MET signaling in colon cancer stem-like cells blunts the therapeutic response to EGFR inhibitors

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Abstract

Metastatic colorectal cancer remains largely incurable, although in a subset of patients survival is prolonged by new targeting agents such as anti-EGFR antibodies. This disease is believed to be supported by a subpopulation of stem-like cells termed colon cancer-initiating cell (CC-ICs), which may also confer therapeutic resistance. However, how CC-ICs respond to EGFR inhibition has not been fully characterized. To explore this question, we systematically generated CC-ICs through spheroid cultures of patient-derived xenografts of metastatic colorectal cancer. These cultures, termed “xenospheres”, were capable of long-term self-propagation in vitro and phenocopied the original patient tumors in vivo, thus operationally defining CC-ICs. Xenosphere CC-ICs retained the genetic determinants for EGFR therapeutic response in vitro and in xenografts: like the original tumors, xenospheres harboring a mutated KRAS gene were resistant to EGFR therapy, while those harboring wild-type RAS pathway genes (RASwt) were sensitive. Notably, the effects of EGFR inhibition in sensitive CC-ICs could be counteracted by cytokines secreted by cancer-associated fibroblasts. In particular, we found the MET receptor ligand HGF was especially active in supporting in vitro CC-IC proliferation and resistance to EGFR inhibition. Ectopic production of human HGF in CC-IC xenografts rendered the xenografts susceptible to MET inhibition, which sensitized the response to EGFR therapy. By showing that RASwt CC-ICs rely on both EGFR and MET signaling, our results offer a strong preclinical proof of concept for concurrent targeting of these two pathways in the clinical setting.
Introduction

The survival outcome of colorectal cancer (CRC) patients at metastatic stage is improving, thanks to agents such as antibodies (cetuximab and panitumumab) targeting the Epidermal Growth Factor Receptor (EGFR). However these tumors are often intrinsically refractory and those that are sensitive almost invariably relapse, mainly due to concomitant genetic alterations that confer EGFR-independent proliferation (reviewed in 1). Such alterations include mutations of KRAS, NRAS, and BRAF, as well as amplification of HER2 and MET (2-5).

A still elusive, but potentially crucial cause of treatment failure is the survival of the so-called “cancer stem cells”. Increasing evidence shows that CRC – as well as other tumors – contains such cells, more appropriately defined as “cancer-initiating cells” (C-ICs), to emphasize their distinctive operational property of driving clonal expansion in xenotransplantation assays (6,7). Consistently, C-ICs retain the ability to long-term self-renew in vitro, and to restore, in vitro and in vivo, a cell hierarchy including both C-ICs and cells devoid of tumorigenic and self-renewal properties (non-C-IC).

Accumulating data indicate that, while non-C-ICs are usually sensitive, C-ICs are inherently resistant to radio-chemotherapy, owing to still poorly understood mechanisms that include efficient DNA repair and self-sustained protection from apoptosis (8-12). The molecular basis of C-IC resistance to targeted therapies is even less characterized, due to lack of models integrating C-IC genetic features with therapeutic responses.

Beside cell-autonomous genetic factors or functional traits typical of C-ICs, the tumor microenvironment contributes to resistance against targeted therapies as well (reviewed in 13): indeed, stromal-derived growth factors can circumvent the proliferative blockade imposed by agents targeting receptor tyrosine kinases or their downstream transducers (14-16). The microenvironment could also promote therapeutic resistance at the C-IC level, by providing factors that may exacerbate intrinsic drug refractoriness, and/or sustain distinctive C-IC properties such as long-term self-renewal.

To unveil the mechanisms underlying sensitivity or resistance of colon C-ICs (CC-ICs) to targeting agents, we generated a model that integrates characterization of CC-IC genetic lesions, biological/tumorigenic properties, and therapeutic responses in vitro and in vivo. Taking advantage of our ample collection of patient-derived xenografts of CRC liver metastasis ("xenopatients") molecularly annotated and characterized for their response to EGFR inhibitors (4), we derived and characterized a panel of CC-ICs ("xenospheres"), and used them to generate secondary tumors in recipient mice ("spheropatients"). We found that,
consistently with clinical data, in the absence of constitutive RAS activation, CC-IC proliferation and survival rely on the EGFR pathway. However, this dependency could be bypassed by other growth factor receptors, mainly wild-type MET activated by a paracrine circuit of HGF. Consistently, inhibition of MET could empower sensitivity to anti-EGFR therapy.
METHODS

Xenosphere generation from xenopatients

Animal experimentation was approved by the Italian Ministry of Health and by the internal Ethical Committee for Animal Experimentation. Cells dissociated from xenopatient tumors (4) were grown in standard stem-cell medium, including basal stem cell medium, i.e. DMEM/F-12 (Sigma), 2 mM glutamine (Sigma), penicillin-streptomycin (EuroClone), N-2 (Life Technologies-GIBCO), 0.4% BSA (Sigma), 4 µg/ml heparin (Sigma), CD Lipid Concentrate (Life Technologies-GIBCO), supplemented with human recombinant EGF (20 ng/ml; Sigma) and bFGF (10 ng/ml; Peprotech).

