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-EGF receptor (anti-EGFR) antibodies. This disease is believed to be supported by a subpopulation of stem-like cells termed colon cancer-initiating cell (CCIC), which may also confer therapeutic resistance. However, how CCICs respond to EGFR inhibition has not been fully characterized. To explore this question, we systematically generated CCICs 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 CCICs. Xenosphere CCICs 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, whereas those harboring wild-type RAS pathway genes (RASWild) were sensitive. Notably, the effects of EGFR inhibition in sensitive CCICs could be counteracted by cytokines secreted by cancer-associated fibroblasts. In particular, we found that the MET receptor ligand hepatocyte growth factor (HGF) was especially active in supporting in vitro CCIC proliferation and resistance to EGFR inhibition. Ectopic production of human HGF in CCIC xenografts rendered the xenografts susceptible to MET inhibition, which sensitized the response to EGFR therapy. By showing that RASWild CCICs 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. Cancer Res; 74(6); 1857–69. © 2014 AACR.

Introduction

The survival outcome of patients with colorectal cancer at metastatic stage is improving, thanks to the agents such as antibodies (cetuximab and panitumumab) targeting the EGFR 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 ref. 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 colorectal cancer—as well as other tumors—contains such cells, more appropriately defined as cancer-initiating cells (CIC), to emphasize their distinctive operational property of driving clonal expansion in xenotransplantation assays (6, 7). Consistently, CICs retain long-term self-renewal ability in vitro, and restore, in vitro and in vivo, a cell hierarchy including both CICs and cells devoid of tumorigenic and self-renewal properties (non–CIC).

Accumulating data indicate that, whereas non–CICs are usually sensitive, CICs are inherently resistant to radiochemotherapy, owing to still poorly understood mechanisms that include efficient DNA repair and self-sustained protection from apoptosis (8–12). The molecular basis of CIC resistance to targeted therapies is even less characterized because of the lack of models that integrate CIC genetic features with therapeutic responses.

Besides cell-autonomous genetic factors or functional traits typical of CICs, the tumor microenvironment contributes to resistance against targeted therapies as well (reviewed in ref. 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 CIC level, by providing factors that may exacerbate intrinsic drug refractoriness, and/or sustain distinctive CIC properties such as long-term self-renewal.

References

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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do i: 10.1158/0008-5472.CAN-13-2340-T ©2014 American Association for Cancer Research.
To unveil the mechanisms underlying sensitivity or resistance of colon CICs (CCIC) to targeting agents, we generated a model that integrates characterization of CCIC genetic lesions, biological/tumorigenic properties, and therapeutic responses in vitro and in vivo. Taking advantage of our ample collection of patient-derived xenografts of colorectal cancer liver metastasis (xenopatients) molecularly annotated and characterized for their response to EGFR inhibitors (4), we derived and characterized a panel of CCICs (xenospheres) and used them to generate secondary tumors in recipient mice (spheropatients). We found that, in consistence with clinical data, in the absence of constitutive RAS activation, CCIC 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 hepatocyte growth factor (HGF). Consistently, inhibition of MET could empower sensitivity to anti-EGFR therapy.

Materials and Methods

Xenosphere generation from xenopatients
Animal experimentation was approved by the Italian Ministry of Health and 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 mmol/L glutamine (Sigma), penicillin–streptomycin (EuroClone), N-2 (Life Technologies-GIBCO), 0.4% bovine serum albumin (Sigma), 4 μg/mL heparin (Sigma), chemically defined lipid concentrate (Life Technologies-GIBCO), supplemented with human recombinant EGF (20 ng/mL; Sigma), and basic fibroblast growth factor (bFGF; 10 ng/mL; Peprotech).

Spheropatient generation and therapy
Dissociated xenosphere cells (10^5 cells/mL) were resuspended in a 1:1 mixture of basal stem-cell medium and Matrigel (BD Biosciences) and were subcutaneously injected into nudeobese diabetic/severe combined immunodeficient (NOD/SCID) female mice (Charles River Laboratories) or human HGF knockin SCID mice. When tumors reached an average volume of 400 mm^3, mice were randomized and treated with 20 mg/kg cetuximab (Merck) twice a week, or 50 mg/kg of JN-38877605 daily (17), or both agents, or vehicle. Tumor size was measured once a week 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
Primary colorectal cancer specimens were processed as described above and grown in adhesive dishes. Fibroblasts were separated from epithelial cells by serial trypsinization. MRC5 fibroblasts were from the American Type Culture Collection; human telomerase reverse transcriptase (hTERT) fibroblasts were kindly provided by Robert A. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, (18). Fibroblasts were cultured to confluence and then kept for 24 hours in basal stem-cell medium. Fibroblast conditioned medium (FCM) was analyzed with the RayBio Human Cytokine Antibody Array 5 (RayBiotech, Inc.).

