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TGF-α and amphiregulin paracrine network promotes resistance to EGFR blockade in colorectal cancer cells

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TRANSLATIONAL RELEVANCE

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 non-mutant 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 TGF-α and amphiregulin. Hence, we have unveiled a paracrine supportive network that is potentially amenable to therapeutic intervention. Blockade of TGF-α and amphiregulin could improve therapies based on EGFR-directed antibodies.

ABSTRACT

Purpose: Targeted inhibition of EGFR with the monoclonal antibodies 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 crosstalk driven by the resistant subpopulation may provide *in trans* protection of surrounding sensitive cells.

Experimental design: Conditioned medium assays and three dimensional co-cultures were used to assess paracrine networks 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: CRC 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: CRC cells that develop resistance to cetuximab and panitumumab secrete TGF-α and amphiregulin, which protect the surrounding cells from EGFR blockade. This paracrine protective mechanism might be therapeutically exploitable.

INTRODUCTION

The epidermal growth factor receptor (EGFR) and its ligands, transforming growth factor alpha (TGF-α), amphiregulin (AR), epidermal growth factor (EGF), betacellulin (BTC), heparin-binding like EGF-factor (HBEGF), and epiregulin (EREG) play a central role in development of epithelial tumors such as colorectal cancers (CRCs) (1). More than half of metastatic colorectal cancers (CRCs) display mutations in members of the RAS signaling pathways such as *KRAS*, *NRAS,* or *BRAF* (2-5). A subset of CRCs 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 CRC patients 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. Secondly, 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 generate 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), the activating ligand for the MET receptor, can protect lung cancer cells from the effect of EGFR inhibitors such as erlotinib and gefitinib (11, 12). Similar effects can be promoted by TGF-β and IL6 (13).

METHODS

Generation of resistant cells

The DiFi and OXCO-2 CRC 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 Prof. Robert Whitehead, Vanderbilt University, Nashville, with permission from the Ludwig Institute for Cancer Research, Zurich, Switzerland. The genetic identity of all cell lines was confirmed by STR profiling (Cell ID, Promega) no longer than six 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, 1x 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, Milan, Italy). Cycle sequencing was carried out using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster

City, CA). 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, State College, PA).

Cell proliferation assays

The proliferation assays were performed by seeding 2000 cells/well in 96 well plates in 100 µl of media. After overnight incubation, 100 µl of media was 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 was supplemented with recombinant human amphiregulin or TGF-α (Abcam, AR [ab104355] TGF-α [ab9587]) at the following concentrations respectively: 0, 5,000 and 10,000 pg/ml and 0, 100, 200, 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 million of 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, 0.5 µg/ml for LIM1215, OXCO2 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 $5x10^4$/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 (Fig. S1). After 6-7 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 co-culture assays

For the three dimensional co-culture 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 LVP300 (Amsbio). A total of 10^5 cells/well were seeded in a 6-well plate (Costar) and infected the following day with lentiviral particles. After four days of incubation, cells were checked for DsRED and

GFP reporter gene expression by fluorescence microscopy. Fluorescent populations were expanded and subjected to puromycin (DsRED) and neomycin (GFP) selection for one week. After selection, cells were expanded and banked in liquid nitrogen until use. One million of fluorescent labeled cells were seeded in 60 mm dishes, 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/well were seeded in 1 ml of 0.6% agarose enriched culture medium. During the two weeks of incubation, 100 µl of medium with or without cetuximab (1 µg/ml) was added every 3-4 days for nutrition and evaporation compensation. After incubation for two weeks, approximately 120 colonies were counted in each well, and their colors were recorded by fluorescence microscopy. Images were acquired with LEICA DMI3000 B microscope and fluorescence images were overlaid by Adobe Photoshop CS2 software. Data points represent mean ± SD of two 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 was conditioned as for CMA (above) with and without cetuximab. After 72 hours of incubation, media were collected, centrifuged, aliquoted and stored at -20^{o}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 (TGF-α), DY262 (AR),

DX236 (EGF), DY259 (HB-EGF), DY377 (NRG1), DY294 (HGF) in 96-well format according to manufacturer's instructions. Well washing was performed with Wellwash^{TM} Versa Microplate Washer instrument (Thermo Scientific). Dual absorbance spectra were measured according to 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 $1.5x10^6$/dish and incubated overnight. At time 0, cells were detached and counted using a Coulter particle counter (Beckman Coulter). After 6, 12, 24, 48, and 72 hour time points, cells were detached and counted and conditioned media was collected, aliquoted, and stored at -20ºC for up to two weeks. Ligand concentrations in non-conditioned 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*V/N)*10^6, where L= measured ligand concentration (pg/ml), V= total volume of media (ml), and N= number of counted cells. Data points represent mean +/- 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% of serum with and without cetuximab (2 µg/ml). After 72 hours of incubation, the

