Cancer Biology and Signal Transduction

ERBB3/HER2 Signaling Promotes Resistance to EGFR Blockade in Head and Neck and Colorectal Cancer Models

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Abstract

EGFR blocking antibodies are approved for the treatment of colorectal cancer and head and neck squamous cell carcinoma (HNSCC). Although ERBB3 signaling has been proposed to limit the effectiveness of EGFR inhibitors, the underlying molecular mechanisms are not fully understood. To gain insight into these mechanisms, we generated potent blocking antibodies against ERBB3 (REGN1400) and EGFR (REGN955). We show that EGFR and ERBB3 are coactivated in multiple HNSCC cell lines and that combined blockade of EGFR and ERBB3 inhibits growth of these cell lines more effectively than blockade of either receptor alone. Blockade of EGFR with REGN955 strongly inhibited activation of ERK in HNSCC cell lines, whereas blockade of ERBB3 with REGN1400 strongly inhibited activation of Akt; only the combination of the 2 antibodies blocked both of these essential downstream pathways. We used a HER2 blocking antibody to show that ERBB3 phosphorylation in HNSCC and colorectal cancer cells is strictly dependent on association with HER2, but not EGFR, and that neuregulin 1 activates ERBB3/HER2 signaling to reverse the effect of EGFR blockade on colorectal cancer cell growth. Finally, although REGN1400 and REGN955 as single agents slowed the growth of HNSCC and colorectal cancer xenografts, the combination of REGN1400 plus REGN955 caused significant tumor regression. Our results indicate that activation of the Akt survival pathway by ERBB3/HER2 limits the effectiveness of EGFR inhibition, suggesting that REGN1400, which is currently in a phase I clinical trial, could provide benefit when combined with EGFR blocking antibodies.

Introduction

EGFR is a validated driver of human cancer. Activating mutations in the EGFR kinase domain are found in a subset of patients with non-small cell lung cancer (NSCLC), and these mutations are associated with responsiveness to EGFR tyrosine kinase inhibitors (1, 2). In addition, antibodies (cetuximab and panitumumab) that block binding of ligands to EGFR are approved for the treatment of colorectal cancer and HNSCC (3–8). However, responses to single-agent EGFR blocking antibodies are relatively infrequent (only ~10% in colorectal cancer and HNSCC) and transient (4, 5, 7, 9). Thus, there is a clear need for agents that can be used in combination with EGFR blocking antibodies to enhance their clinical benefit.

Signaling by ERBB3, a member of the ERBB family of receptor tyrosine kinases (RTK), has recently been identified as a prominent mechanism of tumor resistance to targeted therapies (10–17). Although ERBB3 does not have significant tyrosine kinase activity, it is phosphorylated following heterodimerization with other ERBB family members (18, 19). The regulatory subunit of phosphatidylinositol 3-kinase (PI3K) is recruited to multiple phosphorylation sites in the ERBB3 cytoplasmic domain, resulting in strong activation of the PI3K/Akt survival pathway (20–22).

