). These data demonstrate activation of the EMT process following long-term HER2 inhibition. Targeting ERBB2 by shRNA silencing (Supplementary Figure 2 and 3) or by using high concentrations of trastuzumab (10 and 100µg/mL) for 2 months showed comparable results (Supplementary Figure 4). ERBB inhibition did not induce EMT in HER2-negative EAC cells (Flo-1; Supplementary Figure 5A-D). Remarkably, reactivation of the HER2-HER3 signaling axis by the addition of recombinant NRG-1β in the presence of trastuzumab could reverse this process in both cell lines within 10 days (Figure 1E and Supplementary Figure 6A-F). These results suggest that HER2 positive tumor cells preferentially exist in an epithelial phenotype that is maintained by the same ERBB activation that mediates their oncogenic growth.

Simultaneous inhibition of HER2 and HER3 accelerates the induction of EMT.

As we observed that HER2-HER3 signaling re-activation reversed EMT, we hypothesized that combined HER2-HER3 targeting would in fact accelerate EMT. Indeed, when culturing two cell lines and two HER2-positive primary EAC cultures, with either trastuzumab alone or combined with a specific anti-HER3 antibody or pertuzumab for 2 months, the mesenchymal morphology was more pronounced and more rapidly occurred when both receptors were blocked (Figure 2A). This transition was accompanied by an increased expression of EMT- and the CSC-associated markers determined by FACS (Figure 2B and C), and qRT-PCR (Supplementary Figure 7). To reveal the kinetics of this EMT we used FACS sorting, cell viability assays, and a color-based lineage tracing method.28 We found that a subpopulation of cells is instructed to undergo EMT following ERBB inhibition, and that this population acquires a higher proliferative profile, which contributes to its selection under continued therapeutic pressure (Supplementary Figure 8). This effect was reversible when drug induced pressure was released. Together these data corroborate the dependence of an epithelial tumor phenotype on ERBB signaling, and demonstrate an important role for specifically HER3 in maintaining this cell state.

Inhibition of growth factor receptor signaling increases chemo-resistance, clonogenicity, and cell motility.

As in clinical practice trastuzumab and pertuzumab are usually combined with cytotoxic agents, we studied the effect of various chemotherapeutics in cells cultured for 2 months with or without trastuzumab and pertuzumab. Cell viability assays showed significant chemo-
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Figure 1. HER2 inhibition induces a mesenchymal phenotype that can be mitigated by reactivation of growth factor receptor signaling. (A) OE19 and OE33 cells were continuously cultured with trastuzumab for 6 months, and morphology was assessed by phase-contrast microscopy. Scale bar: 100µm. (B) OE19 cells were cultured as for panel A, and immunofluorescently stained for the indicated epithelial (CDH1) and mesenchymal (VIM, ZEB1) markers. (C-D) Cell surface staining by flow cytometry for epithelial, mesenchymal, and stem cell markers in control or 6 months trastuzumab-incubated OE19 and OE33 cells. Bar graphs show means ± SEM, data were normalized to the control condition, n = 3. *P < .05, **P < .01, and ***P < .001. (E) OE19 and OE33 cultured in the same conditions as for panel A-D, were incubated with 1ng/ml NRG-1β for 10 days. Trastuzumab was maintained during the assays. Scale bar: 100µm.

Resistance to all drugs tested combined with ERBB inhibition (Figure 3A and B). Of note, short term incubation with trastuzumab and pertuzumab did not confer resistance to chemotherapy, supporting our hypothesis that long-term trastuzumab and pertuzumab incubation is needed to induce EMT and resistance. (Supplementary Figure 9A, B). Furthermore, transwell migration assays revealed enhanced migratory capacity for cancer cells following either 6-month trastuzumab or 2-month trastuzumab and pertuzumab incubation compared to the control conditions (Figure 3C). To study the biological relevance of the upregulated CSC markers, limiting dilution assays were performed, which showed increased clonogenicity of the cells incubated with trastuzumab and pertuzumab for 2 months (Figure 3D). Thus, coinciding with ERBB inhibition-induced EMT, cells become more chemo-resistant and increasingly clonogenic. These data imply that combined treatment strategies targeting ERBBs could in the long term be counterproductive, which highlights the need for a clinically applicable solution.
Figure 2. Simultaneous inhibition of HER2 and HER3 accelerates the induction of EMT. (A) OE19, OE33 and primary cultures 007B and 031M were continuously cultured for 2 months with the following therapeutics; trastuzumab, trastuzumab and anti-HER3, or trastuzumab and pertuzumab. Cells cultured without inhibitors were used as control. Morphology was assessed by phase-contrast microscopy. Scale bar: 100 µm. (B-C) Flow cytometric stainings for the indicated epithelial, EMT, and stem cell markers on the same conditions as for panel A using 007B and 031M culture. Bar graphs show means ± SEM, data was normalized to the control condition, n = 3. *P < .05, **P < .01, and ***P < .001.
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**Figure 3.** Inhibition of growth factor receptor signaling increases chemo-resistance, clonogenicity, and cell motility. (A) Cell viability assays were performed on control OE19 cells (gray lines) and OE19 cells cultured with trastuzumab and pertuzumab for 2 months (dark lines), and were subsequently incubated with the indicated chemotherapeutics and concentrations. Graphs show means ± SEM, and normalized to t=0, n = 4. P-values were determined by two-way ANOVA and Bonferroni correction. (B) As for panel A, using OE33 cells. (C) Transwell migration assays on OE19 and OE33 cells in the following conditions: control, 6-month trastuzumab, 2-month trastuzumab and pertuzumab. 1% FCS was used as a chemoattractant. Migration shown is corrected for no-attractant controls (medium without FCS), n = 3. P-values were determined by two-way ANOVA and Tukey’s multiple comparisons correction, one phase exponential curves were plotted, including the SD. (D) Limiting dilution assays were performed using OE19, OE33, and 007B cells. Control (gray bars) and trastuzumab and pertuzumab (TP) incubated cells (colored bars) were sorted into 96 well plates. Bar graphs show means ± SEM, n = 3. *P < .05, **P < .01, and ***P < .001. Trastuzumab or pertuzumab was maintained during all assays.