conditioned media was used for stimulation of 7 million serum-starved (24 hours) parental cells. After 30 minutes of stimulation, cells were lysed with cold lysis buffer (NaCl 150 mM, triton-x100 1%, EDTA 5 mM, glycerol 10%, EGTA 2 mM, HEPES 500 M) containing protease inhibitors (aprotinin, leupeptin, pepstatin, soybean trypsin inhibitor, and phenylmethylsulfonyl 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 (Thr 202/Tyr204), (Cell Signaling Technology); anti-p44/42 ERK (Cell Signaling Technology); anti-P-MEK1/2 (Ser 217/221), anti-MEK1/2, anti-EGFR (clone13G8, Enzo Life Sciences); anti-phospho EGFR (Tyr 1068) (Cell Signaling Technology); anti-vinculin (Sigma-Aldrich).

Statistical analysis

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

RESULTS

CRC cell lines that develop resistance to EGFR-directed therapy exert paracrine *in trans* protection of sensitive cells

We studied three CRC 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). While the parental cells were wt for KRAS, NRAS, and BRAF, resistant derivatives acquired several mutations. Cetuximab resistant LIM1215 (LIM1215-R) displayed KRAS pG12R, KRAS pK117N, NRAS pG12C variants, while OXCO-2 resistant (OXCO-2-R) acquired KRAS pG12D and BRAF pV600E alleles (9, 15). Resistant subpopulations of DiFi (DiFi-R) developed a ~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 (CMA) experiment (Fig. S1). Initially, resistant and sensitive populations conditioned their respective culture medium in the presence of cetuximab for 72 hours as described in the 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 CM (Supplementary Fig. S1). After one week, the supernatant from resistant derivatives increased the viability of parental cells by two-fold when compared to the effect induced by CM collected from the corresponding sensitive cells (Fig. 1B). The influence of

CM from resistant derivatives was evident in all three cell models. This data supports 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 3D 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 two-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 3D 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 intra-colony microenvironment making it permissive for proliferation of sensitive cells, despite cetuximab treatment.

Cetuximab resistant cells secrete TGF-α 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, DiFi) were investigated using ELISA assay. The presence of the EGFR ligands TGF-α, amphiregulin, EGF, HB-EGF, and NRG1 was measured after 72 hours of incubation (Fig. 3A and B and Fig S2). Since 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 TGF-α and amphiregulin compared to 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 TGF-α, while DiFi did not. More

importantly, intrinsically higher secretion of TGF-α by R cells was further stimulated by cetuximab treatment in all three cell models (Fig. 3A). On the other hand, the over-secretion 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 (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 TGF-α 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 TGF-α in both LIM1215-S and R cells (Fig. 3C). In contrast, cetuximab treatment did not increase TGF-α secretion in DiFi S cells but did stimulate its secretion in the R derivatives (Fig. 3D). On the other hand, secretion of amphiregulin increased in DiFi S when exposed to cetuximab (Fig. 3F). Notably, in both LIM1215 and DiFi, R cells treated with cetuximab secreted up to 3 fold more TGF-α and amphiregulin in comparison to their sensitive counterparts (Fig. 3C-F). When drug-treated LIM1215 and DiFi cells are compared to untreated cells, cetuximab triggered a greater than 3-fold increase of TGF-α levels in both S and R cells (Supplementary Fig. S3A and B). These experiments demonstrate that differential secretion profiles of TGF-α and amphiregulin are not due to different cell numbers, but reflect increased secretion of ligands by resistant cells in response to EGFR blockade.

Mutant *KRAS* G12R knock-in cells exert paracrine protection from cetuximab

Our results provide direct evidence for increased production of EGFR ligands by cetuximab resistant derivatives, and these ligands can sustain *in trans* protection of sensitive cells. 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* allele (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 (Fig. S4A), and cetuximab triggered increased secretion of TGF-α (Fig. S4B and C).

TGF-α and amphiregulin protect from cetuximab

While 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 biological experiments in which proliferation assays were performed in the presence of recombinant TGF-α and amphiregulin. In all cell models, the addition of TGF-α reduced the inhibitory effect of cetuximab in a dose dependent manner (Fig. 4A), albeit to a lesser extent in OXCO-2. In contrast, amphiregulin was protective only in DiFi (Fig. 4B). The cell line specific responsiveness to EGFR ligands is intriguing and may reflect differences in EGFR signaling dependency. For example, DiFi are extremely sensitive to cetuximab mediated EGFR blockade

likely due to *EGFR* gene amplification that results in protein overexpression (17-19).

Media conditioned by cetuximab resistant cells sustains 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 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 TGF-α stimulated sensitive cells served as negative and positive control, 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 to media derived from parental cells (Fig. 5). This indicates that paracrine effectors could sustain EGFR signaling in sensitive cells.