Several recent reports suggest that ERBB3 signaling may promote resistance to EGFR blockade in colorectal cancer and HNSCC models. High intratumoral levels of the ERBB3 ligand neuregulin 1 (NRG1) are associated with worse response to cetuximab in patients with colorectal cancer, suggesting the possibility that active ERBB3 signaling can rescue tumor cells from the effects of EGFR blockade (23). Consistent with that possibility, addition of NRG1 to cultured colorectal cancer cells can rescue tumor cells from the effects of EGFR blockade (23). Consistent with that possibility, addition of NRG1 to cultured colorectal cancer cells significantly diminishes the ability of cetuximab to inhibit cell growth (23). Furthermore, combined blockade of EGFR and ERBB3 inhibits HNSCC cell/xenograft growth more effectively than cetuximab (24–26). Consistent with a potential role for ERBB3 signaling in HNSCC, NRG1 expression and ERBB3 phosphorylation seem to be particularly prominent in HNSCC cell lines and primary tumors compared with other tumor types (27, 28).
Although recent findings suggest a role for ERBB3 signaling in limiting the effectiveness of EGFR antibodies in colorectal cancer and HNSCC, only in a few cases has a functional role for ERBB3 in anti-EGFR resistance been substantiated with the use of a specific blocking antibody (24–26). In addition, the molecular basis for the additive effects of combined EGFR/ERBB3 blockade on cancer cell growth/survival is not fully understood. For example, the relative contributions of EGFR and HER2 to ERBB3 activation in HNSCC and colorectal cancer cells remain unclear. In this report, we used novel ERBB3 (REGN1400) and EGFR (REGN955) blocking antibodies, as well as a HER2 blocking antibody, to investigate the functional role of ERBB3 in modulating the response to EGFR inhibition. Our findings demonstrate that ERBB3 partners with HER2 to limit the effects of EGFR blockade in HNSCC and colorectal cancer cell lines and tumor xenografts, suggesting that REGN1400, which is currently in a phase I trial, might potentiate the effects of EGFR-directed therapies in these indications.

Materials and Methods

Generation of ERBB3 and EGFR blocking antibodies

VelocImmune mice (with genes encoding human immunoglobulin heavy and κ light chain variable regions) were immunized with the entire extracellular domain of human ERBB3 or with a human EGFR-expressing cell line. The antibody immune response was monitored by standard antigen-specific immunoassay. Spleens were harvested for generation of hybridomas or for direct isolation of antigen-positive splenocytes. The cloned human immunoglobulin variable region genes from antibodies exhibiting the desired characteristics were joined to human IgC4 (REGN1400) or IgC1 (REGN955) constant region genes for production in CHO cells. REGN1400 and REGN955 were selected as lead antibodies from hundreds of antigen-positive clones, based on in vitro biochemical properties as well as the ability to inhibit tumor xenograft growth.

Molecular characterization of antibodies

The affinities of REGN1400 and REGN955 for human ERBB3 and EGFR, respectively, were measured in Surface Plasmon Resonance Biacore experiments performed on a Biacore T200 instrument using a dextran-coated (CM5) chip at 37°C. Antibodies were captured through their Fc regions by a goat anti-human Fcγ antibody immobilized on the sensor chips and were tested for binding to the extracellular domains of human ERBB3 or EGFR in monomeric or dimeric (fused to mouse Fc) format. ERBB3 or EGFR proteins at a range of concentrations (from 0.78 to 50 nmol/L) were injected over the captured REGN1400 or REGN955 surfaces. Kinetic parameters were obtained by globally fitting the data to a 1:1 binding model using Biacore T200 Evaluation Software. The equilibrium dissociation constant \( K_D \) was calculated by dividing the association rate constant \( k_a \) by the dissociation rate constant \( k_d \) by the association rate constant \( k_a \).

To test the ability of REGN1400 and REGN955 to block ligand binding, increasing concentrations of the antibodies were incubated for 1 hour with the ERBB3 (0.15 nmol/L) or EGFR (2.5 nmol/L) extracellular domains fused to mouse Fc. The mixtures were then transferred to microtiter plates coated with human NRG1 or human EGF. Following incubation and washing, bound ERBB3-mouse Fc and EGFR-mouse Fc were detected using a horseradish peroxidase (HRP)-conjugated anti-mouse Fc polyclonal antibody. Data analysis was performed with Prism software using a sigmoidal dose–response model.

The ability of REGN1400 and REGN955 to block ligand-dependent signaling was tested in an MCF7 (human breast cancer)–derived reporter cell line in which luciferase expression is driven by a serum-response element. Reporter cells were stimulated for 6 hours with either 0.5 nmol/L NRG1 or 50 pmol/LEGF in the presence of increasing amounts of REGN1400 or REGN955, respectively, or isotype control antibodies. Following treatment, luciferase expression was assessed by measuring luminescence.

