TGFα and Amphiregulin Paracrine Network Promotes Resistance to EGFR Blockade in Colorectal Cancer Cells

Sebastijan Hobor¹, Beth O. Van Emburgh¹, Emily Crowley¹,², Sandra Misale¹,³, Federica Di Nicolantonio¹,³, and Alberto Bardelli¹,³

Abstract

**Purpose:** Targeted inhibition of EGFR with the mAbs cetuximab or panitumumab is a valuable treatment for RAS wild-type colorectal cancers. The efficacy of EGFR blockade is limited by the emergence of acquired resistance often attributed to secondary KRAS mutations. Remarkably, tumor biopsies from resistant patients show that only a fraction of the resilient cells carry KRAS mutations. We hypothesized that a paracrine cross-talk driven by the resistant subpopulation may provide in trans protection of surrounding sensitive cells.

**Experimental design:** Conditioned medium assays and three-dimensional cocultures were used to assess paracrine network between cetuximab-sensitive and -resistant cells. Production of EGFR ligands by cells sensitive to cetuximab and panitumumab was measured. The ability of recombinant EGFR ligands to protect sensitive cells from cetuximab was assessed. Biochemical activation of the EGFR signaling pathway was measured by Western blotting.

**Results:** Colorectal cancer cells sensitive to EGFR blockade can successfully grow despite cetuximab treatment when in the company of their resistant derivatives. Media conditioned by resistant cells protect sensitive parental cells from cetuximab. EGFR blockade triggers increased secretion of TGFα and amphiregulin. Increased secretion of ligands by resistant cells can sustain EGFR/ERK signaling in sensitive cells.

**Conclusions:** Colorectal cancer cells that develop resistance to cetuximab and panitumumab secrete TGFα and amphiregulin, which protect the surrounding cells from EGFR blockade. This paracrine protective mechanism might be therapeutically exploitable. *Clin Cancer Res;* 20(24); 6429–38. ©2014 AACR.

Introduction

The EGFR and its ligands, TGFα, amphiregulin, EGF, betacellulin, heparin-binding like EGF-factor, and epiregulin (EREG), play a central role in development of epithelial tumors such as colorectal cancers (1). More than half of metastatic colorectal cancers display mutations in members of the RAS signaling pathways such as KRAS, NRAS, or BRAF (2–5). A subset of colorectal cancers lacking RAS pathway mutations are intrinsically dependent on EGFR, and the ensuing "EGFR addiction" is therapeutically tractable using two EGFR-targeted antibodies, cetuximab and panitumumab (6). After an initial response, secondary resistance invariably ensues, thereby limiting the clinical benefit of these drugs (7, 8). We previously reported the presence of KRAS G12-, G13-, and Q61-mutated alleles in tissue biopsies from patients with colorectal cancer who relapse after EGFR-targeted therapies (9). Notably, highly sensitive methodologies show that "resistant" KRAS-mutant alleles are present only in a fraction of tumor cells with frequencies ranging from 0.4% to 17% (9). Several hypotheses could explain these findings. First, despite efforts to maximize neoplastic cell content, tumor tissues often contain variable proportions of neoplastic and stromal cells. Second, it is plausible that independent subclonal cancer cell lineages, carrying distinct resistance mechanisms, evolve in parallel within the same metastatic lesion. A third possibility, explored in this work, is that a resistant subpopulation may sustain the growth of surrounding sensitive cells through the release of paracrine soluble factors. We hypothesized the existence of protective paracrine interactions, between RAS-mutated (resistant) and the wild-type (WT; therapeutically sensitive) cell subpopulations. This hypothesis is based on evidence that cancer cells are able to produce a plethora of growth factors, thus achieving, in some instances, complete independence from externally provided ligands (10). Furthermore, it has been previously shown that ligands for receptor tyrosine kinases can sustain resistance to targeted therapies. For example, hepatocyte growth factor (HGF),...
Patients with colorectal cancer who receive the EGFR-targeted antibodies cetuximab or panitumumab usually develop resistance within several months of initiating therapy. The emergence of mutations in KRAS, NRAS, and BRAF is associated with acquired resistance to EGFR blockade. Interestingly, cells with these mutations often represent a small fraction of the resistant tumor mass, suggesting that nonmutant cells can also survive the treatment. We report that cells that have acquired resistance to cetuximab can protect sensitive cells through increased secretion of the EGFR ligands TGFβ and amphiregulin. Hence, we have unveiled a paracrine supportive network that is potentially amenable to therapeutic intervention. Blockade of TGFβ and amphiregulin could improve therapies based on EGFR-directed antibodies.