DISCUSSION

Altogether our data indicates that CRC cells that develop resistance through *RAS* pathway mutations produce significantly higher levels of TGF-α and amphiregulin. In patients undergoing treatment based on EGFR directed monoclonal antibodies, 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 TGF-α and amphiregulin. Furthermore, we provide evidence that acquired resistance to cetuximab involves a paracrine network driven by EGFR ligands. It is conceivable that increased secretion of EGFR ligands may also be a mechanism of immediate response to EGFR blockade driven by intracellular pro-survival 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 anti- and pro-proliferative effects of cetuximab and EGFR ligands, respectively, is tipped toward proliferation of sensitive cells, there is little reason for resistant cells to increase their 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 TGF-α 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 pro-proliferating effect of the modified/protective microenvironment.

Previous reports correlated increased mRNA expression of amphiregulin and EREG in metastatic CRC specimens with response to cetuximab treatment (24, 25). These clinical studies support the hypothesis that *KRAS* wild type CRC may respond well to anti-EGFR therapy as a result of dependence on EGFR pathway signaling. In turn, EGFR dependence in RAS wild type 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 also noted that in our work we measured secreted protein ligands, while the studies mentioned above assessed mRNA gene expression (24, 25). Accordingly, protein levels of secreted ligands may more pertinently evaluate their protective potential towards 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* wild type patients (24). In support of our preclinical work, Loupakis and colleagues have reported increased levels of circulating EGFR ligands in the plasma of mCRC patients at the time of the radiological 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 non-transformed 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, TGF-α 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 established intratumor heterogeneity within geographically distinct portions of tumors (33). It is also accepted that intraclonal genetic diversity and genomic instability provides 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 CRC 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 suggest 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 CRC patients 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 re-challenging with EGFR targeted monoclonal antibodies of patients who had become refractory to cetuximab and were subsequently treated with additional lines of therapy (37). We previously reported that CRC 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.

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AUTHOR CONTRIBUTIONS:

- Conception and design: SH, EC, AB
- Development of methodology: SH, EC, BOV
- Acquisition of data: SH, EC, BOV, SM
- Analysis and interpretation of data: SH, EC, BOV, SM, AB, FDN
- Writing, review and/or revision of the manuscript: SH, EC, BOV, SM, AB, FDN
- Study supervision: AB, FDN

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FIGURE LEGENDS

Figure 1

Cells with acquired resistance to cetuximab can protect their drug-sensitive parental counterparts in a paracrine fashion. (A), comparison of sensitive and resistant LIM1215, OXCO-2 and DiFi cells. Cell viability was assayed by the ATP assay. Data points represent the mean ± SD of three independent experiments. (B), conditioned medium assay (CMA) performed by transfer of conditioned media with cetuximab (CTX) from sensitive (S, orange) or resistant (R, green) cells (1:1 – fresh: conditioned) on top of sensitive cell population. Cell viability is normalized to sensitive cells incubated with media conditioned by sensitive cells with cetuximab (LIM1215: 2 µg/ml, OXCO-2: 1 µg/ml and DiFi: 0.5 µg/ml). Symbols and bars, mean ± SD of three independent experiments.

Figure 2.

Cetuximab sensitive parental cells proliferate in 3D culture together with their resistant derivatives in the presence of cetuximab. (A), bright field micrographs of OXCO-2 sensitive (S), resistant (R) and co-culture (S:R-1:1) colonies in 3D culture without and with 1 ug/ml cetuximab (CTX). Scale bar represents 250 µm, magnification 25x. (B), bright field and fluorescence micrographs of OXCO-2 sensitive (RED-S) and resistant (GREEN-R) spheroid colonies in 3D culture. Scale bar represents 62 µm, magnification 250x. (C), count of sensitive (S) and double colored (S/R) colonies observed

after 2 weeks of incubation in 3D culture in the presence or absence (control) of CTX. Symbols and bars, mean ± SD of two independent experiments.

Figure 3.

Cetuximab (CTX) resistant cells secrete higher levels of EGFR ligands than sensitive cells. Ligand secretion is stimulated by CTX treatment in a cell-type dependent manner. (A and B), TGFα and amphiregulin (AR) levels in media conditioned by sensitive and resistant LIM1215, OXCO-2, and DiFi cells after 72 hours of incubation with or without CTX. Error bars represent the mean ± SD of three independent experiments. * $p \leq 0.05$, ** $p < 0.01$. (C-F), secretion of TGFα and AR by LIM1215 and DiFi sensitive and resistant cells during a 72 hour time course with and without CTX treatment. Ligand levels were normalized to number of cells for each time point and expressed as pg/10^6 cells. Error bars represent mean ± s.d of two independent experiments. S: sensitive, R: resistant, CTX: CTX treated. * $p \leq 0.05$, between cell populations in the same column (Student's *t* test, two tailed).

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 to CTX treatment. Error bars represent mean ± SD of three independent experiments.