**TGFB1** and EMT-related genes associate with growth factor receptor-low EACs.

To gain insight in the possible mechanism of the observed phenotypic transition, we first performed GSEA of published EMT signatures on a publicly available gene expression set containing non-pretreated resected EAC specimens (https://gdc-portal.nci.nih.gov/projects/TCGA-ESCA). Based on the well-defined association of HER3 with an epithelial cancer cell state, samples were dichotomized by median **ERBB3** expression, and analysis showed a significant association of two pooled previously published EMT gene sets with low **ERBB3**
expression (Figure 4A). Furthermore, high ERBB3 expressing tumors associated with an epithelial signature (Supplementary Figure 10A), corroborating our experimental data pointing to a role for HER3 in maintaining an epithelial phenotype. Previous studies identified TGF-β as an important mediator of EMT. To confirm this in the TCGA EAC set, TGFB1 gene expression was correlated with EMT related genes, which all showed highly significant correlations (Supplementary Figure 10B). To determine a potential association of TGFB1 with EMT in EAC following ERBB inhibition, ERBB3 and TGFB1 gene expression correlation was performed on the same TCGA gene expression set. This revealed a significant negative correlation between TGFB1 and ERBB3 (Figure 4B), and a positive correlation between ERBB3 and the epithelial marker EPCAM (Supplementary Figure 10C). Together, these data point to TGF-β as a likely candidate to induce EMT following ERBB inhibition.

**EMT following growth factor receptor inhibition is induced via TGF-β in a non-cell autonomous manner.**

To functionally test if TGF-β was indeed responsible for ERBB inhibition-mediated EMT, we cultured 6-month trastuzumab incubated cells for 10 days with a low concentration of TGFβ receptor inhibitor (A83-01, 100nM) in the presence of trastuzumab, and indeed observed a return to an epithelial phenotype (Figure 5A). Previous studies reported a role for microRNAs (miRs) of the miR-200 family to regulate the balance between epithelial and mesenchymal states. We indeed observed decreased miR-200 family expression following ERBB inhibition (Supplementary Figure 11). Both reactivation of the HER2-HER3 signaling axis by NRG-1β, and TGF-β blocking rescued the epithelial phenotype and restored miR-200 expression levels (Supplementary Figure 11). Furthermore, autocrine TGF-β signaling is reported to be required to mediate sustained ZEB expression, and maintain a stable mesenchymal phenotype, supporting the pivotal role of TGF-β as a mediator of EMT in our model system.

To further investigate if TGF-β was secreted by long-term ERBB-inhibited tumor cells, supernatants of OE19 and 007B cells, either control or incubated with trastuzumab and...
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pertuzumab for 2 months, were used to determine phospho-SMAD2-3 activation in 293T cells. These cells lack endogenous TGF-β pathway-activating ligands. Supernatant of the cells cultured with ERBB inhibitors effectively induced SMAD2-3 activation (Figure 5B). Using TGF-β1-2-3 neutralizing antibody, we confirmed specificity (Figure 5C). These results show that tumor cells can produce and secrete active TGF-β ligands following ERBB-targeting. We reasoned that if TGF-β ligand production is indeed the mechanism mediating EMT in EAC cells, stimulating control (naive) cancer cells with recombinant TGF-β should show the same effect. Indeed, following incubation of OE19 and 007B cells for 10 days with TGF-β ligand, all cells underwent EMT (Figure 5D). As supernatants of ERBB-inhibited cells were able to activate the SMAD2-3 pathway in 293T cells (Figure 5B), we reasoned that TGF-β signaling could also act in a paracrine manner on other tumor cells. To confirm this, control and 2-month trastuzumab and pertuzumab incubated cells were transduced with constructs for green and red fluorophores. These populations were then mixed and co-cultured for 10 days before the two populations were again separated by FACS sorting (Figure 5E). RNA was isolated and the expression of EMT related genes revealed that the control (naive) population, which was mixed with resistant TGF-β-producing cells, now also showed an increased expression of EMT related genes (Figure 5F). Unmixed transduced populations were subjected to the same sorting procedure as a control.