Spheropatient generation and therapy

Dissociated xenosphere cells (10^6 cells/ml) were resuspended in a 1:1 mixture of basal stem-cell medium and matrigel (BD Bioscience), and subcutaneously injected into NOD/SCID female mice (Charles River Laboratories) or human HGF knock-in SCID mice. When tumors reached an average volume of 400 mm^3, mice were randomized and treated with 20 mg/kg cetuximab (Merck) twice-weekly, or 50 mg/kg of JNJ-38877605 daily (17), or both agents, or vehicle. Tumor size was measured once-weekly by caliper, and volume was calculated using the formula \(4/3(d/2)^2D/2\), where \(d\) is the minor and \(D\) is the major tumor axis.

Fibroblast Conditioned Medium (FCM)

Primary CRC specimens were processed as described above and grown in adhesive dishes. Fibroblasts were separated from epithelial cells by serial trypsinization. MRC5 fibroblasts were from ATCC; human hTERT-fibroblasts were kindly provided by R.A. Weinberg (18). Fibroblasts were cultured to confluence and then kept for 24h in basal stem-cell medium. FCM was analyzed with the RayBio® Human Cytokine Antibody Array 5 (RayBiotech, Inc.).

Flow-cytometric analysis

Cells (2x10^5) were incubated with the following mouse monoclonal antibodies: anti-CD133/1 (MiltenyiBiotec GmbH), anti-MET (R&D Systems Inc.), anti-CD24 and anti-CD44 (both Invitrogen), and analyzed in a CyAN ADP (Dako Cytomation).

Cell viability and apoptosis assays

Cells were plated at clonal density (10 cells/µl) in basal stem-cell medium. Growth factors, cetuximab (10 µg/ml), JNJ-38877605 (0,5 µM), or gefitinib (0,5 µM) were added at day 0.
ATP production and Caspase 3/7 activity were measured using respectively Cell Titer Glow® (Promega) and Caspase-Glow® 3/7 Assay (Promega), and a GloMax 96 Microplate Luminometer (Promega).

**Western blot**

Immunoblotting were analyzed with the following antibodies: mouse monoclonal anti-MET (DL21, ref. 19), goat polyclonal anti-HGF (R&D Systems), rabbit monoclonal anti-EGFR, rabbit anti-phospho-ERK1/2 (Thr202/204), rabbit anti-ERK1/2, rabbit anti-phospho-Akt (Ser473), rabbit anti-Akt, rabbit anti-phospho-S6 (Ser235/236), rabbit anti-S6 (all Cell Signaling Technology), rabbit anti-phospho-EGFR (Tyr1068, Abcam). Mouse monoclonal anti-Hsp70 (Santa Cruz Biotechnology) and anti-vinculin (Sigma Aldrich) antibodies were used as controls of equal protein loading.

**Immunohistochemistry**

Tumor sections were formalin-fixed, paraffin-embedded, processed according to standard procedures, and analyzed with the following antibodies: anti-phospho-S6 rabbit monoclonal (Ser235/236, Cell Signaling Technology) and anti-phospho-ERK1/2 rabbit monoclonal (Thr202/204; Cell Signaling Technology).

**Statistical analysis**

Results were expressed as means ± standard error of the mean (SEM). Statistical significance was evaluated using two-tailed Student’s t-tests. p<0.05 was considered statistically significant. Statistical analysis of HGF expression in a publicly available dataset (GEO dataset GSE5851) (20) was performed by Fisher exact test on data extracted from KRASwt patients, taking the 75th percentile as threshold and comparing patients with reported Progressive Disease versus those with Complete/Partial Response and Stable Disease.

**For more detailed methods see Supplementary Experimental Procedures**
RESULTS

Xenospheres retain genetic mutations of xenopatients

To obtain CC-ICs, xenopatient tumors were dissociated and selected in standard stem-cell medium, thus establishing sphere cultures, which, as previously shown, are enriched in stem/progenitor cells (11,21,22). To specify their origin from xenopatients rather than from fresh surgical specimens, these spheres were named “xenospheres” (Supplementary Fig. S1A). Eight distinct xenosphere lineages were established from as many xenopatients, and displayed the ability to long-term self-propagate in vitro (see below). Together with their respective xenopatient tumors, xenospheres underwent analysis of gene alterations typically associated with CRC resistance to EGFR targeted therapy (\textit{KRAS}, \textit{NRAS}, \textit{BRAF}, and \textit{PIK3CA} gene mutations; \textit{HER2} and \textit{MET} gene amplifications). Xenospheres retained the same lesions detected in the matched xenopatients (Supplementary Table S1), allowing grouping in two main subsets: (i) xenospheres lacking alterations of \textit{KRAS}, \textit{NRAS}, \textit{BRAF}, \textit{HER2}, or \textit{MET}, hereafter indicated as RAS\textsuperscript{wt} (M016, M049, M195, M199); ii) xenospheres harboring a mutated \textit{KRAS} gene - invariably at residue G12 - hereafter indicated as RAS\textsuperscript{mut} (M119, M126, M138, M211). Although the efficiency of xenosphere derivation was relatively low (10-15%), the frequency of \textit{KRAS} mutation was similar in xenospheres, xenopatients and patients, ruling out a selection bias for this mutation. Mutational analysis was extended to \textit{APC} gene hotspots, and, again, concordant frequency among xenospheres, xenopatients and human patients was observed (Supplementary Table S1).