Figure 5

Conditioned medium (CM) from resistant cells sustains ERK signaling. Western blot analysis of protein expression after 30 minute stimulation of LIM1215 sensitive cells with the indicated treatments. CONTROL: serum free media; CTX: serum free media with 2 µg/ml cetuximab, TGFα: serum free media with 5 ng/ml TGF-alpha; CM-S CTX: CM from sensitive cells treated with 2 µg/ml CTX; CM-R CTX: CM from resistant cells treated with 2 µg/ml CTX; CM-S CTX later: CM from sensitive cells with CTX added after conditioning; CM-R CTX later: CM from resistant cells with CTX added after conditioning; CM-S: CM from sensitive cells, CM-R: CM from resistant cells. Asterisks indicate the bands to be compared in individual exposures.

Figure 6

Conceptual representation of the impact of intercellular paracrine protection during cetuximab therapy in colorectal cancer.

Fig. 1

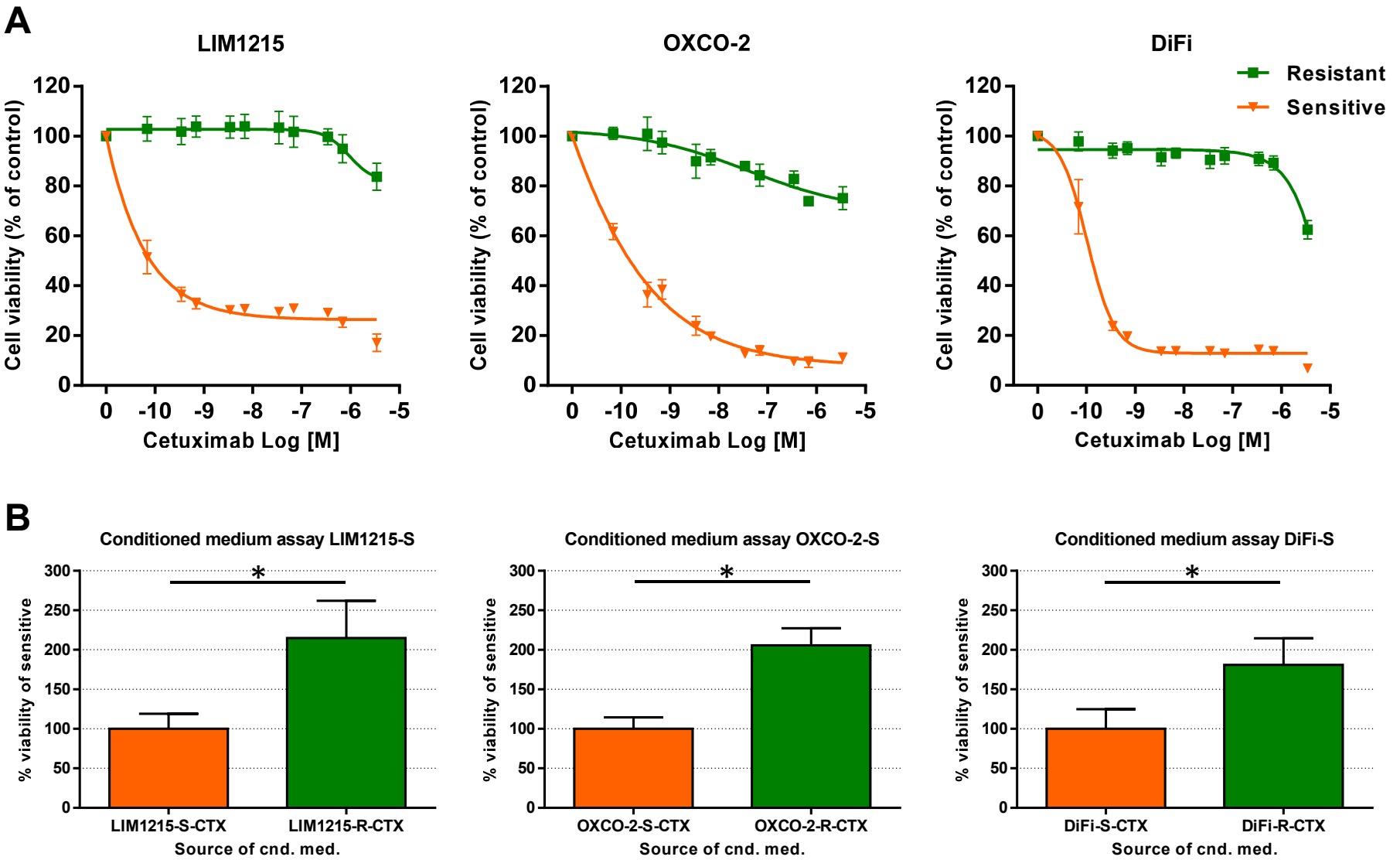

A
LIM1215
OXCO-2
DiFi
Resistant
Sensitive
Cell viability (% of control)
120
100
80
60
40
20
0
0 -10 -9 -8 -7 -6 -5
Cetuximab Log [M]
B
Conditioned medium assay LIM1215-S
Conditioned medium assay OXCO-2-S
Conditioned medium assay DiFi-S
% viability of sensitive
300
250
200
150
100
50
0
*
LIM1215-S-CTX
LIM1215-R-CTX
OXCO-2-S-CTX
OXCO-2-R-CTX
DiFi-S-CTX
DiFi-R-CTX
Source of cnd. med.