The tumor xenograft experiments and some of the in vitro experiments used an afucosylated version of REGN955. The affinities and ligand blocking activities of the afucosylated and normal fucose versions of REGN955 are essentially identical (the affinity and ligand blocking data shown are for the normal fucose version). In addition, the normal fucose version of REGN955 exhibits the same activity as the afucosylated version in the FaDu and LIM1215 xenograft models (data not shown).

To assess the role of HER2 in our models, we generated a HER2 blocking antibody using the heavy and light chain variable sequences of pertuzumab as set forth in US Patent 7,537,931 B2. The ability of this antibody to bind HER2 with the expected affinity and to potently block HER2 signaling was confirmed.

Human tumor cell lines

FaDu, Cal27, SCC-15, and SCC-9 HNSCC cells and A431 epidermoid carcinoma cells were obtained from American Type Culture Collection (ATCC). LIM1215 colorectal cancer cells were obtained from CellBank Australia. All cell lines were authenticated in 2012 or 2013 by short tandem repeat profiling at ATCC/Promega. All experiments were performed with low-passage cell cultures (<passage 10). All cell lines were cultured in the medium and supplements recommended by the vendor.

Receptor tyrosine kinase arrays

The tyrosine phosphorylation status of 49 human RTKs was assessed using the Human Phospho-RTK Array Kit (R&D Systems). Briefly, tumor cells were plated in 6-well plates in serum-containing medium and cultured for 1 to 3 days until the cells were almost confluent. Cells were then washed with PBS and scraped into 0.2 mL of lysis buffer (supplied in the kit) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific).
Phosphorylated RTKs in the cell lysates were detected according to kit instructions.

Cell growth assays

To assess the effects of REGN1400 and REGN955 on tumor cell growth, 2 to 5,000 cells were plated in 96-well plates (n = 8 replicate wells per treatment group) in serum-containing medium. The day after plating, the baseline (0 hour) cell number was determined by MTS assay using the CellTiter96 Aqueous One Solution Cell Proliferation Assay (Promega). Cells were treated at 0, 24, and 48 hours with control antibody, REGN1400, REGN955, HER2 blocking antibody, or various combinations of the antibodies at the concentrations indicated in the figure legends. For the LIM1215 growth assay testing the effect of exogenous NRG1, cells were also treated with 1 nmol/L NRG1 at 0, 24, and 48 hours. At 72 hours, the final cell number was determined by MTS assay (except for SCC-9 cells, which were assayed after 5 days of growth). The relative cell growth for each treatment group was determined by subtracting the baseline MTS reading from the final MTS reading.

Effects of antibodies on tumor cell signaling

To assess the effects of REGN1400 and REGN955 on basal tumor cell signaling, cells were grown in serum-containing medium and treated with antibodies for 2 hours at the concentrations indicated in the figure legend. To test antibody effects on ligand-dependent signaling, cells were serum starved in medium containing 0.5% FBS for 1 hour and then treated with ligands for 30 minutes as indicated in the figure legends. Human NRG1 and EGF were from R&D Systems and were used at 1 and 5 nmol/L, respectively. REGN1400, REGN955, and the HER2 blocking antibody were used at the concentrations indicated in the figure legends. Following treatment, cell lysates were subjected to Western blot analysis with the following antibodies: phospho-ERBB3 (cat. # 4791), EGFR (cat. # 2646), phospho-EGFR (cat. # 2234), Akt (cat. # 9272), phospho-Akt (cat. # 9271), ERK (cat. # 4695), phospho-ERK (cat. # 9101), HER2 (cat. # 2242), and phospho-HER2 (cat. # 2249) antibodies were from Cell Signaling Technology. The ERBB3 antibody (clone 2F12) was from Thermo Scientific.