To confirm inhibition of downstream ERBB signaling, (p)ERK and (p)AKT levels were determined. pAKT is known to be activated upon HER2-HER3 dimerization. Indeed, in the control condition we observed that pAKT was induced, whereas following 2-month trastuzumab and pertuzumab incubation pAKT levels were low. The induction of EMT following ERBB inhibition was accompanied by a shift in the downstream oncogenic signaling pathway towards pERK, suggesting that in this situation pathways other than the canonical HER2-HER3 signaling are activated. This was accompanied by an increase in pSMAD2-3 levels (Figure 5G). Together these results show that TGF-β secretion can non-cell autonomously induce EMT in a population of cells incubated with ERBB inhibitors and thereby confer resistance. This also suggests that although ERBB inhibitors may not reach every cell within the tumor efficiently, the resulting mesenchymal phenotype can be conferred to cells across a wide range in the tissue, amplifying the potential for resistance and metastasis.

**Combined targeting of growth factor receptor and TGF-β signaling inhibits tumor growth.**

To further investigate the druggability of this mechanism we identified to mediate EMT, we blocked both the ERBB pathway using trastuzumab and pertuzumab as well as TGF-β signaling using either a TGF-β neutralizing antibody or a TGF-β receptor inhibitor. Data obtained in naive OE19 and 007B cells, receiving this triple combination, showed up to 80% cell death by dye exclusion (Figure 6A), and a strong anti-proliferative effect as determined by cell counting (Figure 6B).

To confirm these findings *in vivo*, tumor-bearing mice (007B) were injected once a week intraperitoneally with PBS control, single inhibition using trastuzumab, pertuzumab, or TGFβ pathway inhibitor (A83-01), double inhibition using trastuzumab and pertuzumab or triple inhibition using trastuzumab and pertuzumab+A83-01. Weight of the animals and tumor growth were measured twice a week. As expected, an inhibitory effect of trastuzumab on
Figure 5. EMT following growth factor receptor inhibition is induced via TGF-β in a non-cell autonomous manner.

Panel A: Immunofluorescence images showing OE19 and OE19 trastuzumab + A83-01.

Panel B: Western blot analysis of 293T + OE19 sup and 007B sup with bands for pSMAD2/3, SMAD2/3, and GAPDH.

Panel C: Western blot analysis of 293T control with bands for pSMAD2/3, SMAD2/3, and GAPDH.

Panel D: OE19 and OE19 + TGF-β (10 days) images.

Panel E: Flowchart showing cell culture, plating, sorting, and RNA isolation.

Panel F: Graph showing relative to GAPDH for untreated, trastuzumab + pertuzumab, mix untr. population, mix TP population, and mix TP population.

Panel G: Western blot analysis of OE19 and 007B with bands for pAKT, AKT, pERK, ERK, and GAPDH.
tumor growth could be observed (Figure 6C). This effect was the same for the single agent pertuzumab. Combined injection with trastuzumab and pertuzumab delayed tumor growth more efficiently than the single agents. However, the most significant effect on tumor growth delay was observed in mice receiving the triple drug combination of trastuzumab and pertuzumab+A83-01. In contrast, single A83-01 injection showed no tumor inhibitory effect at all. The triple combination was well tolerated and no weight loss was observed (Supplementary Figure 13A).

To exclude that the trastuzumab and pertuzumab+A83-01 combination enriched a dormant population that could mediate tumor outgrowth at later passages, we performed serial grafting experiments (Figure 6F). These demonstrated that whereas control tumors consistently grafted, the tumors which received the triple combination occasionally failed to take, arguing against the enrichment of a dormant cell population that could mediate tumor regrowth. Also, the tumors of the control group grew faster compared to the group which received the triple combination. Further analysis corroborated that tumors which received trastuzumab and pertuzumab had increased levels of EMT- and CSC-related genes, and a downregulation of epithelial markers compared to the control mice. This effect could also be mitigated by the addition of A83-01 to the trastuzumab and pertuzumab combination (Figure 6D, E and Supplementary Figure 13B). Finally, the morphology of the 007B PDX tumors was analyzed (Supplementary Figure 12), and a poorly differentiated morphology was observed after injection with trastuzumab and pertuzumab, which was partly rescued by adding A83-01 to trastuzumab and pertuzumab. These data suggest an efficient antitumor activity of combined HER2-HER3 and TGF-β signaling inhibition, thereby suggesting this triple combination could constitute a new treatment strategy.