Flow-cytometric analysis
Cells (2 × 10^3) were incubated with the following mouse monoclonal antibodies—anti-CD133/1 (Miltenyi Biotec GmbH), anti-MET (R&D Systems Inc.), anti-CD24, and anti-CD44 (both Invitrogen)—and were 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), JN-38877605 (0.5 μmol/L), or gefitinib (0.5 μmol/L) were added at day 0. ATP production and caspase-3/7 activity were measured using CellTiter-Glo (Promega) and Caspase-Glo 3/7 Assay (Promega), respectively, and a GloMax 96 Microplate Luminometer (Promega).

Western blot analysis
Immunoblottings 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), and 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 the 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 mean ± SEM. Statistical significance was evaluated using two-tailed Student t tests. P < 0.05 was considered statistically significant. Statistical analysis of HGF expression in a publicly available dataset [Gene Expression Omnibus (GEO) dataset GSE5851; ref. 20] was performed by the Fisher exact test on data extracted from KRAS WT 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 CCICs, 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 these displayed the ability for long-term self-propagation in vitro (see below). Together with their respective xenopatient tumors, xenospheres underwent analysis of gene alterations typically associated with colorectal cancer resistance to EGFR-targeted therapy (KRAS, NRAS, BRAF, and PIK3CA gene mutations; HER2 and 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 KRAS, NRAS, BRAF, HER2, or MET, hereafter indicated as RASwt (M016, M049, M195, and M199); and (ii) xenospheres harboring a mutated KRAS gene— invariably at residue G12—hereafter indicated as RASmut (M119, M126, M138, and M211). Although the efficiency of xenosphere derivation was relatively low (10%–15%), the frequency of KRAS mutation was similar in xenospheres, xenopatients, and patients, ruling out a selection bias for this mutation. Mutational analysis was extended to 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 the originary tumor. This confirms the histogenetic and pseudodifferentiating ability of CICs (6, 7, 23, 24) and indicates xenospheres were maintained only in the presence of exogenous growth factors, that is, EGF-bFGF, whereas representative RASwt xenospheres (M016 and M049) proliferated only in the presence of exogenous growth factors. However, proliferation of RASmut xenospheres (M126 and M138) were stimulated also by bFGF alone, although less efficiently than by EGF, and responded to the EGF–bFGF combination (Supplementary Fig. S1B and data not shown).

Xenospheres were then analyzed for expression of markers previously used to isolate and characterize CCICs, 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 CCICs from primary colorectal cancer (6, 7), was very heterogeneous among and within the remaining xenospheres. This is not surprising, as in metastatic colorectal cancer both CD133^+ and CD133^- 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, HER2, and HER3), 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 RASwt but not RASmut xenospheres requires exogenous growth factors**

In growth curve assays, representative RASwt xenospheres (M016 and M049) proliferated only in the presence of exogenous growth factors, that is, EGF-bFGF, whereas representative RASmut xenospheres (M126 and M138) displayed the same proliferation rate irrespective of exogenous growth factors (Fig. 2B), even though EGF (stem-cell medium) could induce EGFR phosphorylation (Supplementary Fig. S1E). Similarly, after dissociation and embedding in soft agar, RASwt cells formed spheres only in the presence of exogenous growth factors, whereas RASmut cells grew even in their absence (Supplementary Fig. S1F).

Next, the xenospher proliferative response to individual EGF or bFGF was assessed. All four RASmut xenospheres were significantly stimulated by EGF; three of them (M016, M49, and M195) were stimulated also by bFGF alone, although less efficiently than by EGF, and responded to the EGF–bFGF combination with an additive effect (Fig. 2C). Conversely, proliferation of RASmut 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 CCICs in vitro. As expected, in representative RASmut xenospheres M126 and M138, cetuximab did not affect cell viability (Fig. 3A) or apoptosis (Fig. 3B), irrespective of the presence of growth factors. However, although cetuximab induced a therapeutic response in RASwt xenosphere xenospheres (see above; Fig. 1B; ref. 4), it did not significantly affect proliferation or apoptosis of the corresponding RASmut xenospheres cultured in standard stem-
cell medium (Fig. 3C and D). This could be explained by the presence of (i) an excess EGF (20 ng/mL) that likely out-competed 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 also showed 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 and G).