Tumor xenograft models

Tumor cells (5 × 10⁶ FaDu cells; 10⁷ LIM1215 cells) were implanted subcutaneously into the hind flank of 6- to 8-week-old C.B.-17 SCID mice. Once tumors were established (~200 mm³ in volume), mice were randomized into treatment groups (n = 6–10 mice per group) and injected subcutaneously twice per week with human Fc control protein, REGN1400, REGN955, or the combination of REGN1400 plus REGN955 at the doses indicated in the figure legend. Average tumor growth for each treatment group was calculated by subtracting the tumor volume at the start of treatment from the tumor volume on the day that the Fc-treated control mice were sacrificed (day 41 for FaDu tumors and day 87 for LIM1215 tumors). Growth of combination-treated tumors was monitored for a longer period of time. Average tumor growth in the different treatment groups was compared using one-way ANOVA and Tukey multiple comparison test. All procedures were conducted according to the guidelines of the Regeneron Institutional Animal Care and Use Committee.

Results

Generation of potent, fully-human ERBB3 and EGFR blocking antibodies

Human antibodies against ERBB3 and EGFR were generated using Velocimmune mice as described in the Materials and Methods. The ERBB3 lead antibody REGN1400 and the EGFR lead antibody REGN955, selected from hundreds of antigen-positive clones, exhibit high-affinity binding to their respective targets (~0.3 nmol/L for REGN1400, ~1.0 nmol/L for REGN955; Supplementary Tables S1 and S2) and potently block ligand binding. REGN1400 blocked the binding of NRG1 to dimeric ERBB3 with an IC₅₀ of 0.14 nmol/L (Fig. 1A), whereas REGN955 blocked the binding of EGF to dimeric EGFR with an IC₅₀ of 0.28 nmol/L (Fig. 1B). In a luciferase reporter assay in MCF7 breast cancer cells, REGN1400 and REGN955 potently blocked signaling induced by NRG1 and EGF, respectively (REGN1400 IC₅₀ = 31 pmol/L, REGN955 IC₅₀ = 28 pmol/L; Supplementary Fig. S1).

REGN1400 completely inhibited NRG1-dependent phosphorylation of ERBB3 in A431 epidermoid carcinoma cells (Fig. 1C) and REGN955 blocked EGF-dependent phosphorylation of EGFR in FaDu HNSCC cells (Fig. 1D), confirming that these antibodies inhibit receptor activation in tumor cells. Thus, REGN1400 and REGN955 potently block ligand binding and activation of ERBB3 and EGFR, respectively.

Combined blockade of ERBB3 and EGFR inhibits tumor cell growth more effectively than blockade of either receptor alone

To identify HNSCC and colorectal cancer cell lines in which EGFR and/or ERBB3 are active, we used a phospho-RTK array to simultaneously assess the tyrosine phosphorylation levels of 49 human RTKs. FaDu and Cal27 cells exhibited phosphorylation of both EGFR and ERBB3, as well as HER2 (Fig. 2A and B); SCC-15 cells exhibited strong phosphorylation of EGFR, but very weak phosphorylation of ERBB3 (Fig. 2C); LIM1215 cells exhibited phosphorylation of EGFR, but not of ERBB3 (Fig. 2D); and SCC-9 cells did not have detectable levels of either phospho-EGFR or phospho-ERBB3 (Fig. 2E). In addition to ERBB family RTKs, other phosphorylated RTKs in these cells included MET in Cal27 and SCC-15 cells, IGF-IR in SCC-15 and LIM1215 cells, Axl in Cal27 and SCC-9 cells, and insulin receptor in LIM1215 cells (Fig. 2).

To assess the effect of combined EGFR and ERBB3 blockade on the growth of these tumor cell lines, we
treated cultured cells with either control antibody, REGN955, REGN1400, or the combination of REGN955 plus REGN1400 for 3 days and determined the relative amount of cell growth in each treatment group. Combined blockade of ERBB3 and EGFR inhibited growth/survival of FaDu and Cal27 cells significantly more than blockade of either receptor alone (Fig. 3A and B). In SCC-15 cells, EGFR blockade alone had a significant effect on growth/survival, and this effect was moderately but significantly enhanced by concomitant ERBB3 blockade (Fig. 3C), consistent with the observation that EGFR is strongly phosphorylated in this cell line whereas ERBB3 phosphorylation is weak (Fig. 2C). These results indicate that in HNSCC cell lines in which both ERBB3 and EGFR are active, blockade of both receptors inhibits cell growth more potently than blockade of either receptor alone. EGFR blockade by itself had a very strong effect on LIM1215 colorectal cancer cell growth (Fig. 3D), and this effect was not potentiated by ERBB3 blockade, consistent with the observation that these cells exhibit EGFR, but not ERBB3, phosphorylation (Fig. 2D). None of the treatments had an effect on SCC-9 cell growth (Fig. 3E), consistent with our inability to detect phosphorylated EGFR or ERBB3 in this cell line (Fig. 2E).