Figure 5. EMT following growth factor receptor inhibition is induced via TGF-β in a non-cell autonomous manner. (A) 6-month trastuzumab cultured OE19 and OE33 cells, were incubated for 10 days with a TGF-β receptor inhibitor (A83-01 at 100nM), and morphology was assessed by phase-contrast microscopy. Trastuzumab was maintained during the assay. Scale bar: 100µm. (B) OE19 and 007B cells were incubated with trastuzumab and pertuzumab (TP) for 2 months, supernatant was used to stimulate 293T cells. TGF-β neutralizing antibody was used as a control for TGF-β-induced phosphorylation of SMAD2-3. Supernatant of the control cancer cells was used as control. (C) As for panel B using control medium, 1ng/ml recombinant TGF-β as a positive control, and with TGF-β neutralizing antibody as a control for TGF-β ligand specificity of the effect on pSMAD2-3 observed. (D) OE19 and 007B cells were incubated with 5ng/ml TGF-β for 10 days, and morphology was assessed by phase-contrast microscopy. Scale bar: 100µm. (E) Diagram explaining experimental setup: control cells were transduced with a Venus fluorescent construct (green), and the 2-month trastuzumab and pertuzumab (TP) incubated cells with an mCherry fluorescent construct (magenta). These cells were mixed, (except for the control conditions), and cultured for 10 days, prior to fluorescence-activated cell sorting, separating the former control and incubated conditions by color. This was followed by RNA isolation. (F) Quantitative Reverse Transcription-PCR for EMT genes in OE19 cells cultured as shown in E, open bars represent the unmixed control conditions, filled bars the mixed conditions. Bars show the mean ± SD, technical triplicates of two experiments. (G) OE19 and 007B cells were cultured with trastuzumab and pertuzumab (2 months), and downstream components of ERBB and TGF-β signaling were analyzed.
Chapter 6

Figure 6. Combined targeting of growth factor receptor and TGFβ signaling inhibits tumor growth.

A

OE19

007B

B

OE19

007B

C

007B PDX

D

ZEB1  VIM  SNAI2  CDH2

E

007B PDX serial passaging 2

F

007B PDX serial passaging 3


**Discussion**

Previous studies have shown dichotomous roles for ERBB signaling and their contribution to EMT. In breast cancer, HER2 targeting has been described to inhibit as well as induce EMT.\(^{20, 21, 35, 36}\) The same holds true for HER3, for which studies have shown both an inhibitory and a stimulatory effect on EMT.\(^{37, 38}\) Here, we investigated the effect of single HER2 and dual HER2-HER3 targeting, and found that ERBB signaling functions to retain an epithelial phenotype in EAC. Inhibiting signaling through these receptors induced a mesenchymal phenotype, which could be rescued by re-activation of the HER2-HER3 signaling axis. Silencing *ERBB2* by RNA interference induced the same mesenchymal phenotype, but also resulted in decreased *ERBB3* expression. We speculate that when cells have fully transitioned to a mesenchymal state, they no longer require the HER2-HER3 signaling axis, and downregulate both components.

Besides maintaining an epithelial morphology, HER3 is reported to mediate resistance towards different targeted agents in various cancer types.\(^{15-18, 39}\) HER3 or HER2-HER3 dimer targeting is currently investigated in several clinical trials. We found, however, that combined HER2-HER3 targeting accelerated EMT, highlighting the dynamic nature of cancer cells and urging caution: Targeting a protein that mediates acquired resistance to an oncogene-targeted drug can, in the long run, induce an aggressive phenotype.

Studying the sequence of EMT induction following ERBB targeting we found that a subpopulation of cells exists that is instructed to undergo EMT following ERBB inhibition. This population subsequently acquires a higher proliferative profile, which then contributes to its selection under continued therapeutic pressure. This effect is reversible when drug induced pressure is released. Given the plasticity of EMT we observe in our model system, drug holidays could be considered to achieve an optimal treatment regimen during which patients will be less at risk for developing resistance or metastatic disease.