Interestingly, when cultured without growth factors (Fig. 3C and D), RAS\textsuperscript{mut} 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 and D). At variance, M049 and M195 kept in the absence of growth factors were poorly viable and irreversibly poised to apoptosis, attitudes that were unaffected by cetuximab (Fig. 3C and 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 growth factors, and, on the other side, likely sensitized CCICs to EGFR inhibition. Results were supported by the use of gefitinib, an alternative EGFR-specific small-molecule inhibitor (Supplementary Fig. S2C; ref. 28).

Taken together, these experiments indicate that EGFR inhibition blocked proliferation and induced apoptosis of RASwt CCIC. The in vitro 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 RASwt CCICs is additively sustained by EGF and bFGF suggests that one or more such growth factors might compensate for the interception of EGFR signaling by cetuximab.

Colorectal cancer–associated fibroblasts (CAF) 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 representative experiment ± SEM (n = 3; *, P < 0.05).

Figure 3. Xenosphere response to EGFR inhibition hinges on RAS mutation and on paracrine/autocrine EGF signaling. A–D, cell viability (A and C) and caspase-3/7 activity (B and D) assays of xenospheres kept for 4 days in standard (EGF-bFGF) or basal [no growth factors (GF)] stem-cell medium. Caspase activity was normalized versus CellTiter-Glo values. Columns, relative viability or caspase activity versus xenospheres kept in EGF-bFGF and vehicle of a representative experiment ± SEM (n = 3; *, P < 0.05). E, F, and G, cell viability (E and F) or caspase-3/7 activity (G) of xenospheres kept for 4 days in basal stem-cell medium alone (no growth factors) or with increasing doses of growth factors (ng/mL). Columns, relative viability or caspase activity versus xenospheres kept with no growth factor of a representative experiment ± SEM (n = 3; *, P < 0.05).
MET expression in xenospheres

Significant expression of MET was verified in all xenospheres by Western blot analysis (Fig. 5E) and flow cytometry (Fig. 5F). As an exception, among RAS<sup>wt</sup> xenospheres, M199 displayed lower levels of MET and, consistently, weaker proliferative response to HGF (Fig. 5A). Low MET expression also contributed to explain why this xenosphere was poorly sensitive to MET inhibitors when grown in FCM (Supplementary Fig. S3B and S3C). In RAS<sup>mut</sup> 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<sup>mut</sup> 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, and M138) and increased levels in the remaining five (M049, M195, M119, M126, and M211; Fig. 5E).

**RAS<sup>wt</sup> 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 CCIC primary resistance to cetuximab, which can be counteracted by MET inhibition. Consistently, we observed that HGF expression in tumors of patients who progressed on cetuximab was significantly higher than that of responders, as assessed by analyzing publicly available gene expression datasets of human KRAS<sup>wt</sup> metastatic colorectal cancers annotated for response to cetuximab monotherapy (Fig. 6A; ref. 20).

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). Consequently, M049 spheropatient did not respond to treatment with JNJ-38877605 as a single agent (as previously observed in RAS<sup>wt</sup> 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. First, M049 xenospheres were transduced with a lentiviral construct expressing the human 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 and S5D). 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 6 weeks. MET inhibition alone slightly, but significantly, delayed tumor growth, although without inducing regression or stabilization. On the contrary, cetuximab caused a 70% tumoral regression that stabilized after 3 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 *Hgf* gene with the human gene (*hHGF-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).
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 CAFs at passage 3 (p3) or 9 (p9). B, cell viability assay of xenospheres cultured in basal stem-cell medium [no growth factors (GF)] or in the same conditioned media analyzed in A, and treated with JNJ-38877605 (0.5 μmol/L) or cetuximab (10 μg/mL), or equal volume of vehicle for 4 days. (Continued on the following page.)
As shown by immunohistochemistry of sections from tumors formed by M049-HGF xenospheres, JNJ-38877605 modestly affected the 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), according to in vitro findings (Fig. 5D).

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.

(Continued.) Columns, relative cell viability versus xenospheres kept in no growth factors ± SEM (n = 3; *, P < 0.05). C, micrographs (magnification, × 100) of xenospheres cocultured with hTERT and treated as above. Cell viability (D) and caspase-3/7 activity (E) of xenospheres kept in basal (no growth factors) or standard (EGF-bFGF) stem-cell medium or in the presence of MRC5-CM or hTERT-CM and treated as above. Columns, relative viability and caspase activity versus xenospheres kept in EGF-bFGF and vehicle of a representative experiment ± SEM (n = 3; *, P < 0.05).
Figure 6. RAS™ spheropatients display enhanced response to combined EGFR and MET inhibition. A, heatmap showing preferential association of HGF expression with progressive disease (PD) rather than complete response (CR) or partial response (PR) or stable disease (SD) in patients treated with cetuximab as monotherapy. Fisher exact test $= 0.0281$. (Continued on the following page.)
In these experiments, the groups initially treated with vehicle or JNJ-38877605 alone started to receive cetuximab when tumors reached a volume of 1,400 or 1,300 mm³, 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 with 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 and S6B).