To provide further support for the contention that ERBB3 signaling limits the effect of EGFR inhibition, we tested whether addition of NRG1 to LIM1215 cells, which do not exhibit basal phosphorylation of ERBB3, could reverse the potent inhibition of cell growth caused by EGFR blockade. As shown in Fig. 3F, NRG1 treatment completely reversed the effect of REGN955 on LIM1215 cell growth, indicating that ligand-dependent activation of ERBB3 in these cells promotes cell growth/survival in an EGFR-independent fashion.

**ERBB3 partners with HER2 to limit the effectiveness of EGFR blockade in HNSCC and colorectal cancer cells**

To investigate the molecular mechanisms underlying the effects of REGN1400 and REGN955 on HNSCC cell growth, we assessed the ability of these antibodies to inhibit the PI3K/Akt and RAS/RAF/ERK signaling pathways, which play major roles in the regulation of tumor cell survival and proliferation downstream of ERBB receptors (19). In FaDu and Cal27 cells, REGN1400, but not REGN955, strongly inhibited Akt phosphorylation (Fig. 4A and B). In contrast, REGN955 strongly inhibited ERK phosphorylation in both cell
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The observation that REGN1400 and REGN955 have different effects on Akt and ERK activation suggested that ERBB3 and EGFR signal as components of distinct ERBB receptor complexes. Ligand binding to ERBB family receptors stabilizes them in an untethered conformation that promotes formation of homo- and heterodimers (HER2, which has no known ligand, is constitutively available to form heterodimers; refs. 19 and 29–31). To determine the nature of the ERBB receptor signaling complexes that are active in FaDu cells, we assessed the effects of REGN1400, REGN955, and a HER2 blocking antibody on receptor activation. To inhibit HER2 signaling, we generated a HER2 dimerization blocking antibody as a research tool using the heavy and light chain variable sequences of pertuzumab (ref. 32; see Materials and Methods for details). FaDu cells were either unstimulated or stimulated with NRG1 plus EGF, in the presence of control antibody or the ERBB receptor blocking antibodies as single agents or in various combinations. ERBB3 phosphorylation was completely inhibited by REGN1400 and by the HER2 blocking antibody, but was not inhibited at all by REGN955, indicating that ERBB3 phosphorylation is dependent on association with HER2 but not with EGFR (Fig. 4C).

EGFR phosphorylation was completely inhibited by REGN955 but was not inhibited by REGN1400, the HER2 blocking antibody or the combination of REGN1400 plus the HER2 blocker, indicating that EGFR is capable of signaling as a homodimer in these cells (Fig. 4C). However, this observation does not necessarily imply that EGFR signals as a homodimer if HER2 is available for partnering.