\[\text{Figure 6. Combined targeting of growth factor receptor and TGF-\(\beta\) signaling inhibits tumor growth. (A) Representative flow cytometry images of OE19 and 007B cells, cultured in the indicated conditions for 2 weeks; control, A83-01, trastuzumab, pertuzumab, trastuzumab and pertuzumab (TP), trastuzumab and pertuzumab+A83-01 (TP+A83-01) (TGF-\(\beta\) receptor inhibitor). Cell death was determined by flow cytometry using propidium iodide (PI). (B) Cell proliferation was determined by calculating the amount of cells in the life fraction of the conditions as in A, using CountBright Counting Beads. (C) Early passage 007B cells were injected subcutaneously in the flank of NSG mice. Mice with tumors reaching a size of 50-100 mm\(^3\) were injected intraperitoneally with the following compounds: PBS control, 1mg/kg trastuzumab, 1mg/kg pertuzumab, 1mg/kg TGF-\(\beta\) pathway inhibitor (A83-01), 1mg/kg trastuzumab and pertuzumab (TP), or 1mg/kg trastuzumab and pertuzumab+A83-01 (TP+A83-01), once a week (5 mice per group). Tumor growth was measured twice a week, and values were normalized to tumor size at the start of the injections with the inhibitors. Survival analysis, ****\(P < .0001\). (D) qRT-PCR for selected EMT genes in 007B patient-derived xenograft (PDX) tumors injected with the inhibitors as for panel C. (E) Flow cytometry for stem cell markers in the same PDX tumors. Bar graphs show means ± SEM, \(n = 3\). *\(P < .05\), **\(P < .01\), and ***\(P < .001\) were determined by two-sided unpaired \(t\) tests and analyzed against the control group. (F) Serial passaging of the control and trastuzumab and pertuzumab+A83-01 (TP+A83-01) PDX tumors as shown in panel C. Tumor pieces of 4mm\(^3\) were re-grafted subcutaneously and grown for 5 weeks. For the third and final grafting, tumor pieces of 4mm\(^3\) from the previous passaging were again inserted subcutaneously and grown for 4 weeks.}\]
EMT is associated with poor disease outcome. This has been attributed to the migratory capacity of these cells, and resulting propensity for metastasis.\textsuperscript{40, 41} Recently, two studies reported that the mesenchymal phenotype is related to chemo-resistance rather than metastatic dissemination.\textsuperscript{42, 43} We found proof for both increased migration and chemo-resistance upon EMT following ERBB inhibition. Importantly, targeted therapy is regularly combined with cytotoxic agents, making the latter finding of great clinical importance. For instance, conventional chemo-therapeutics combined with trastuzumab are currently investigated in phase III clinical trials in the neo-adjuvant setting; RTOG 1010 study (NCT01196390, National Cancer Institute (NCI)) and the INNOVATION study (NCT02205047, European Organization for Research and Treatment of Cancer (EORTC)).

Remarkably, we found EMT-associated chemo-resistance to several chemotherapeutics. The exact mechanism that mediates this resistance remains to be determined, however, previous studies have identified purported CSC populations to harbor general resistance mechanisms like efflux pumps.\textsuperscript{44} Additionally, EMT induction has previously been linked to the stem-like state.\textsuperscript{45} Here we report the increased expression of CSC markers upon ERBB inhibition, which could explain the broad resistance to therapeutics but also the increased clonogenicity. Observing enhanced migration, chemo-resistance, and clonogenicity following initially successful treatment in our models is alarming, considering that these all contribute to resistant and metastatic disease. However, the identification of a single targetable molecule that mediates all these processes is of great clinical value. As EMT induction is mediated by TGF-\textbeta\textsuperscript{27}, a factor predicting prognosis, we considered TGF-\textbeta a likely candidate. Targeting TGF-\textbeta signaling in trastuzumab-induced EMT-like cells indeed reversed this transition. Furthermore, we showed that TGF-\textbeta activation was mediated in a non-cell autonomous manner and was produced by the cancer cells themselves rather than stromal cells, which are absent from the majority of our models. Therefore, we hypothesized that combined targeting of both the HER2-HER3 pathway and TGF-\textbeta signaling would result in decreased tumor growth. Indeed, both \textit{in vitro} as \textit{in vivo} assessment of this combination, showed an effective anti-tumor response. Targeting TGF-\textbeta is currently investigated in clinical trials using either ligand or receptor inhibitors.\textsuperscript{46}

In summary, our data elucidate the role of ERBB signaling in the maintenance of an epithelial phenotype in HER2-positive EAC and demonstrate that TGF-\textbeta, secreted by tumor cells following HER2-HER3 targeting, induces EMT non-cell autonomously. Targeting both signaling pathways resulted in a marked anti-tumor effect, thereby providing a new treatment strategy for EAC. Given the availability of TGF-\textbeta targeting agents, these results can readily be translated in clinical studies.
EMT mediates resistance to ERBB targeting

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Conflicts of interest
The authors declare no conflict of interest. HWML has acted as a consultant for Eli Lilly and Company, and Nordic Pharma Group, and has received unrestricted research grants from, Amgen, Bayer Schering Pharma AG, Celgene, Eli Lilly and Company, GlaxoSmithKline Pharmaceuticals, Nordic Pharma Group, Roche Pharmaceuticals. MFB has received research funding from Celgene. None of these were involved in drafting of the manuscript.

Abbreviations
CSC: cancer stem cells; EAC: esophageal adenocarcinoma; EMT: epithelial to mesenchymal transition; Erb-b2/HER/ERBB: human epidermal growth factor receptor; GSEA: gene Set Enrichment Analysis; MiRs: microRNAs; PBS: phosphate-buffered saline; NSG mice: NOD. Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice; RTK: receptor tyrosine kinase; TGF-beta/TGF-β1: transforming growth factor beta; VIM: vimentin

Author contributions
• Conception and design of the study; EAE, JPM, MFB, HWML
• Generation, collection, assembly, analysis and/or interpretation of data; EAE, AS, EF, PS, JL, KKK, LV, MFB, HWML
• Drafting or revision of the manuscript; EAE, MFB, HWML
• Approval of the final version of the manuscript; EAE, AS, EF, PS, JL, KKK, LV, JPM, MFB, HWML

Supplementary Material
Supplementary data accompany this paper.
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REFERENCES

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epithelial-to-mesenchymal transition and stemness maintenance of cancer cell lines. Stem Cells 2013;31:2827-32.