Materials and Methods

**Generation of resistant cells**

The DiFi and OXCO-2 colorectal cancer cell lines were received from Dr. J. Baselga in November 2004 (Oncology Department of Vall d’Hebron University Hospital, Barcelona, Spain) and Dr. V. Cerundolo in March 2010 (Weatherall Institute of Molecular Medicine, University of Oxford, UK), respectively. The LIM1215 parental cell line (14) was obtained from professor Robert Whitehead, Vanderbilt University, Nashville, with permission from the Ludwig Institute of Molecular Medicine, University of Oxford, UK. The genetic identity of all cell lines was confirmed by short tandem repeat profiling (Cell ID; Promega) no longer than 6 months before execution of the experiments. Cetuximab-resistant derivatives of LIM1215, OXCO-2, and DiFi cell lines were generated as described in our previous publications (9, 15). Briefly, cells were cultured in RPMI, ISCOVES (Sigma), and F12 (Gibco) for LIM1215, OXCO-2, and DiFi, respectively. Cells were treated with increasing concentrations of cetuximab (1, 5, 50 μg/mL for DiFi and 1, 5, 50, 200 μg/mL for LIM1215 and OXCO-2) until resistance was achieved as per Fig. 1A. The cetuximab concentration was escalated every 3 to 4 passages. Resistant derivatives were subsequently cultured in their respective media with 200 μg/mL of cetuximab for LIM1215-R, OXCO-2-R and 50 μg/mL of cetuximab for DiFi-R.

**DNA sequence analysis**

Mutational analysis was performed as described before (9). Briefly, DNA was extracted, using Wizard SV genomic DNA Purification System (Promega). PCR amplifications were performed using 0.25 mmol/L deoxynucleotide triphosphates, 1 μmol/L each of the forward and reverse primers, 6% DMSO, 1 x PCR reaction buffer, 0.05 unit/μL Platinum Taq, and 1 ng/μL genomic DNA (Invitrogen/Life Technologies). Primer sequences were previously reported (15). PCR products were purified using AMPure (Agencourt Bioscience Corp.; Beckman Coulter S.p.A). Cycle sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were purified using CleanSeq (Agencourt Bioscience; Beckman Coulter) and analyzed on a 3730 DNA Analyzer, ABI capillary electrophoresis system (Applied Biosystems). Sequence traces were analyzed using the Mutation Surveyor software package (SoftGenetics).

**Cell proliferation assays**

The proliferation assays were performed by seeding 2,000 cells/well in 96-well plates in 100 μL of media. After overnight incubation, 100 μL of media were added with a titrated concentration of cetuximab to achieve final concentrations between 0 and 500 μg/mL. Cell viability was assessed by ATP content using the CellTiter-Glo luminescent assay (Promega). Measurements were recorded using Victor-X4 plate reader (PerkinElmer). Treated cells were normalized to the untreated. Data points represent mean ± SD of three independent experiments.

In stimulation experiments, the proliferation assay described above was modified. The cetuximab titrated media added on the second day were supplemented with recombinant human amphiregulin or TGFβ (Abcam; AR [ab104355] TGF-β [ab9587]) at the following concentrations respectively: 0, 5,000, and 10,000 pg/mL and 0, 100, 200, and 400 pg/mL. Cell viability was assessed by ATP content using the CellTiter-Glo luminescent assay (Promega). Measurements were recorded using Victor-X4 plate reader (PerkinElmer). Treated wells were normalized to untreated. Data points represent mean ± SD of three independent experiments.