Discussion

Over decades, cell lines contributed to unravel the genetic basis of cancer pathogenesis and to identify therapeutic targets. Nevertheless, their validity for the preclinical screening of anticancer drugs has been challenged (reviewed in refs. 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 colorectal cancer (7, 11, 23, 24). These cells display the operational properties of cancer stem cells, or CICs. 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 colorectal cancer, or "colospheres," were used to demonstrate CIC 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 colorectal cancer metastases xenografted in mice, the so-called "xenopatients" (4), we derived spheres named "xenospheres." Colorectal cancer 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 histologic 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 with the response observed in the corresponding xenopatients, in accordance with the mutational status of KRAS. We, thus, conclude that xenospheres are a reliable, molecularly annotated, in vitro model of CCICs, able to retain from the tumor of origin, and pass to a secondary tumor (spheropatient), the genetic determinants of therapeutic response.

We then confirmed, for the first time in CCIC, the relevance of the KRAS gene status for proliferation and survival. Indeed, xenospheres harboring a KRAS mutation self-sustain their

(Continued.) B and 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 (HGF/K; C) and treated as indicated. Graphs, tumor volume increase versus day 0 ± SEM. (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 gray lines, arrows indicate when cetuximab was added. Graphs, tumor volume increase versus day 0 ± SEM (n = 3; *P < 0.05). F, immunohistochemical analysis with anti-pERK or anti-pS6 antibodies of histologic tumor sections derived from M049-HGF spheropatients treated as indicated in D. Scale bar, 100 μm.)
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<sup>wt</sup> xenospheres are exquisitely sensitive to EGF; indeed, they proliferate at an EGF molar concentration one log lower than that required for normal 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 CCICs: 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 downregulated 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 have recently reported that MET is expressed in glioblastoma stem cells and promotes their self-renewal (39–41). Concerning colorectal cancer, it was previously shown that HGF can sustain the WNT self-renewal pathway (42, 43). Yet, the ability of HGF to sustain long-term CCIC proliferation was unknown. We show here that HGF can promote RAS<sup>wt</sup> CCIC 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 finding with salient implications for colorectal cancer pathogenesis and therapy.

Although MET amplification likely represents a rare mechanism of primary resistance to EGFR inhibition (1% of xenopatients; ref. 3), intrinsic expression of wild-type MET, together with physiologic secretion of HGF by CAFs, may provide a more widespread mechanism. Indeed, we show that, in the microenvironment generated by conditioned media or cocultures of CAFs, HGF is so abundant that it may take the lead over other growth factors, including EGF, to regulate CCIC 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 CCIC. Primary resistance provided by HGF may be rooted in CCICs: if RAS pathway genes are intact, EGFR inhibition, regression was boosted and stabilized for 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 CCIC tumorigenicity, we generated two RAS<sup>wt</sup> spheropatients 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, in vitro and in vivo, indicating empowered targeting of CCICs.

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

### Disclosure of Potential Conflicts of Interest

M. Han has ownership interest (including patents) in AVEO Pharmaceuticals. T. Perera is an employee and shareholder of Johnson and Johnson and has ownership interest (including patents) in the same. P.M. Comoglio is a consultant/advisory board member of Metheresis Translational Research SA. No potential conflicts of interest were disclosed by the other authors.

### Authors’ Contributions

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### Acknowledgments

The authors thank Giorgia Migliardi for help with in vivo experiments, Daniela Gramaglia, Antonella Cignetto, and Francesca Natale for assistance. P.L. and F.S. are recipients of a YIP Fondazione Umberto Veronesi Fellowship.

### Grant Support

This study was supported by AIRC, Italian Association for Cancer Research (Special Program Molecular Clinical Oncology 5+Mille, N. 9970; Investigator Grants N. 10446 to C. Boccaccio, N. 11852 to P.M. Comoglio, and N. 10116 to L.Trusolino); MIUR, Italian Ministry of Education (Future in Research to A. Bertotti); and AACR, American Association for Cancer Research (Fight Colorectal Cancer Career Development Award to A. Bertotti).

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Received August 15, 2013; revised December 9, 2013; accepted December 28, 2013; published OnlineFirst January 21, 2014.

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