Fig. 2

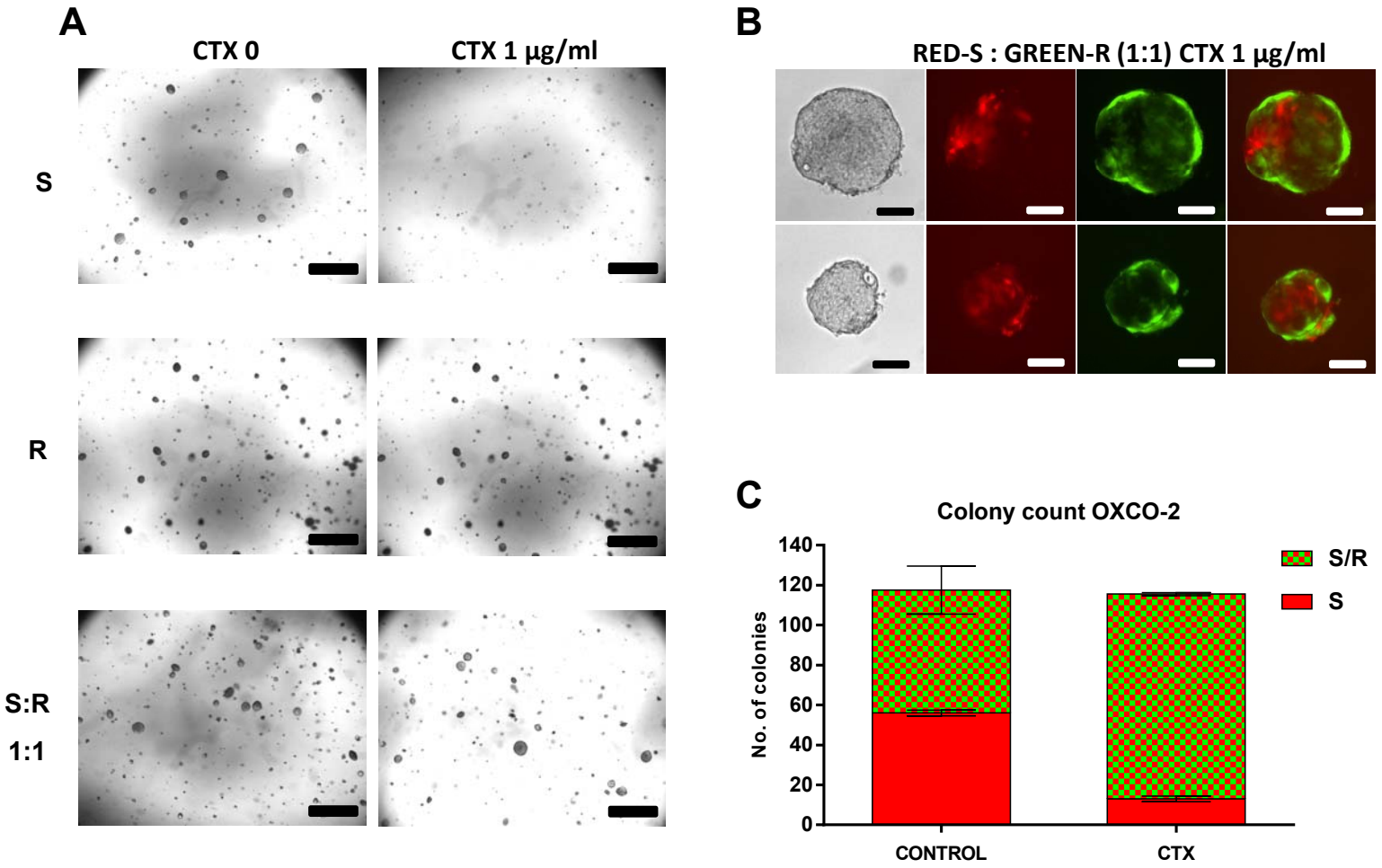

A
CTX 0
CTX 1 µg/ml
S
R
S:R
1:1
B
RED-S : GREEN-R (1:1) CTX 1 µg/ml
C
Colony count OXCO-2
No. of colonies
140
120
100
80
60
40
20
0
S/R
S
CONTROL
CTX