HER2 phosphorylation was completely inhibited by the HER2 blocking antibody, was partially inhibited by REGN955, and was not inhibited at all by REGN1400 (Fig. 4C). However, the combination of REGN955 plus REGN1400 completely blocked HER2 phosphorylation (Fig. 4C). These observations suggest that HER2 can partner with either EGFR or ERBB3 and that if only one of the potential partners is blocked, HER2 can be phosphorylated in a complex with the other partner. The finding that HER2 phosphorylation is partially inhibited by REGN955, but slightly increased by REGN1400 (Fig. 4C), suggests that HER2 phosphorylation is stimulated more strongly when it dimerizes with EGFR than when it dimerizes with ERBB3. Phosphorylation of HER2 following association with the inactive kinase ERBB3 may result from the formation of higher order ERBB3/HER2 complexes in which HER2 phosphorylates both itself and ERBB3 (33). Taken together, our signaling data indicate that the ability of the REGN955 plus REGN1400 combination to inhibit tumor cell growth more potently than either single agent is attributable to the inhibition of several distinct ERBB receptor complexes (summarized in the model in Fig. 4D): an EGFR/EGFR homodimer and an EGFR/HER2 heterodimer that signal via the ERK pathway and an ERBB3/HER2 heterodimer that strongly (and uniquely) activates Akt. The ERBB3/HER2 heterodimer, via phosphorylation of HER2, may also contribute to ERK activation in some cells.

To confirm the functional relevance of HER2 as an activator of ERBB3 in HNSCC cells, we assessed the effect of the HER2 blocking antibody on cell growth. Consistent with the observation that either REGN1400 or the HER2 blocking antibody is able to inhibit ERBB3 phosphorylation in FaDu cells, combined treatment with REGN955 and EGFR, although ERBB3 may contribute to ERK activation in some cell lines. The observation that REGN1400 and REGN955 have different effects on Akt and ERK activation suggested that ERBB3 and EGFR signal as components of distinct ERBB receptor complexes. Ligand binding to ERBB family receptors stabilizes them in an untethered conformation that promotes formation of homo- and heterodimers (HER2, which has no known ligand, is constitutively available to form heterodimers; refs. 19 and 29–31). To determine the nature of the ERBB receptor signaling complexes that are active in FaDu cells, we assessed the effects of REGN1400, REGN955, and a HER2 blocking antibody on receptor activation. To inhibit HER2 signaling, we generated a HER2 dimerization blocking antibody as a research tool using the heavy and light chain variable sequences of pertuzumab (ref. 32; see Materials and Methods for details). FaDu cells were either unstimulated or stimulated with NRG1 plus EGF, in the presence of control antibody or the ERBB receptor blocking antibodies as single agents or in various combinations. ERBB3 phosphorylation was completely inhibited by REGN1400 and by the HER2 blocking antibody, but was not inhibited at all by REGN955, indicating that ERBB3 phosphorylation is dependent on association with HER2 but not with EGFR (Fig. 4C).

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To confirm the functional relevance of HER2 as an activator of ERBB3 in HNSCC cells, we assessed the effect of the HER2 blocking antibody on cell growth. Consistent with the observation that either REGN1400 or the HER2 blocking antibody is able to inhibit ERBB3 phosphorylation in FaDu cells, combined treatment with REGN955
plus either REGN1400 or the HER2 blocker strongly inhibited FaDu cell growth (Fig. 5A). Like REGN1400, the HER2 blocking antibody alone had no effect on cell growth (Fig. 5A), consistent with its inability to inhibit EGFR signaling (Fig. 4C). Essentially the same effects were observed in Cal27 cells, where combined treatment with REGN955 plus either REGN1400 or the HER2 blocking antibody inhibited cell growth to the same extent (Fig. 5B). Thus, in 2 HNSCC cell lines, combined blockade EGFR and HER2 (or ERBB3) potently inhibited cell growth, confirming the importance of HER2 as an activator of ERBB3 signaling.

Consistent with the hypothesis that ERBB3 partners with HER2 to limit the effect of EGFR blockade, NRG1 stimulation of LIM1215 colorectal cancer cells (which reverses the effect of REGN955 on cell growth, Fig. 3F) resulted in phosphorylation of both ERBB3 and HER2 (Fig. 5C). Phosphorylation of both ERBB3 and HER2 was inhibited by REGN1400 and the HER2 blocking antibody, but not by REGN955, indicating that ERBB3 phosphorylation in these cells is dependent on dimerization with HER2 but not with EGFR (Fig. 5C). The complete dependence of HER2 phosphorylation on ERBB3 in this setting (compared with the dual dependence of HER2 phosphorylation on EGFR and ERBB3 in FaDu cells stimulated with both NRG1 and EGF) probably reflects a comparatively low level of EGFR activation by endogenous ligand. NRG1 stimulated the phosphorylation of both Akt and ERK in LIM1215 cells, and this was inhibited by both REGN1400 and the HER2 blocking antibody, consistent with the effects of these antibodies on receptor phosphorylation (Fig. 5C). Thus, similar to our observations in HNSCC cells, NRG1-induced phosphorylation of ERBB3 is dependent on association of ERBB3 with HER2.