Supplementary Figure 1. Increased expression of EMT related genes following HER2 inhibition. (A) qRT-PCR for EMT genes in OE19 cells cultured as for Figure 1C (trastuzumab, T). Bar graphs show means ± SEM, n = 3. *P < .05, **P < .01, and ***P < .001 were determined by two-sided unpaired t tests and analyzed against the control. (B and C) Cytofluorimetric dot plots belonging to Figure 1C and D. (D) HER2 SISH of control and 6-month trastuzumab cultured OE19 and OE33 cells. Scale bar: 200 µm.
Supplementary Figure 2. Increased expression of EMT-related genes upon growth factor receptor targeting by shRNA. (A) Hairpins against ERBB2, and scrambled control (shc002), were obtained from the Mission TRC library and targeting sequences were cloned into the inducible pLKO-Tet-On vector. Stable OE19 and OE33 ERBB2 knockdown lines were established using lentiviral delivery. Following induction by doxycycline, cells were cultured for 2 weeks, and morphology was assessed by phase-contrast microscopy. Scale bar: 100 µm. (B) qRT-PCR for selected EMT genes in OE19 cells in the same conditions as for panel A. Bar graphs show means ± SEM, n = 3. *P < .05, and **P < .01 were determined by two-sided unpaired t tests and analyzed against the shc002 control. (C) Cell surface staining by flow cytometry for epithelial, mesenchymal, and stem cell markers in OE19 cells transduced with the hairpins as in panel A. Bar graphs show means ± SEM, data was normalized to the control condition, n = 3. *P < .05, **P < .01, and ***P < .001. (D) As for panel C, using OE33 cells. (E) qRT-PCR for ERBB2 and ERBB3 in OE19 cells in the same conditions as for panel A. To exclude a non-specific effect of doxycycline, control silenced cells were also incubated with doxycycline.
Supplementary Figure 3. Dot plots from flow cytometry for the CSC markers as show in Supplementary Figure 2. (A and B) For OE19 cells stably transduced with shRNA clone #1 (A) and #2 (B). (C and D) As panel A, for the OE33 cell line.