Fig. 3

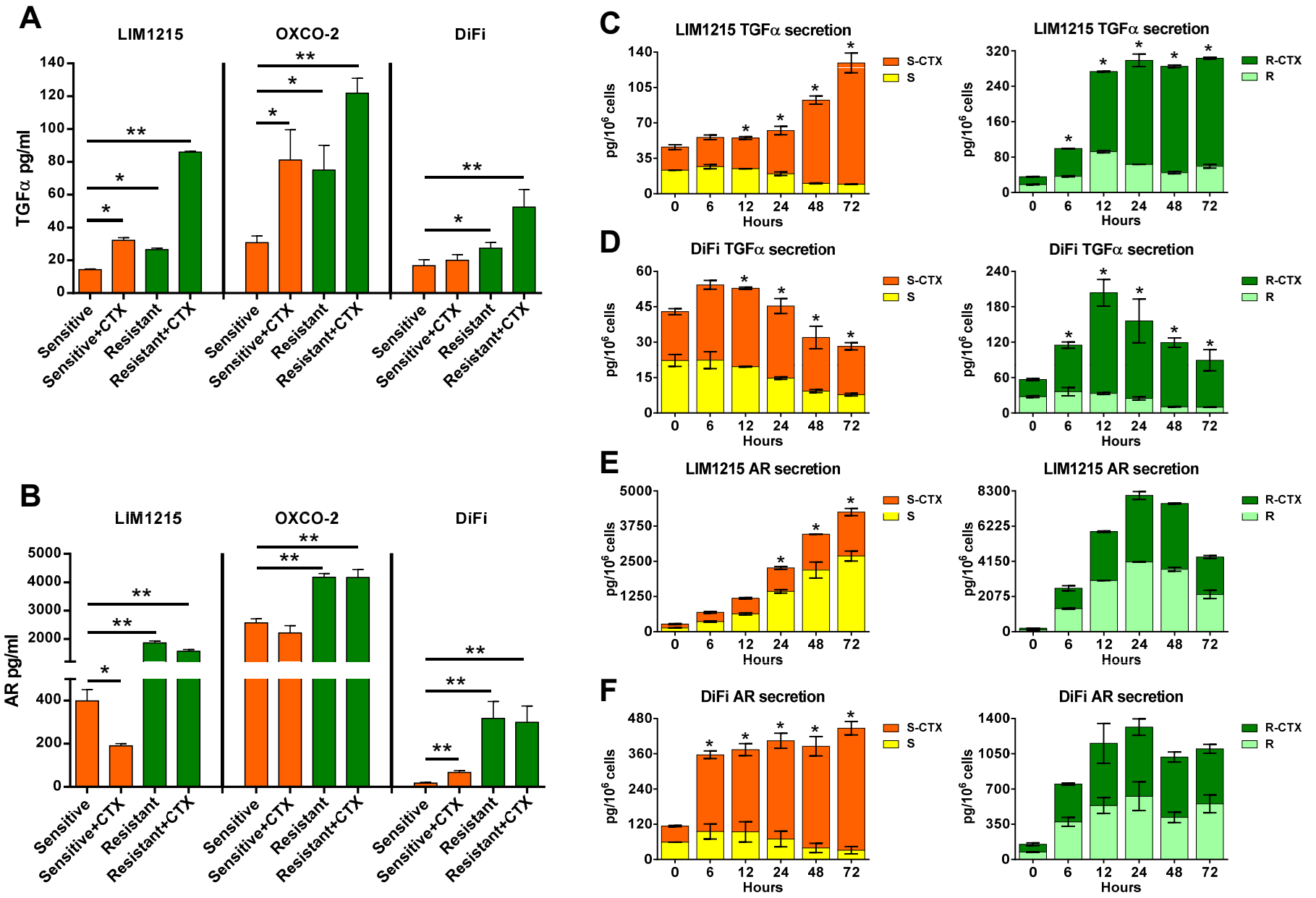

A
LIM1215
OXCO-2
DiFi
TGFα pg/ml
Sensitive
Sensitive+CTX
Resistant
Resistant+CTX
B
AR pg/ml
C
LIM1215 TGFα secretion
pg/10^6 cells
Hours
S-CTX
S
R-CTX
R
D
DiFi TGFα secretion
E
LIM1215 AR secretion
F
DiFi AR secretion