Consistent with the signaling data, the ability of NRG1 to rescue LIM1215 cell growth from EGFR blockade was blocked by either REGN1400 or the HER2 blocking antibody (Fig. 5D), indicating that NRG1 rescue of cell growth requires association of ERBB3 with HER2. Thus, ERBB3
limits the effectiveness of EGFR blockade in both colorectal cancer and HNSCC cell lines via partnering with HER2.

**Combined blockade of ERBB3 and EGFR promotes regression of HNSCC and colorectal cancer xenografts**

To assess the effect of combined EGFR/ERBB3 blockade on the growth of tumor xenografts, we treated established FaDu or LIM1215 tumors with human Fc control protein, REGN1400, REGN955, or REGN1400 plus REGN955. Although both REGN1400 and REGN955 as single agents moderately delayed the growth of FaDu tumors, the combination treatment caused a dramatic tumor regression (Fig. 6A). The superior effect of the combination treatment is consistent with our *in vitro* data and suggests that tumor regression requires blockade of both the Akt and ERK pathways.
Similarly, although both REGN1400 and REGN955 as single agents delayed the growth of LIM1215 tumors, the combination was able to promote significant tumor regression (Fig. 6B). Interestingly, the response of LIM1215 tumor xenografts to combined EGFR and ERBB3 blockade was more similar to the \textit{in vitro} cell growth response in the presence of exogenous NRG1 (Fig. 5D) than to the cell growth response in the absence of NRG1 (Fig. 3D), where EGFR blockade alone had a very strong inhibitory effect. This suggests that in the tumor xenograft setting either LIM1215 cells or stromal cells express an ERBB3 ligand. Thus, in both HNSCC and colorectal cancer tumor xenograft models, inhibition of either ERBB3 or EGFR only slows tumor growth, whereas combined blockade of ERBB3 and EGFR results in substantial tumor regression. Interestingly, the combination treatment did not generally result in long term "cures" in these tumor models, and many of the tumors eventually regrew, even in the face of continued treatment (as seen in Fig. 6B, starting at around day 130). Although the mechanisms that promote tumor regrowth are unknown, this remains an interesting topic for future investigation.

Discussion
Given that EGFR blocking antibodies provide clinical benefit in colorectal cancer and HNSCC (3–8), but that the responses are relatively infrequent and transient...
Figure 6. Combined blockade of ERBB3 and EGFR promotes regression of HNSCC and colorectal cancer xenografts. Mice bearing established FaDu (A) or LIM1215 (B) tumors were treated twice per week with the indicated doses (mg/kg) of human Fc control protein, REGN1400, REGN955, or the combination of REGN1400 plus REGN955. In the FaDu model, the last administration of the REGN1400 plus REGN955 combination was on day 52. In the LIM1215 model, administration of the combination was switched to a once per week schedule after day 87. The line graphs depict the average tumor volumes over the course of treatment. The bar graphs depict the average tumor growth from the start of treatment to the day when the control-treated mice were sacrificed (from day 27 to day 41 for FaDu tumors; from day 59 to day 87 for LIM1215 tumors). Error bars, SD. Tumor growth was compared by one-way ANOVA with Tukey multiple comparison test (*, P < 0.05; **, P < 0.01; ***; P < 0.001).