**Conditioned medium assay**

The conditioned medium assay (CMA) was performed in two phases. In the first phase, 1.5 x 10⁶ sensitive or resistant cells were seeded in 100-mm culture dishes in 10 mL of their respective medium supplemented with cetuximab at a drug concentration of 2, 1, and 0.5 μg/mL for LIM1215, OXCO-2, and DiFi, respectively. Medium was conditioned for 72 hours. In the second phase, sensitive cells were seeded in 6-well plates at a density of 5 x 10⁴ per well. After overnight incubation, attached cells were washed with PBS and covered with 4 mL of media prepared from half conditioned and half fresh medium (Supplementary Fig. S1). After 6 to 7 days of incubation, the viability of sensitive cells was assessed by ATP content using the CellTiter-Glo luminescent assay (Promega). Luminescence was measured by Victor-X4 plate reader (PerkinElmer). Results were normalized to viability of sensitive cells incubated with conditioned media from sensitive cells with cetuximab. Data points represent mean ± SD of three independent experiments.
Three-dimensional coculture assays

For the three-dimensional coculture experiment in soft agar, sensitive and resistant derivatives of OXCO-2 were labeled by lentivirus-mediated transduction with DsRED and GFP. The reporter plasmid vector, pLemiR (Empty Vector) with DsRED (Open Biosystems) was packaged into lentiviral particles using HEK293 cells. The GFP reporter lentivirus was obtained as ready to use lentiviral particles labeled by lentivirus-mediated transduction with DsRED (LVP300 (Amsbio)). A total of 10^5 cells per well were seeded in a 6-well plate (Costar) and infected the following day with lentiviral particles including parental (P-DsRED), resistant (R-GFP), or the mixture of both populations. Cells were incubated overnight and then detached by short trypsinization, counted, and seeded in soft agar. The soft agar assay was performed in 12-well plates (Costar) where the bottom of the wells was covered with culture medium enriched with agarose (1%). A total of 20,000 cells per well were seeded in 1 mL of 0.6% agarose-enriched culture medium. During the 2 weeks of incubation, 100 μL of medium with or without cetuximab (1 μg/mL) was added every 3 to 4 days for nutrition and evaporation compensation. After incubation for 2 weeks, approximately 120 colonies were counted in each well, and their colors were recorded by fluorescence microscopy. Images were acquired with LEICA DM13000 B microscope, and fluorescence images were overlaid by Adobe Photoshop CS2 software. Data points represent mean ± SD of three independent experiments.

Measurements of EGFR ligands by ELISA

Measurements of ligands’ concentrations were performed by ELISA. The respective media for the three cell line models were conditioned as for CMA (above) with and without cetuximab. After 72 hours of incubation, media were collected, centrifuged, aliquoted, and stored at −20°C for up to 2 weeks. Each aliquot was thawed and used only once. Ligand measurements were performed by R&D DuoSet ELISA assays, DY239 (TGFβ), DY262 (amphiregulin), DX236 (EGF), DY259 (HB-EGF), DY377 (NRG1), DY294 (HGF) in 96-well format according to the manufacturer’s instructions. Well washing was performed with Wellwash Versa Microplate WASHER instrument (Thermo Scientific). Dual absorbance spectra were measured according to the manufacturer’s instructions using Victor-X4 plate reader (PerkinElmer). Data points represent mean ± SD of three independent experiments.
Ligand concentration measurements in the time course experiments were recorded using the same ELISA DuoSet system. Sensitive or resistant derivative cells were seeded in six 100-mm dishes at a density of $1.5 \times 10^6$/dish and incubated overnight. At time 0, cells were detached and counted using a Coulter particle counter (Beckman Coulter). After 6, 12, 24, and 72-hour time points, cells were detached and counted, and conditioned media were collected, aliquoted, and stored at $-20^\circ C$ for up to 2 weeks. Ligand concentrations in nonconditioned media (for time point 0) or conditioned media (for other time points) were normalized to the number of counted cells and expressed as pg/10^6 cells according to formula $(L \times V/N) \times 10^6$, where $L$ is the measured ligand concentration (pg/mL), $V$ is the total volume of media (mL), and $N$ is the number of counted cells. Data points represent mean $\pm$ SD of two independent experiments.