Fig. 4

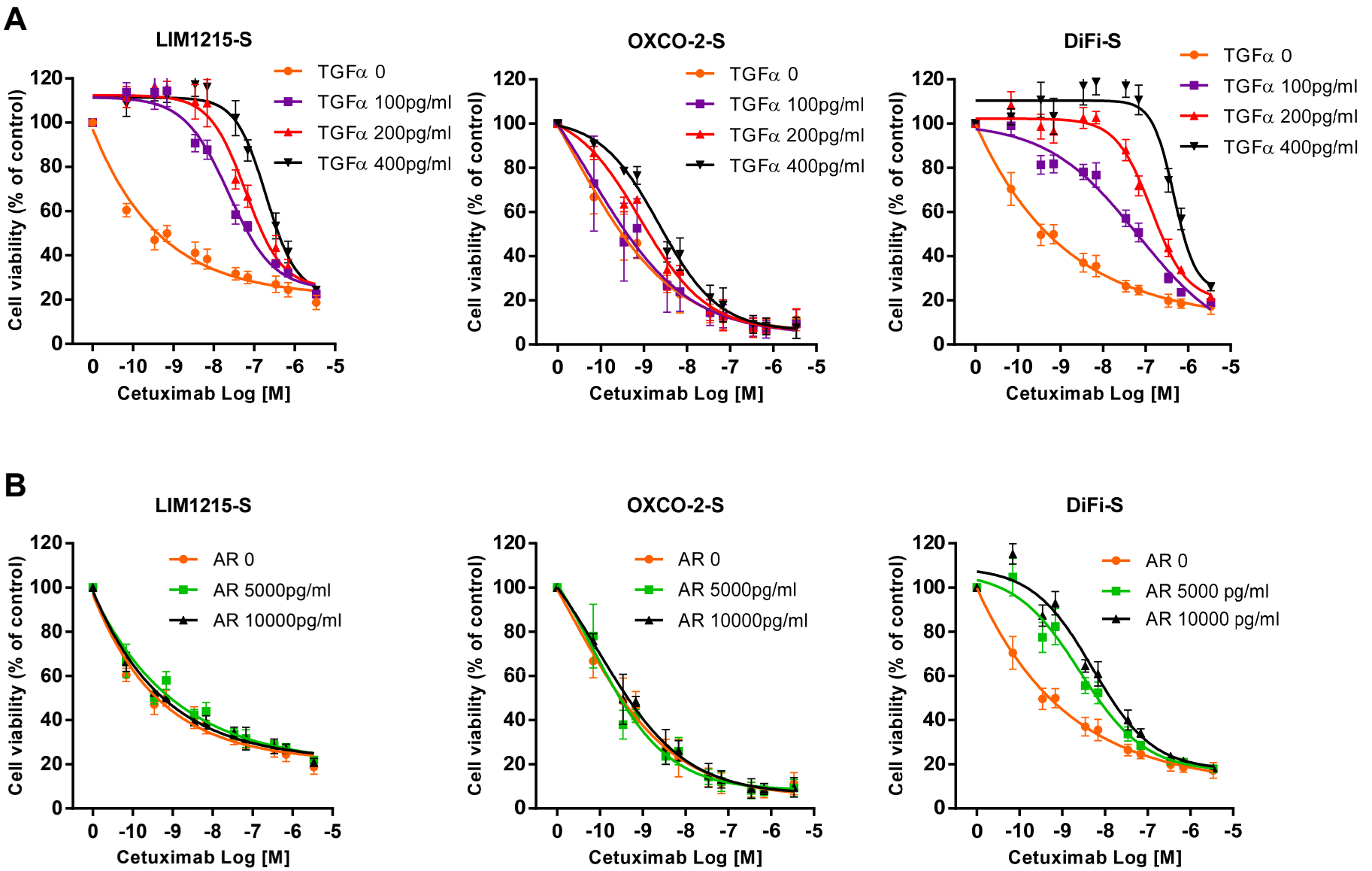

A
LIM1215-S
OXCO-2-S
DiFi-S
TGFα 0
TGFα 100pg/ml
TGFα 200pg/ml
TGFα 400pg/ml
Cell viability (% of control)
Cetuximab Log [M]
B
LIM1215-S
OXCO-2-S
DiFi-S
AR 0
AR 5000pg/ml
AR 10000pg/ml
AR 5000 pg/ml
AR 10000 pg/ml
Cell viability (% of control)
Cetuximab Log [M]