(4, 5, 7, 9), identification of pathways that limit the efficacy of EGFR inhibitors is critical. In this report, we use novel ERBB3 and EGFR blocking antibodies (REGN1400 and REGN955, respectively) to demonstrate that combined blockade of these 2 receptors inhibits the growth of HNSCC and colorectal cancer cell lines/tumors more potently than blockade of either receptor alone. Specifically, we show that ligand-activated ERBB3 partners with HER2 to limit the effects of EGFR blockade on cell growth/survival. Activation of the Akt survival pathway in the HNSCC cell lines we studied is driven exclusively by ERBB3/HER2 signaling, with no apparent contribution from EGFR, underscoring the limitation of single agent EGFR blockade. Importantly, the combination of REGN1400 plus REGN955, but neither single agent, caused substantial regression of HNSCC and colorectal cancer xenografts. Our results suggest that dual blockade of EGFR and ERBB3 in HNSCC and colorectal cancer, by inhibiting distinct ERBB family receptor complexes and downstream pathways, may provide more dramatic and/or durable clinical responses than blockade of EGFR alone.

Because multiple mechanisms can promote resistance to EGFR blocking antibodies in colorectal cancer and HNSCC, the effective translation of our findings to the clinic will require identification of the subset of patients most likely to benefit from combined EGFR/ERBB3 blockade. Recent data suggest the possibility that measurement of NRG1 levels might be a useful way to identify such patients. Pretreatment circulating NRG1 protein levels and intratumoral NRG1 RNA levels were significantly higher in patients with colorectal cancer that subsequently failed to respond to cetuximab than in patients that exhibited a partial response (23). Furthermore, in a small set of paired pretreatment and postresistance plasma samples from patients that initially responded to cetuximab and then developed resistance, NRG1 levels were significantly higher after resistance (23). These findings suggest the possibility that NRG1/ERBB3 signaling promotes resistance to EGFR blockade and therefore that patients with colorectal cancer with high NRG1 levels might benefit from combined EGFR/ERBB3 blockade.

NRG1 also seems to be a promising biomarker in HNSCC. In a large set of primary human cancers, HNSCC had the highest median level of NRG1 RNA compared with other cancers, including lung, colorectal, and breast (28). Furthermore, elevated NRG1 RNA expression was correlated with detection of phospho-ERBB3 by immunohistochemistry in fresh pretreatment patient samples (28). Consistent with these findings, NRG1 expression is particularly prominent in HNSCC cell lines, where it correlates with active ERBB3/HER2 signaling and sensitivity to the HER2 inhibitor lapatinib (27). In addition, a dual action EGFR/ERBB3 blocking antibody (MEHD945A) caused partial responses in 2 HNSCC patients in a phase I trial and these 2 tumors expressed high levels of NRG1 RNA (34). Thus, analysis of NRG1 expression may enable identification of patients whose tumors have active ERBB3 signaling and that might benefit from treatment with an ERBB3 blocking antibody.
Interestingly, a recent report identified somatic ERBB3 mutations in 11% (11/100) of primary colorectal cancer samples (35). Most of the point mutations, including several recurrent mutations, were in the ERBB3 extracellular domain. These mutations increased the ability of ERBB3 to cooperate with HER2 to transform colon epithelial cells, potentially by altering the conformation of the ERBB3 extracellular domain. Importantly, the activity of several of these ERBB3 mutants was inhibited by a ligand-blocking ERBB3 antibody, indicating that their function is not completely ligand-independent (35). Thus, assessment of ERBB3 mutation status may be an additional means to identify tumors in which ERBB3/HER2 signaling is active and that might benefit from anti-ERBB3 therapy.

Multiple ERBB3 blocking antibodies have been described in the literature (24, 25, 36, 37) and several have entered clinical development (34, 38). REGN1400 is currently in a phase I trial (NCT01727869), which includes testing of REGN1400 in combination with EGFR inhibitors. The findings presented in this study suggest that REGN1400 could potentially enhance the efficacy of EGFR blockers in both colorectal cancer and HNSCC.

Disclosure of Potential Conflicts of Interest
D. MacDonald, G. Thurston, and C. Daly have ownership interest in patent applications. No potential conflicts of interest were disclosed by the other authors.

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