Supplementary Figure 3. Increased expression of EMT related genes following growth factor receptor inhibition by shRNA.
Supplementary Figure 4. High concentrations of trastuzumab induce EMT. (A) OE19 and OE33 cells were continuously cultured for 2 months in the presence of 10µg/ml or 100µg/ml trastuzumab. Cells cultured without inhibitors were used as control. Morphology was assessed by phase-contrast microscopy. Scale bar: 100 µm. (B) qRT-PCR for EMT genes in OE19 cells cultured as for panel A. (C) Cell surface staining for stem cell markers of OE19 cells cultured as for panel A. Bar graphs show means ± SEM, n = 3. *P < .05, **P < .01, and ***P < .001. Statistical significance was determined by two-sided unpaired t tests and compared against the control. (D) Cytofluorimetric dot plots for data shown in panel C.
Supplementary Figure 5. Growth factor receptor inhibition does not induce EMT in a HER2-negative EAC cell line. (A) Flo-1 cells were continuously cultured for 2 months in the presence of 1 µg/ml trastuzumab or 1 µg/ml trastuzumab and pertuzumab. Cells cultured without inhibitors were used as control. Morphology was assessed by phase-contrast microscopy. Scale bar: 100 µm. (B) qRT-PCR for EMT genes in Flo-1 cells as for panel A. (C) Cell surface staining for stem cell markers on cells as shown in panel A. Bar graphs show means ± SEM, n = 3. *P < .05, **P < .01, and ***P < .001. Statistical significance was determined by two-sided unpaired t tests and compared against the control. (D) Cytofluorimetric dot plots for panel C.
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Supplementary Figure 6. Reactivation of growth factor receptor signaling reduces stem cell marker expression and cell viability. (A) Cell surface staining for stem cell markers of OE19 cells cultured for 6 months with 1µg/ml trastuzumab (T), followed by an additional incubation with 1ng/ml NRG-1β for 10 days (T+NRG-1β) (as for Figure 1 panel E). Bar graphs show means ± SEM, n = 3. *P < .05, **P < .01, and ***P < .001 were determined by two-sided unpaired t tests and analyzed against the control. (B) As for panel A, using OE33 cells. (C and D) Cyt fluorometric dot plots for panels A and B. (E) Cell viability of OE19 cells as for panel A. (F) Cell viability of OE33 cells as for panel B. Trastuzumab was maintained during the assay in those conditions where ‘trastuzumab’ is indicated.
Supplementary Figure 7. Increased expression of EMT related genes following simultaneous HER2-HER3 inhibition. (A) qRT-PCR for selected EMT genes in 007B cultures as for Figure 2 panel A. Bar graphs show means ± SEM, n = 3. *P < .05, **P < .01, and ***P < .001 were determined by unpaired t tests and analyzed against the control for each gene. (B) As for panel A, using 031M cultures.
Supplementary Figure 8. Acquired resistance following growth factor receptor inhibition is reversible. (A) OE19 cells were cultured in the following conditions: control, trastuzumab and pertuzumab (TP) for 48h, continuous 2-month trastuzumab and pertuzumab, continuous 2-month trastuzumab and pertuzumab followed by withdrawal of trastuzumab and pertuzumab for 1 week, and withdrawal of trastuzumab and pertuzumab for 2 weeks. Bar graphs are means ± SEM, and normalized to the control condition, n = 3. P-values were determined by two-sided unpaired t tests. (B) As for panel A, using 007B cultures. (C) Control and long-term trastuzumab and pertuzumab (TP) cultured OE19 cells were sorted in a 10% EPCAM high and low population. These cells were either kept on trastuzumab and pertuzumab, or no inhibitors, and EPCAM expression was measured at the indicated time points. Graphs are means ± SEM, n = 3. (D) As for panel C, using 007B cultures. (E) Colored-based lineage tracing of cell lines under drug pressure. OE19 and OE33 cells were transduced with a mixture of three lentiviral constructs containing Venus, mCherry, and Cerulean to create a population of cells with unique colors. Cells were then cultured for 2 months in the following conditions; control, trastuzumab (T), trastuzumab and anti-HER3 (T+a-3), and trastuzumab with pertuzumab (TP) as for Figure 2. Cells were imaged on an EVOS (Thermo Fisher Scientific, Waltham, MA, USA) fluorescence microscope, and the number of distinct cell clusters as identified by color was quantified per optical field. For this a dedicated script was developed in MatLab. (F) Size of distinct cell clusters is shown relative to imaged optical field. To minimize the loss of clones culturing, cells were passed twice a week at high density (1:3 for OE19, 1:5 for OE33) instead of once a week at low density (1:6 for OE19, 1:10 for OE33). Graphs show first quartile, median, third quartile, n = 3.
Supplementary Figure 9. Short-term growth factor receptor inhibition does not confer resistance to chemotherapy. (A) Cell viability of OE19 cells incubated with the following conditions; acute chemotherapy on naïve cells (Chemotherapy only), cells exposed continuously to trastuzumab and pertuzumab for 2 months and then incubated with chemotherapy (either carboplatin, cisplatin or paclitaxel) for 144 hours (Long term trastuzumab and pertuzumab + chemo), and naïve cells which received trastuzumab and pertuzumab + chemotherapy simultaneously (Acute trastuzumab and pertuzumab + chemo). Chemotherapeutic agents were used at concentrations indicated on X-axis. Graphs show means ± SEM, and are normalized to the control condition, n = 3. P-values were determined by two-way ANOVA and Bonferroni correction. (B) As for panel A, using OE33 cells.
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Supplementary Figure 10. High ERBB3 expression positively correlates with an epithelial gene signature. (A) EAC samples from the TCGA set, dichotomized by median ERBB3 expression, were used. ES, enrichment score; NES, normalized enrichment score. Epithelial gene signature consisted of the following genes: CDH1, MAL2, MAP7, RAB25, CLDN4, ELF3, SPINT2, MARVELD2, ST14, CD24, AP1M2, MAPK13, DSP, ERBB3, GALNT3, AP1M2, CLDN7, ST14, KRT19, TMPRSS4, GPX2, TOX3, PRSS8, PKP3, SPINT1, MARVELD3, CEACAM5, CGN, MYH14, FXYD3, MAP7, CEACAM6. (B) TGFB1 gene expression was correlated with indicated EMT-related genes using the EAC TGCA set. (C) Gene expression correlation between ERBB3 and EPCAM was performed on the same TCGA tumor set. P-value and the R-value were determined by linear regression analysis.
Supplementary Figure 11. EMT following HER2 inhibition is accompanied by downregulated miR200-expression, which is rescued by TGF-β pathway inhibition or reactivation of HER2-HER3 signaling. (A) OE19 cells cultured with trastuzumab for 6 months were either incubated with or without 500nM TGF-β receptor inhibitor (A83-01) for 10 days. Expression of miR-200 family members was measured by qRT-PCR. Shown here are values relative to SNORD44. Bar graphs show means ± SEM, n = 3. *P < .05, **P < .01, and ***P < .001 were determined by two-sided unpaired t tests and analyzed against the trastuzumab condition for each miR. (B) As for panel A, using OE33 cells. (C) As for panel A, treating the cells with 1ng/ml NRG-1β for 10 days instead of A83-01. (D) As for panel C, using OE33 cells. Trastuzumab was maintained during the assays.
Supplementary Figure 12. Histology of 007B patient-derived xenograft (PDX) tumors following injections. 007B PDX tumors from experiments shown in Figure 6 panel C were stained with hematoxylin and eosin (HE) and morphology was assessed by brightfield microscopy. Scale bar: 200 μm.
Supplementary Figure 13. Combined targeting of growth factor receptor and TGF-β signaling in vivo. (A) Early passage 007B cells were injected subcutaneously in the flank of NSG mice. Mice with tumors reaching a size of 50-100 mm³ were injected intraperitoneally with the following compounds: PBS control, 1mg/kg trastuzumab, 1mg/kg pertuzumab, 1mg/kg TGF-β receptor inhibitor (A83-01), 1mg/kg trastuzumab and pertuzumab (TP), or 1mg/kg trastuzumab and pertuzumab+A83-01 (TP+A83-01), once a week (5 mice per group). Weight of the animals was measured twice a week, graph show the weight per group of the animals before they were sacrificed. (B) Cytofluorimetric dot plots belonging to Figure 6 panel E.
## Supplementary Tables