**Western blot analysis**

Biochemical activation of EGFR and its downstream effector ERK1/2 by conditioned media was assessed by Western blot. Two million cells were seeded in 100-mm dishes with 10 mL of respective media containing 1% serum with and without cetuximab (2 μg/mL). After 72 hours of incubation, the conditioned media were used for stimulation of 7 $\times$ 10^5 serum-starved (24 hours) parental cells. After 30 minutes of stimulation, cells were lysed with cold lysis buffer (NaCl 150 mmol/L, triton-x100 1%, EDTA 5 mmol/L, glycerol 10%, EGTA 2 mmol/L, HEPES 500 mol/L) containing protease inhibitors (aprotinin, leupeptin, pepstatin, soybean trypsin inhibitor, and phenylmethylsulfonfyl fluoride) and phosphatase inhibitors (sodium orthovanadate and sodium fluoride). Western blot detection was done by enhanced chemiluminescence (GE Healthcare).

The following antibodies were used for Western blotting: anti–phospho-p44/42 ERK (Thr202/Tyr204; Cell Signaling Technology); anti-p44/42 ERK (Cell Signaling Technology); anti–P-MEK1/2 (Ser217/221), anti-MEK1/2, anti–phospho-p44/42 ERK (Tyr1068; Cell Signaling Technology); anti–phospho-p44/42 ERK (Tyr1068; Cell Signaling Technology); anti-vinculin (Sigma-Aldrich).

**Statistical analysis**

All statistical analyses were completed using the Student’s $t$ test (two tailed). $P$ value of $\leq 0.05$ was considered significant.

**Results**

**Colorectal cancer cell lines that develop resistance to EGFR-directed therapy exert paracrine protection of sensitive cells**

We studied three colorectal cancer cell lines (LIM1215, OXCO-2, and DiFi) that are highly sensitive to cetuximab, from which we previously derived resistant subpopulations by continuous exposure to the antibody (9, 15; Fig. 1A). Although the parental cells were WT for KRAS, NRAS, and BRAF, resistant derivatives acquired several mutations. Cetuximab-resistant LIM1215 (LIM1215-R) cells displayed KRAS pG12R, KRAS pK117N, NRAS pG12C variants, whereas OXCO-2–resistant (OXCO-2-R) cell lines acquired KRAS pG12D and BRAF pV600E alleles (9, 15). Resistant subpopulations of DiFi (DiFi-R) developed an approximately 50-fold amplification of WT KRAS and lost amplified WT EGFR (9).

To investigate whether the resistant cells, in addition to the genetic alterations described above, also developed the ability to create a permissive microenvironment for sensitive cells, we performed a conditioned medium assay experiment (Supplementary Fig. S1). Initially, resistant and sensitive populations conditioned their respective culture medium in the presence of cetuximab for 72 hours as described in the Materials and Methods. Subsequently, sensitive cells were seeded in a 1:1 ratio of conditioned media (CM) and fresh culture media to avoid the negative effect of partial depletion of conditioned media (Supplementary Fig. S1). After 1 week, the supernatant from resistant derivatives increased the viability of parental cells by two-fold when compared with the effect induced by conditioned media collected from the corresponding sensitive cells (Fig. 1B). The influence of conditioned media from resistant derivatives was evident in all three cell models. These data support the hypothesis that resistant cells carrying genetic alterations in the RAS pathway produce paracrine-acting factors that could shield sensitive WT cells from the anti-EGFR antibody cetuximab.