Xenospheres generate tumors that recapitulate morphology and therapeutic sensitivity of the original xenopatients

To assess the tumorigenic potential of xenospheres, $10^5$ cells were injected subcutaneously into NOD/SCID mice to produce “spheropatients” (100% engraftment efficiency) (Supplementary Fig. S1A). Spheropatient tumors reproduced the same morphology as the corresponding xenopatients (Fig. 1A), and thus as the original tumor. This confirms the histogenetic and pseudodifferentiating ability of C-ICs (6,7,23,24) and indicates that tumors from xenospheres properly recapitulate intra- and inter-tumor heterogeneity. From spheropatients, secondary xenospheres could be re-derived, which retained the same in vitro properties and tumorigenicity of the primary xenosphere, namely the ability to sustain serial transplantation (Supplementary Fig. S1A).
To ascertain whether the therapeutic responses of the original tumors were retained, spheropatients were treated with the anti-EGFR antibody cetuximab. Like the matched xenopatients, RAS\textsuperscript{wt} spheropatients M016, M049 and M195 underwent tumor regression (Fig. 1B and Supplementary Table S2), while RAS\textsuperscript{mut} spheropatients M119, M126, and M138 experienced tumor progression similarly to controls (Fig. 1B). Of note, CC-ICs of spheropatient M016 (as well as the bulk of the corresponding xenopatient) exhibited a \textit{PIK3CA} exon 20 mutation (Supplementary Table S1), whose role as resistance biomarker is still debated (25). In our context both M016 xenopatient and spheropatient responded to cetuximab with evident tumor shrinkage (4), confirming their concordance.

\textbf{Xenospheres display in vitro properties and markers of CC-ICs}

CC-ICs are defined not only by the ability to regenerate tumors with the same morphology (and therapeutic response) as the original tumors, but also by the ability to self-renew \textit{in vitro}, and to switch towards growth arrest and differentiation in appropriate culture conditions (24). Accordingly, xenospheres could be propagated in standard stem-cell medium for more than 60 passages, without alteration of proliferative or tumorigenic potential. Conversely, when cultured in prodifferentiating conditions, xenospheres ceased to grow and died, although RAS\textsuperscript{mut} survived longer than RAS\textsuperscript{wt} xenospheres (Supplementary Fig. S1B, and data not shown).

Xenospheres were then analyzed for expression of markers previously used to isolate and characterize CC-ICs, such as CD133, CD44 and CD24 (24). Flow-cytometric analysis revealed high and consistent CD24 expression, and lower and more heterogeneous CD44 expression (Fig. 2A). Unexpectedly, CD133 expression, previously used to prospectively isolate CC-IC from primary CRC (6,7), was very heterogeneous within and among the remaining xenospheres. This is not surprising, as in metastatic CRC both CD133\textsuperscript{+} and CD133\textsuperscript{-} cells display tumor-initiating ability (26).

Xenospheres were also analyzed for expression of receptors activated by ligands present in standard stem-cell medium, including EGFR family members (EGFR, HER-2 and HER-3), and FGFR2. Transcripts for the four receptors were significantly expressed in all xenospheres (Supplementary Fig. S1C) as was EGFR protein, albeit at variable levels (Supplementary Fig. S1D).
**Proliferation of RAS\textsuperscript{wt} but not RAS\textsuperscript{mut} xenospheres requires exogenous growth factors**

In growth curve assays, representative RAS\textsuperscript{wt} xenospheres (M016 and M049) proliferated only in the presence of exogenous growth factors, i.e EGF-bFGF (GFs), while representative RAS\textsuperscript{mut} xenospheres (M126 and M138) displayed the same proliferation rate irrespectively of exogenous GFs (Fig. 2B), even though EGF (stem-cell medium) could induce EGFR phosphorylation (Supplementary Fig. S1E). Similarly, after dissociation and embedding in soft agar, RAS\textsuperscript{wt} cells formed spheres only in the presence of exogenous GFs, while RAS\textsuperscript{mut} cells grew even in their absence (Supplementary Fig. S1F).

Next, the xenosphere proliferative response to individual EGF or bFGF was assessed. All four RAS\textsuperscript{wt} xenospheres were significantly stimulated by EGF; three of them (M016, M49 and M195) were stimulated also by bFGF alone, although less than by EGF, and responded to the EGF-bFGF combination with additive effect (Fig. 2C). Conversely, proliferation of RAS\textsuperscript{mut} xenospheres was not further increased by either EGF or bFGF with the (partial) exception of M119 (Fig. 2C).