### Supplementary Table 1. List of antibodies and dilutions used.

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<td>197G2, Cell Signaling</td>
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</tr>
<tr>
<td>anti-ERK1/2</td>
<td>1:1000</td>
<td>137F5, Cell Signaling</td>
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<tr>
<td>anti-pAKT, Ser473</td>
<td>1:1000</td>
<td>D9E, Cell Signaling</td>
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<tr>
<td>anti-AKT</td>
<td>1:1000</td>
<td>9272, Cell Signaling</td>
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<tr>
<td>anti-GAPDH</td>
<td>1:5000</td>
<td>6C5, BioConnect</td>
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<tr>
<th>Secondary</th>
<th>Dilution</th>
<th>Source</th>
<th>Cat. No.</th>
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<tbody>
<tr>
<td>HRP-conjugated Goat anti rabbit</td>
<td>1:5000</td>
<td>7074, Cell Signaling</td>
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<td>HRP-conjugated Goat anti mouse</td>
<td>1:5000</td>
<td>1031-05, Southern Biotech</td>
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\(^a\)These antibodies detect (p)SMAD3 as well, due to high sequence homology.

Supplementary Table S2. Primer sequences for quantitative RT-PCR.

<table>
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<tr>
<th>Transcript</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>GAPDH–forward:</td>
<td>5'-AATCCCATCACCATCTTTCCA-3'</td>
</tr>
<tr>
<td>GAPDH–reverse:</td>
<td>5'-TGGACTCCAGCATGCTACTCA-3'</td>
</tr>
<tr>
<td>ZEB1–forward:</td>
<td>5'-GCACAAGAAGGCCACAGTA-3'</td>
</tr>
<tr>
<td>ZEB1–reverse:</td>
<td>5'-GCAAGACAGTTCAGGGTGTC-3'</td>
</tr>
<tr>
<td>VIM–forward:</td>
<td>5'-CCCTCAGCTGATAGGGAT-3'</td>
</tr>
<tr>
<td>VIM–reverse:</td>
<td>5'-TCCAGACGGTCATGCTAGT-3'</td>
</tr>
<tr>
<td>SNAI2–forward:</td>
<td>5'-GGCCAAGATCTTTCAAGC-3'</td>
</tr>
<tr>
<td>SNAI2–reverse:</td>
<td>5'-CAGTGGCCACTGCAAGG-3'</td>
</tr>
<tr>
<td>CDH2–forward:</td>
<td>5'-ACAGTGCGCCACCATGAAAG-3'</td>
</tr>
<tr>
<td>CDH2–reverse:</td>
<td>5'-CCGAGATGCGGCTGATAAG-3'</td>
</tr>
<tr>
<td>ERBB3–forward:</td>
<td>5'-TGGGGAACCTTGAAGATTGT-3'</td>
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<tr>
<td>ERBB3–reverse:</td>
<td>5'-GAGGAGTGGGCAATGCTAGAG-3'</td>
</tr>
<tr>
<td>CDH1–forward:</td>
<td>5'-TGGCCAGAAAAATGAAAAGG-3'</td>
</tr>
<tr>
<td>CDH1–reverse:</td>
<td>5'-GGTATGTGGCACAATGGTC-3'</td>
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<tr>
<td>KRT19–forward:</td>
<td>5'-CTTGAGATCTTCAATGGTTG-3'</td>
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<tr>
<td>KRT19–reverse:</td>
<td>5'-CTAGGAGAGATGATGGGGA-3'</td>
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<td>CD44–forward:</td>
<td>5'-TGGCAAGACACAACTCTTG-3'</td>
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<td>CD44–reverse:</td>
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<td>CD133–forward:</td>
<td>5'-TCCAGAGAATTTCCTACATG-3'</td>
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<td>CD133–reverse:</td>
<td>5'-CAGCAAGACAGCAATGGAC-3'</td>
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<tr>
<td>LGR5–forward:</td>
<td>5'-ACAGAATGCGCTTTGGAAC-3'</td>
</tr>
<tr>
<td>LGR5–reverse:</td>
<td>5'-TCCCAGGGAGTGATTCTAT-3'</td>
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