**Cetuximab-resistant cells create a permissive microenvironment for sensitive cells**

To directly observe the protective influence of resistant (R) cells on sensitive (S) cells, we developed a three-dimensional culture system in which S and R cell populations differentially expressed the fluorescent markers, DsRED in S (RED-S) and GFP in R (GREEN-R). OXCO-2 cells were most conducive for the experiments as they were readily transduced with the lentivirus and formed spheroid colonies. After a 2-week incubation period, spheroids were documented by light and fluorescence microscopy (Fig. 2A and B). As expected, the RED-S population did not produce viable colonies in the presence of cetuximab. In contrast, the GREEN-R population readily formed colonies. Interestingly, when mixed colonies were generated by seeding RED-S and GREEN-R populations in a 1:1 ratio, RED-S cells were successfully growing together with GREEN-R derivatives despite cetuximab (Fig. 2B). To provide quantitative measurements, colonies were counted and grouped according to their colors. In the presence of cetuximab, there was an increase of dual-colored colonies and a small number of RED-S (~90% RED-S/GREEN-R vs. ~10% RED-S; Fig. 2C). The small number of RED-S colonies can be potentially explained by incomplete fluorescent labeling of cells, resulting in a portion of unlabeled cells in the spheroids. Incomplete labeling is evident in the micrographs (Fig. 2B), where portions of spheroids are neither red nor green.

In summary, the three-dimensional assay enabled us to directly observe proliferation of S cells together with R derivatives in the presence of cetuximab. Proliferation of S cells despite cetuximab treatment can only be attributed to...
the presence of the R cells. We conclude that resistant cells substantially modified the intracolony microenvironment making it permissive for proliferation of sensitive cells, despite cetuximab treatment.

**Cetuximab-resistant cells secrete TGFα and amphiregulin, whose production is further increased by cetuximab treatment**

The experiments above suggest that protective paracrine interactions could be mediated by soluble factors. To identify such factors, media conditioned by S and R cells from each of the three cell models (LIM1215, OXCO-2, and DiFi) were investigated using ELISA assay. The presence of the EGFR ligands TGFα, amphiregulin, EGF, HB-EGF, and NRG1 was measured after 72 hours of incubation (Fig. 3A and B and Supplementary Fig. S2). Because HGF, the MET receptor ligand, has been previously shown to confer resistance to EGFR inhibitors (16), its concentration was also evaluated.

Media conditioned by R populations revealed significantly higher concentrations of TGFα and amphiregulin compared with their S counterparts, even when not exposed to cetuximab (Fig. 3A and B). However, when cells were treated with cetuximab, the ligand secretion was, at least partially, cell-type specific. In the presence of cetuximab, sensitive LIM1215 and OXCO-2 significantly increased secretion of TGFα, whereas DiFi did not. More importantly, intrinsically higher secretion of TGFα by R cells was further stimulated by cetuximab treatment in all three cell models (Fig. 3A). On the other hand, the oversecretion of amphiregulin in R cells did not further increase under cetuximab treatment (Fig. 3B). No differences in the concentration of other assessed ligands were noted (Supplementary Fig. S2).

In the previous assay, ligand levels were measured after 72 hours of incubation. Therefore, only the final cumulative concentration was revealed. These results may be influenced by differences in cell numbers and the temporal heterogeneity of secretion. To further analyze the production of TGFα and amphiregulin by S and R populations, we performed a time course experiment and normalized ligand concentration to the number of cells in two cell models (LIM1215 and DiFi). Consistent with the above observations, cetuximab triggered an increased secretion of TGFα in both LIM1215-S and R cells (Fig. 3C). In contrast, cetuximab treatment did not increase TGFα secretion in DiFi.
Levels were normalized to number of cells for each time point and expressed as pg/10^6 cells. Error bars, mean ± SD of three independent experiments.

As discussed above, the development of resistance in patients is associated with the emergence of "secondary" KRAS genetic alterations. To formally link the acquisition of KRAS mutations to the increased secretion of ligands, we exploited LIM1215 cells in which a mutant KRAS (G12R) was introduced in the endogenous KRAS locus making them resistant (9). Medium conditioned by the knock-in (mutant) population had protective properties similar to that of cells, which had acquired resistance (Supplementary Fig. S4A), and cetuximab triggered increased secretion of TGFα (Supplementary Fig. S4B and S4C).