**Xenosphere response to EGFR inhibition hinges on RAS mutation and on paracrine/autocrine EGF signaling**

We then investigated whether cetuximab could block proliferation and induce apoptosis of CC-ICs \textit{in vitro}. As expected, in representative RAS\textsuperscript{mut} xenospheres M126 and M138, cetuximab did not affect cell viability (Fig. 3A) or apoptosis (Fig. 3B), irrespectively of the presence of GFs. However, although cetuximab induced a therapeutic response in RAS\textsuperscript{wt} xeno- and spheropatients (see above, Fig. 1B) (4), it did not significantly affect proliferation or apoptosis of the corresponding RAS\textsuperscript{wt} xenospheres cultured in standard stem-cell medium (Fig. 3C,D). This could be explained by the presence of (i) an excess EGF (20 ng/ml) that likely outcompeted the effective dose of the antibody (10 µg/ml) for binding the EGFR receptor extracellular domain (5,27), and (ii) bFGF (10 ng/ml) that could compensate for EGFR inhibition in sustaining viability. We then verified that the minimal EGF concentration required to achieve a plateau proliferative effect in RAS\textsuperscript{wt} xenospheres was as low as 0.2 ng/ml (Supplementary Fig. S2A); at this EGF concentration (and up to 2 ng/ml), cetuximab potently impaired viability of representative RAS\textsuperscript{wt} xenospheres (Fig. 3E), in accordance with the \textit{in vivo} data. In dose-response experiments, we showed also that bFGF concentrations higher than those present in the standard stem-cell medium (at least 20 ng/ml), could significantly, although partly, restore viability and protect RAS\textsuperscript{wt} xenospheres from apoptosis induced by cetuximab (Fig. 3F,G).
Interestingly, when cultured without GFs (Fig. 3C,D), RAS<sup>wt</sup> xenospheres exhibited different basal viability and apoptotic rates, as well as different sensitivity to EGFR blockade. M016 and M199 displayed self-sustained viability and low levels of apoptosis, but were extremely sensitive to EGFR inhibition (Fig. 3C,D). At variance, M049 and M195 kept in the absence of GFs were poorly viable and irreversibly poised to apoptosis, attitudes that were unaffected by cetuximab (Fig. 3C,D). The behavior of M016 and M199 was explained by EGF autocrine expression (Supplementary Fig. S2B), which, on one side, could sustain viability even in the absence of exogenous GFs, and, on the other side, likely sensitized CC-ICs to EGFR inhibition. Results were supported by the use of gefitinib, an alternative EGFR specific small-molecule inhibitor (28) (Supplementary Fig. S2C).

Taken together, these experiments indicate that EGFR inhibition blocked proliferation and induced apoptosis of RAS<sup>wt</sup> CC-IC. The <i>in vitro</i> response to cetuximab was masked by the standard experimental conditions used to grow xenospheres (excess EGF); however, response could be restored by lowering exogenous EGF concentration to doses sufficient to fully sustain cell proliferation and survival.

**Xenosphere proliferation and resistance to EGFR inhibition are sustained by fibroblast-derived HGF**

Recent reports highlighted that stromal-derived cytokines mediate resistance to targeted therapies (16). Our observation that growth of RAS<sup>wt</sup> CC-ICs is additively sustained by EGF and bFGF suggests that one or more such growth factors might compensate for interception of EGFR signaling by cetuximab.

CRC-associated fibroblasts (CAFs) are known to secrete several growth factors, cytokines and chemokines (29,30). To investigate the CAF ability to sustain xenosphere propagation, a primary culture of CAFs derived from a CRC sample, and two human fibroblast cell lines (MRC5 and hTERT, ref. 18) were used to produce conditioned media (FCM). These were analyzed by multiplex cytokine arrays, revealing high amounts of IL-6, IL-8, Rantes, Gro-α, and HGF, but negligible quantities of EGF and FGFs (Fig. 4A).

Using the representative RAS<sup>wt</sup> xenosphere M049, we noticed that FCM from different fibroblast cell lines induced cell proliferation proportionally to the amount of HGF secreted. Indeed, the FCM from senescent CAFs (p9), where most cytokines were still abundant, but HGF was significantly reduced, did not induce proliferation (Fig. 4A,B). At odds, changes in contents of other cytokines (e.g. IL-6, Rantes or Gro-α) did not correlate with changes in xenosphere viability (Fig. 4A,B). Moreover, FCM-induced proliferation was
completely blocked by JNJ-38877605, a specific inhibitor of the HGF receptor, MET (17) (Fig. 4B), or by the anti-MET monovalent antibody DN30-FAB (31) (Supplementary Fig. S3A). Importantly, FCM not only sustained the growth of xenospheres, but it also protected them from cetuximab (Fig. 4B). Similarly, also co-culture with hTERT fibroblasts sustained xenosphere growth and viability, which were impaired after addition of JNJ-38877605, but not of cetuximab (Fig. 4C).