Fig. 5

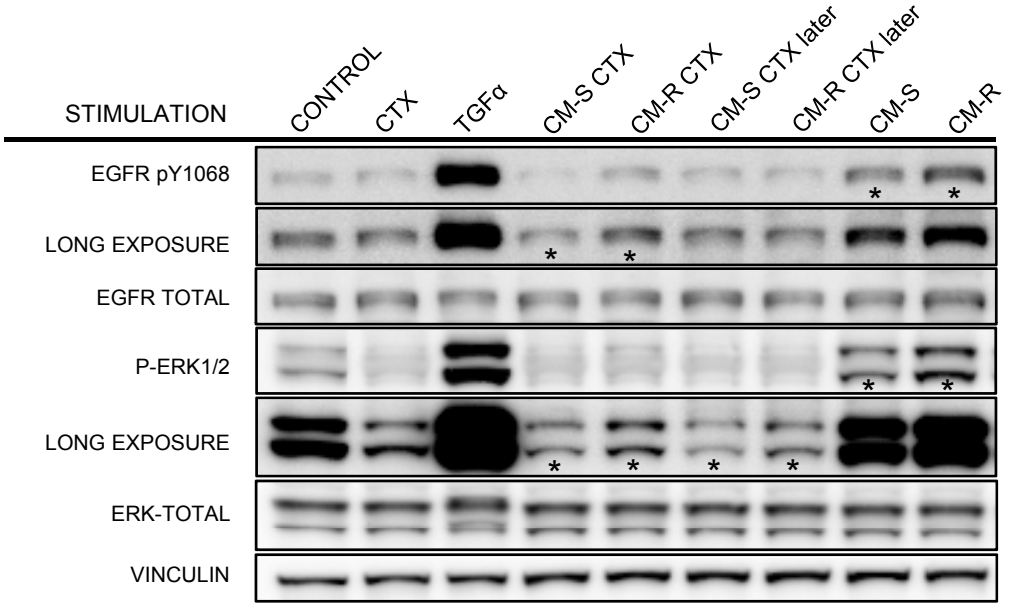
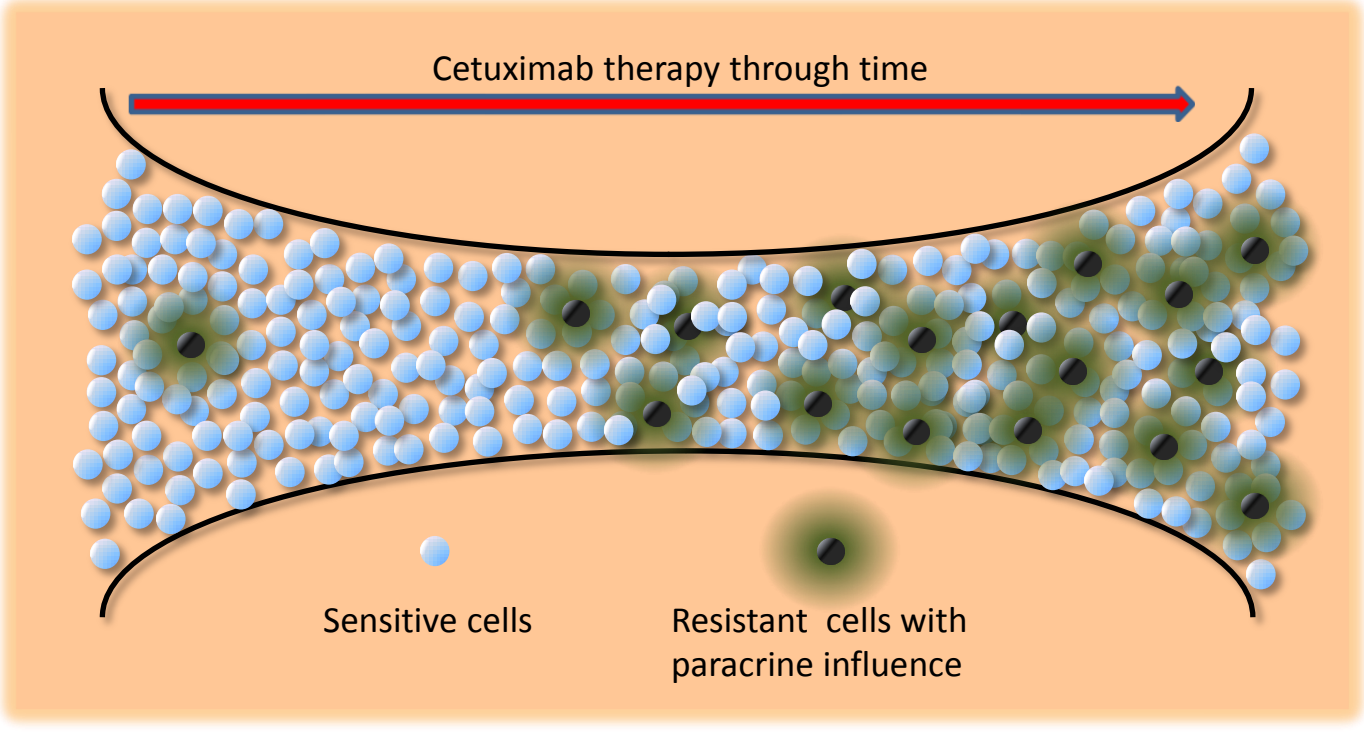

STIMULATION
CONTROL
CTX
TGFα
CM-S CTX
CM-R CTX
CM-S CTX later
CM-R CTX later
CM-S
CM-R
EGFR pY1068
LONG EXPOSURE
EGFR TOTAL
P-ERK1/2
LONG EXPOSURE
ERK-TOTAL
VINCULIN

Fig. 6


Cetuximab therapy through time
Sensitive cells
Resistant cells with paracrine influence