**TGFα and amphiregulin protect from cetuximab**

Although these data point to paracrine protection against cetuximab, they do not formally prove that EGFR ligands are directly responsible for this effect. Accordingly, we implemented forward biologic experiments in which proliferation assays were performed in the presence of recombinant TGFα and amphiregulin. In all cell models, the addition of TGFα reduced the inhibitory effect of cetuximab in a dose-
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Media conditioned by cetuximab-resistant cells sustain ERK signaling in sensitive cells

To provide a mechanistic link between paracrine factors produced by resistant cells and increased resilience of sensitive cells to cetuximab, we studied whether and how EGFR intracellular signaling was affected by conditioned media. As a model, we used parental S LIM1215 cells that were serum-starved and then stimulated with conditioned media from S parental or R derivatives in the presence or absence of cetuximab. Untreated and TGFα-stimulated sensitive cells served as negative and positive controls, respectively. After 30 minutes of stimulation, the activation of EGFR downstream signaling was determined by Western blot. Media conditioned by resistant derivatives fostered greater phosphorylation of EGFR and ERK1/2 compared with media derived from parental cells (Fig. 5). This indicates that paracrine effectors could sustain EGFR signaling in sensitive cells.

Discussion

Altogether, our data indicate that colorectal cancer cells that develop resistance through RAS pathway mutations produce significantly higher levels of TGFα and amphiregulin. In patients undergoing treatment based on EGFR-directed mAbs, tumor cells are continuously exposed to cetuximab for several months. Our in vitro data suggest that tumor cells initially sensitive to cetuximab respond to EGFR blockade by increasing the secretion of TGFα and amphiregulin. Furthermore, we provide evidence that acquired resistance to cetuximab involves a paracrine network driven by EGFR ligands (Fig. 6). It is conceivable that increased secretion of EGFR ligands may also be a mechanism of immediate response to EGFR blockade driven by intracellular prosurvival signaling cascades. The ensuing signaling network would then be maintained after the acquisition of EGFR downstream pathway mutations (in RAS and other effectors). Overall, our results support the possibility of paracrine in trans protection of sensitive cells by their mutated resistant derivatives.

Microenvironmental concentrations of EGFR ligands in tumor tissue may very well rise to high enough levels to counteract the inhibitory concentration of cetuximab (20). Speculatively, when the balance between antiproliferative and proproliferative effects of cetuximab and EGFR ligands, respectively, is tipped toward proliferation of sensitive cells, there is little reason for resistant cells to increase their

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Figure 4. Cetuximab (CTX) sensitivity in sensitive cells can be overcome by exogenous EGFR ligands. A and B, cell proliferation assay comparing the effects of recombinant human TGFα and amphiregulin (AR) on LIM1215 (left), OXCO-2 (middle), and DiFi (right) sensitive parental cells with cetuximab treatment. Error bars, mean ± SD of three independent experiments.
numerical proportion to achieve tumor resistance as a whole. Just as the anti-EGFR antibody concentration gradients are established in tumor tissue (21), ligand concentration gradients are equally probable, radiating outward from resistant cells. Proliferation of sensitive cells would therefore be limited to permissive zones within the tumor. Amphiregulin and TGFα binding to EGFR cause longer retention time of the receptor on the surface of the plasma membrane and can redirect EGFR to the recycling pathway rather than to proteasomal degradation (22, 23). This can potentially strongly enhance the proproliferating effect of the modified/protective microenvironment.

Previous reports correlated increased mRNA expression of amphiregulin and EREG in metastatic colorectal cancer specimens with response to cetuximab treatment (24, 25). These clinical studies support the hypothesis that KRAS WT colorectal cancer may respond well to anti-EGFR therapy as a result of dependence on EGFR pathway signaling. In turn, EGFR dependence in RAS WT tumors may be mediated by expression of EGFR ligands that trigger constitutive receptor activation. Instead, in cells that become refractory to anti-EGFR therapy, abnormal production of ligands overcomes the effects of cetuximab or panitumumab. It should be noted that in our work we measured secreted protein ligands, whereas the studies mentioned above assessed mRNA gene expression (24, 25). Accordingly, protein levels of secreted ligands may more pertinently evaluate their protective potential toward the surrounding sensitive cells than ligands mRNA levels in the original cancerous tissue. Of further note, in previous studies, the predictive effect was not noted in patients with KRAS mutations, but only in KRAS WT patients (24). In support of our preclinical work, Loupakis and colleagues have reported increased levels of circulating EGFR ligands in the plasma of patients with

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metastatic colorectal cancer at the time of the radiologic progression to cetuximab and irinotecan, suggesting their potential role as a mechanism of acquired resistance to drug treatment (26).