The proliferative effect of FCM, as well as FCM contribution to resistance against EGFR inhibition, was confirmed on RASwt M016, M049 and M195: in the presence of FCM, cetuximab and gefitinib did not affect viability (Fig. 4D and Supplementary Fig. S3B), nor did they promote caspase activation (Fig. 4E and Supplementary Fig. S3C). Conversely, JNJ-38877605 completely blocked proliferation and induced apoptosis (Fig. 4D,E and Supplementary Fig. S3B,C), attesting that HGF mediates the protective activity of FCM. Interestingly, in RASwt M199, FCM promoted viability, but inhibition by JNJ-38877605 was attenuated (Supplementary Fig. S3B,C) likely by the expression of a strong EGF autocrine loop (Supplementary Fig. S2B). Consistently, this xenosphere maintained sensitivity to EGFR inhibitors (Supplementary Fig. S3B,C).

The role of HGF in sustaining CC-IC proliferation was further confirmed by supplying purified HGF as a single growth factor (Fig. 5A). RASwt M016 and M049 were treated with cetuximab in the presence of HGF or IL-8, another cytokine particularly abundant in FCM (Fig. 4A). HGF, but not IL-8, significantly protected both xenospheres from cetuximab, by preserving cell viability and inhibiting apoptosis (Fig. 5B,C). Protection was likely mediated by activation of MAP kinase and AKT pathways, which remained high when HGF was supplied together with cetuximab, and was extinguished when the MET inhibitor was also added (Figure 5D). Similar to our observations with FCM, protection against cetuximab by purified HGF was dose-dependent (Supplementary Fig. S4A).

**MET expression in xenospheres**

Significant expression of MET was verified in all xenospheres by Western blot (Fig. 5E) and flow-cytometry (Fig. 5F). As an exception, among RASwt xenospheres, M199 displayed lower levels of MET and, consistently, weaker proliferative response to HGF (Fig. 5A). Low MET expression contributed also to explain why this xenosphere was poorly sensitive to MET inhibitors when grown in FCM (Supplementary Fig. S3B,C). In RASmut xenospheres, which also expressed high levels of MET, HGF stimulated MET activation (Supplementary Fig. S4C), but did not increase their autonomous proliferation rate (not shown). Interestingly,
in all xenospheres cultured in prodifferentiating conditions, except in RAS\textsuperscript{mut} M126, MET protein expression decidedly decreased (Fig. 5E), indicating preferential association of MET expression with the stem/progenitor status. Notably, serum-induced differentiation affected EGFR expression randomly, with reduced protein levels in three xenospheres (M016, M199, M138) and increased levels in the remaining five (M049, M195, M119, M126, M211) (Fig. 5E).

**RAS\textsuperscript{wt} spheropatients display enhanced response to combined EGFR and MET inhibition**

Data obtained in vitro support the hypothesis that HGF secretion by CAFs provides a mechanism of CC-IC primary resistance to cetuximab, which can be counteracted by MET inhibition. Consistently, we observed that HGF expression in tumors of patients that progressed on cetuximab, was significantly higher than that of responders, as assessed by analyzing publicly available gene expression datasets of human KRAS\textsuperscript{wt} metastatic CRCs annotated for response to cetuximab monotherapy (20) (Fig. 6A).

The efficacy of MET inhibitors cannot be assessed in a classical xenograft model, as murine HGF does not activate the human MET receptor (32,33). Consistently, M049 spheropatient did not respond to treatment with JNJ-38877605 as single agent (as previously observed in RAS\textsuperscript{wt} xenopatients, ref. 34), or in combination with cetuximab (Supplementary Fig. S5A). We thus generated two experimental settings to include human HGF in the murine tumor microenvironment. In the first, M049 xenospheres were transduced with a lentiviral construct expressing the human \textit{HGF} gene (M049-HGF), to induce an autocrine loop (Supplementary Fig. S5B). This loop was functional, as shown by administration of JNJ-38877605, which decreased viability of M049-HGF, and, in the presence of EGF, increased their sensitivity to cetuximab (Supplementary Fig. S5C,D). Spheropatients were then obtained by subcutaneous injection of M049-HGF into NOD/SCID mice, and treated with placebo, cetuximab, JNJ-38877605, or both inhibitors, for six weeks. MET inhibition alone slightly, but significantly, delayed tumor growth, although without inducing regression or stabilization. On the contrary, cetuximab caused a 70% tumor regression that stabilized after three weeks. Interestingly, the concomitant administration of cetuximab and JNJ-38877605 resulted in more pronounced tumor regression, which continued up to 6 weeks of treatment, reaching 90% (Fig. 6B).

In the second experimental setting, mice were genetically engineered by replacing the endogenous \textit{Hgf} gene with the human gene (h\textit{HGF}-Ki), used as recipients of parental M049
xenospheres, and treated as above. In this case, JNJ-38877605 was ineffective as a single agent, but enhanced the rate of tumor shrinkage when combined with cetuximab, again inducing up to 90% regression after 6 weeks (Fig. 6C).

As shown by immunohistochemistry of sections from tumors formed by M049-HGF xenospheres, JNJ-38877605 modestly affected MAPK and AKT pathways, while cetuximab inhibited completely the MAPK and only partially the AKT pathway. Combination of the two inhibitors effectively impaired both signal transduction pathways (Fig. 6D), accordingly to in vitro findings (Fig. 5D).

In order to assess long-term therapeutic outcomes, M049-HGF spheropatients were treated up to 6 months with cetuximab and JNJ-38877605, alone or in combination (Fig. 6E) as above. In these experiments, the groups initially treated with vehicle or JNJ-38877605 alone started to receive cetuximab when tumors reached a volume of 1400 mm$^3$ or 1300 mm$^3$, respectively. By this strategy, we could monitor the consequences of delayed cetuximab administration in mice that were either naïve for any inhibitor or “primed” by prior MET inhibition. Tumors treated with cetuximab alone from the beginning experienced a maximum regression of 70% that stabilized after 6 weeks and, at 12 weeks, started to display a slight but continuous trend to regrowth, regaining 40% of the baseline volume after 24 weeks. Mice treated with the two inhibitors from the beginning showed a decidedly more pronounced tumor regression than mice treated with cetuximab alone, reaching a maximum 94% tumor shrinkage after 9 weeks, which stabilized without any statistically significant change until 24 weeks. The outcomes of the groups with delayed administration of cetuximab were comparable to the corresponding “early” treatment groups (Supplementary Table S3): tumors that received delayed cetuximab monotherapy reached a maximum 75% regression 4 weeks after treatment initiation, and, at the end of the experiment (17 weeks after treatment initiation) regained 58% of the baseline volume. Tumors initially treated with JNJ-38877605 and experiencing delayed addition of cetuximab displayed continuous regression, reaching 90% reduction of their maximum volume (Fig. 6E).

Consistently, tumors explanted after 6 months of treatment with cetuximab alone exhibited basally (re)activated MAPK and AKT pathways, whereas tumors treated with both inhibitors did not show any sign of pathway activity (Fig. 6F). Moreover tumors treated with both inhibitors showed signs of increased pseudodifferentiation, such as increased stromal density, and reduced epithelial cellularity, with adenomatous phenotype (Fig. 6F) and increased expression of cytokeratin 20 (Supplementary Fig. S6A). Interestingly, tumors treated with both inhibitors, showed decreased expression of stem-cell markers such as LGR5.
and β-catenin, as well as the corresponding xenospheres treated in vitro showed decreased expression of CD24, CD133, CD44 and MET (Supplementary Fig. S6A,B).
Discussion

Over decades cell lines contributed to unravel the genetic basis of cancer pathogenesis, and to identify therapeutic targets. Nevertheless, their validity for the pre-clinical screening of anti-cancer drugs has been challenged (reviewed in 35,36). Indeed, cancer cell lines may fail to recapitulate the overall features of the original tumor, because, after the several in vitro passages required to achieve immortalization, they might have genetically drifted, and lost differentiation plasticity (37).

The quest for cells maintaining the genetic and histogenetic properties of the original tumors has led to tumor “spheres”, primary cultures established by a protocol initially set up for neural stem cells (38), and later successfully applied to several tumor tissues, including CRC (7,11,23,24). These cells display the operational properties of “cancer stem cells”, or “cancer-initiating cells” (C-ICs). Indeed, they retain long-term self-renewal ability in vitro, and, when implanted in immunocompromised mice, form tumors almost indistinguishable from the originals (7,37). Notably, tumor spheres retain the genetic make-up of the original cancer far more faithfully than standard cell lines (37), thus – regardless of their “stem” properties – they should be more suitable than cell lines to identify the mechanisms responsible for sensitivity and resistance to molecular cancer therapeutics.

Spheres obtained from CRC, or “colospheres”, were used to demonstrate C-IC resistance to conventional therapies (12); however, to our knowledge, little has been done so far to characterize their genetic profiles, and to relate them to response to targeted therapies.

In this work, from human CRC metastases xenografted in mice, the so-called “xenopatients” (4), we derived sphere named “xenospheres”. CRC xenopatients allowed (i) to reproduce the therapeutic response to anti-EGFR antibodies observed in human patients, (ii) to validate negative predictors of response, such as mutations in RAS pathway genes, (iii) to discover HER2 amplification as a novel mechanism of primary resistance, and (iv) to validate HER2 as an effective therapeutic target in combination with EGFR inhibitors (4). More recently, xenopatients contributed to demonstrate that amplification of the MET oncogene is a mechanism of both primary and secondary resistance to anti-EGFR therapies (3).

Taking advantage of data available in the original corresponding patients and xenopatients, here we show that xenospheres (i) retain the same genetic lesions of the tumor of origin, and (ii) generate tumor xenografts (“spheropatients”) that recapitulate the histological heterogeneity of the corresponding xenopatients (and thus of the corresponding patients). We also show that (iii) the spheropatient response to the anti-EGFR antibody is...
comparable to the response observed in the corresponding xenopatients, accordingly with the mutational status of KRAS. We thus conclude that xenospheres are a reliable, molecularly annotated, in vitro model of CC-ICs, able to retain from the tumor of origin, and pass to a secondary tumor (spheropatient), the genetic determinants of therapeuic response.