KRAS-activating mutations were previously associated with increased production of EGFR ligands (27) and increased radiation resistance due to paracrine/autocrine protection (28). The ability of amphiregulin to sustain growth of cancer cells was previously reported in association with low serum in vitro where neutralization of amphiregulin in conditioned media inhibited cell growth (29–31). Studies of the nontransformed breast-derived cell line MCF-10A transduced with inducible RAF/estrogen/GFP fusion protein have shown that RAF activation results in high ERK activation (32). Sustained ERK signaling was shown to boost secretion of the EGFR ligands, HB-EGF, TGFα, and amphiregulin, which, in turn, activated EGFR in an autocrine fashion (32). Most interestingly, conditioned medium from RAF-transduced cells successfully prevented anoikis in the original parental MCF-10A population (32).

Recent studies identified intratumor heterogeneity within geographically distinct portions of tumors (33). It is also accepted that intrac nal genetic diversity and genomic instability provide a substrate for therapeutic clonal Darwinian selection of the fittest (34, 35). However, Darwinian selection does not explain the low prevalence of RAS/RAF-mutated resistant cells in relapsed colorectal cancer tumors. The in trans paracrine protection effect described here offers at least a partial explanation for this phenomenon. Conceptually, targeted therapy may offer a selective advantage to resistant cells, but paracrine protection of sensitive subpopulations could significantly widen the selective bottleneck (Fig. 6). This could help preserve initial clonal heterogeneity at the time of relapse and substantially increase it during subsequent tumor regrowth. The ability of low-frequency KRAS mutations to persist through anti-EGFR therapy and sustain surrounding sensitive cells suggests the importance of improving the sensitivity of RAS mutation detection, as it is possible that low prevalence mutations may affect (the duration of) responses in patients (36).

The persistence of sensitive cells could have clinical implications for further lines of therapy. Indeed, when patients with colorectal cancer suspend anti-EGFR therapy, they are often offered an additional line of chemotherapy with agents that act via an EGFR-independent pathway. It is possible that the subsequent treatments allow the outgrowth of sensitive cells over resistant clones when the pressure on the EGFR pathway is relieved. The ultimate outcome would be the regrowth of neoplastic cells sensitive to EGFR treatment. In support of this hypothesis, a clinical report recently described successful rechallenging with EGFR-targeted mAbs of patients who had become refractory to cetuximab and were subsequently treated with additional lines of therapy (37). We previously reported that colorectal cancer cells that develop KRAS mutations as a mechanism of resistance to EGFR blockade are sensitive to the combination of EGFR–MEK blockade (15). It is possible that cells in which the ligand paracrine network contributes to anti-EGFR resistance may be equally sensitive to this combination and this aspect should be further explored.

The concept of protective paracrine interactions between genetically distinct subclonal cell populations is most likely transferable to other cancer types and to acquired resistance against other types of therapy. We believe that additional research into perturbation of paracrine interactions, such as by ligand neutralization, could lead to improvement of existing therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Hobor, E. Crowley, F. Di Nicolantonio, A. Bardelli
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Hobor, B.O. Van Emburgh, E. Crowley, F. Di Nicolantonio, A. Bardelli
Writing, review, and or revision of the manuscript: S. Hobor, B.O. Van Emburgh, E. Crowley, S. Misale, F. Di Nicolantonio, A. Bardelli
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Hobor, B.O. Van Emburgh, E. Crowley, S. Misale, F. Di Nicolantonio, A. Bardelli
Study supervision: F. Di Nicolantonio, A. Bardelli

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