We then confirmed, for the first time in CC-IC, the relevance of the KRAS gene status for proliferation and survival. Indeed, xenospheres harboring a KRAS mutation self-sustain their growth and are insensitive to exogenous growth factors. Conversely, xenospheres with wild type KRAS, NRAS and BRAF cannot grow and survive unless supplied with exogenous growth factors. Although the number of cases examined was limited, we show that RAS\textsuperscript{wt} xenospheres are exquisitely sensitive to EGF: indeed, they proliferate at an EGF molar concentration one log lower than that required for other growth factors such as FGF or HGF. In some cases, xenospheres express EGF autocrine loops able to maintain basal survival signals, although insufficient to sustain autonomous proliferation. We therefore show that the response of the original xenopatients (and patients) to EGFR inhibition is rooted in CC-ICs level: if RAS pathway genes are intact, EGFR inhibitors can stop tumor growth by hitting the tumorigenic cell subpopulation.

However, we also found that, in this subpopulation, EGFR may be the dominant but not the unique regulator of proliferation. The HGF receptor MET was expressed in all xenospheres, and, unlike EGFR, it was down-regulated in their in vitro differentiated derivatives, suggesting a specific association of MET expression with – and possibly a specific function within – the stem/progenitor compartment. Consistently, we and others recently reported that MET is expressed in glioblastoma stem cells and promotes their self-renewal (39-41). Concerning CRC, it was previously shown that HGF can sustain the WNT self-renewal pathway (42,43). Yet, HGF ability to sustain long-term CC-IC proliferation was unknown. We show here that HGF can promote RAS\textsuperscript{wt} CC-IC proliferation, and that inherent expression of the wild-type MET gene in these cells is a factor of primary resistance to anti-EGFR therapy, a knowledge with salient implications for CRC pathogenesis and therapy.

While MET amplification likely represents a rare mechanism of primary resistance to EGFR inhibition (1% of xenopatients) (3) intrinsic expression of wild-type MET, together with physiologic secretion of HGF by cancer-associated fibroblasts, may provide a more widespread mechanism. Indeed, we show that, in the microenvironment generated by conditioned media or co-cultures of cancer-associated fibroblasts, HGF is so abundant that it may take the lead over other growth factors, including EGF, to regulate CC-IC proliferation.
and survival. As a result, in the presence of excess HGF, MET inhibition, but not EGFR inhibition, is sufficient to stop the growth of CC-IC. Primary resistance provided by HGF may be relevant in human patients, as suggested by our analysis of data from metastatic CRC patients that received cetuximab as monotherapy (20): indeed, high HGF expression in KRAS<sup>wt</sup> tumors associates with poor response to cetuximab.

The ability of MET to support CC-IC growth <em>in vitro</em> prompted us to reconsider previous results showing that, in xenopatients, MET inhibition failed to block metastatic CRC featuring wild-type <em>MET</em> (34). Indeed, those experiments were performed in regular immunocompromised mice (NOD/SCID), where murine HGF exerts negligible effects, as it does not cross-react with human MET (32,33). To better evaluate the contribution of HGF to CC-IC tumorigenicity, we generated two RAS<sup>wt</sup> spheropatient models by engineering either the xenospheres or the mice to express human HGF. In these models, MET inhibition alone could not induce significant tumor regression; however, when it was combined with EGFR inhibition, regression was boosted and stabilized for long-time, delaying, and possibly preventing, tumor relapse. Importantly, the combined treatment resulted in decreased expression of cancer stem cell markers, <em>in vitro</em> and <em>in vivo</em>, indicating empowered targeting of CC-ICs.

Previous studies have shown that HGF sustain resistance against EGFR inhibition in cell lines (44,45). We now suggest that concomitant inhibition of the two targets – MET and EGFR – hits CC-ICs that feature defined genetic traits such as wild-type <em>RAS</em> pathway genes. This approach may help to prevent resistance to targeted therapies and lead to a virtually complete and durable tumor regression.
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FIGURE LEGENDS

Figure 1. Xenospheres generate tumors that recapitulate morphology and therapeutic sensitivity of the original xenopatients.
A, Tumor sections (H&E staining). Scale bar: 100 μm. B, Tumor growth curves. Graphs represent tumor volume fold increase/decrease vs. day 0 ± s.e.m. (n = 6 for M016 and M049; n = 3 for M195, M119, M126, and M138, *P < 0.05).

Figure 2. Xenospheres display in vitro markers of CC-ICs, and different growth factor requirements depending on RAS mutational status.
A, Flow cytometric analysis of CD24, CD44, and CD133 in xenospheres. B, Growth curves of xenospheres. Graphs represent relative viability increase vs. day 0 ± s.e.m (n = 3, *P < 0.05). C, Cell viability of xenospheres kept in basal stem-cell medium either alone (NO GFs) or with the indicated growth factors for 6 days. Columns represent relative cell viability vs. NO GFs ± s.e.m (n = 3, *P < 0.05).

Figure 3. Xenosphere response to EGFR inhibition hinges on RAS mutation, and on paracrine/autocrine EGF signaling.
A,C, Cell viability and B,D, Caspase 3/7 activity assays of xenospheres kept for 4 days in standard (EGF-bFGF) or basal (NO GFs) stem-cell medium. Caspase activity was normalized vs. CellTiter-Glow® values. Columns represent relative viability or caspase activity vs. xenospheres kept in EGF-bFGF and vehicle of a representative experiment ± s.e.m (n = 3, *P < 0.05). E, F, G, Cell viability (E,F) or caspase 3/7 activity (G) of xenospheres kept for 4 days in basal stem-cell medium alone (NO GFs) or with increasing doses of growth factors (ng/ml) Columns represent relative viability or caspase activity vs. xenospheres kept with NO GF of a representative experiment ± s.e.m (n = 3, *P < 0.05).

Figure 4. Xenosphere proliferation and cetuximab resistance are sustained by fibroblast-derived HGF.
A, Cytokine array of conditioned media (CM), produced from human fibroblast cell lines MRC5 and hTERT, or freshly isolated CRC Associated Fibroblasts (CAF) at passage 3 (p3) or 9 (p9). B, Cell viability assay of xenospheres cultured in basal stem-cell medium (NO GFs) or in the same CM analyzed in A, and treated with JNJ-38877605 (0.5 μM) or cetuximab (10 μg/ml), or equal volume of vehicle for 4 days. Columns represent relative cell
viability vs. xenospheres kept in NO GFs ± s.e.m ($n = 3$, **$P < 0.05$). C, Micrographs (magnification: x100) of xenospheres co-cultured with hTERT and treated as above. D, Cell viability and E, Caspase 3/7 activity of xenospheres kept in basal (NO GFs) or standard (EGF-bFGF) stem-cell medium, or in the presence of MRC5-CM, or hTERT-CM, and treated as above. Columns represent relative viability and caspase activity vs. xenospheres kept in EGF-bFGF and vehicle of a representative experiment ± s.e.m ($n = 3$, **$P < 0.05$).

Figure 5. HGF activity and MET expression in xenospheres.
A, Cell viability of xenospheres kept in basal stem-cell medium either alone (NO GFs) or with HGF (20 ng/ml) for 6 days. Columns represent the relative viability vs. NO GFs ± s.e.m ($n = 3$, **$P < 0.05$). B, Cell viability and C, Caspase 3/7 activity assays of M016 and M049 xenospheres kept for 4 days in basal stem-cell medium alone (NO GFs), or supplemented with EGF (0.2 ng/ml) and increasing doses of HGF (0.2, 20 and 50 ng/ml), and treated either with cetuximab (10 μg/ml) or equal volume of vehicle. Columns represent the relative viability and caspase activity vs. NO GFs and vehicle of a representative experiment ± s.e.m ($n = 3$, **$P < 0.05$). D, E, Western Blot analysis of total protein extracts (D) from M049 xenospheres cultured in basal stem-cell medium supplemented with combinations of EGF (0.2 ng/ml) or HGF (20 ng/ml), treated with cetuximab (10 μg/ml) or JNJ-38877605 (0.5 μM) as indicated, or (E) from the indicated xenospheres or their differentiated derivatives (serum). F, Flow cytometric analysis of MET.

Figure 6. RASwt spheropatients display enhanced response to combined EGFR and MET inhibition.
A, Heatmap showing preferential association of HGF expression with PD (progressive disease) rather than complete response (CR), or partial response (PR), or stable disease (SD), in patients treated with cetuximab as monotherapy. Fisher’s exact test = 0.0281. B, C, Growth curves (log scale) of tumors generated by M049 expressing a HGF autocrine loop (B), or by parental M049 injected in mice expressing human HGF (hHGFki) (C), and treated as indicated. Graphs represent tumor volume increase vs. day 0 ± s.e.m. ($n = 5$, **$P < 0.05$). D, Immunohistochemical analysis with anti-pERK (pERK) or anti-pS6 (pS6) antibodies of tumor sections derived from M049-HGF spheropatients treated as indicated in B. Scale bar 100 μm. E, Long-term tumor growth curves (log scale) of M049-HGF spheropatients. Blue and red lines: treatments started at day 0. Black and grey lines: arrows indicate when cetuximab was added. Graphs represent tumor volume increase vs. day 0 ± s.e.m. ($n = 3$, **$P}$
< 0.05). **F**, Immunohistochemical analysis with anti-pERK or anti-pS6 antibodies of histological tumor sections derived from M049-HGF spheropatients treated as indicated in **D**. Scale bar: 100 μm.
MET signaling in colon cancer stem-like cells blunts the therapeutic response to EGFR inhibitors

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