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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 (RAS^{wt}) 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 RAS^{wt}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 EGF 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.

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 (10^6 cells/mL) were resuspended in a 1:1 mixture of basal stem-cell medium and Matrigel (BD Biosciences) and were subcutaneously injected into nonobese 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 mm³, mice were randomized and treated with 20 mg/kg cetuximab (Merck) twice a week, or 50 mg/kg of JNJ-38877605 daily (17), or both agents, or vehicle. Tumor size was measured once a week by caliper, and volume was calculated using the formula $4/3(d/2)^2 D/2$, 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 (2×10^5) 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), JNJ-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 RAS[wt] (M016, M049, M195, and M199); and (ii) xenospheres harboring a mutated *KRAS* gene—invariably at residue G12—hereafter indicated as RAS[mut] (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 as the original tumor. This confirms the histogenetic and pseudodifferentiating ability of CICs (6, 7, 23, 24) and indicates that tumors from xenospheres properly recapitulate intra- and intertumor heterogeneity. From spheropatients, secondary xenospheres could be rederived, which retained the same *in vitro* properties and tumorigenicity of the primary xenosphere, namely the ability to sustain serial transplantation (Supplementary Fig. S1A).
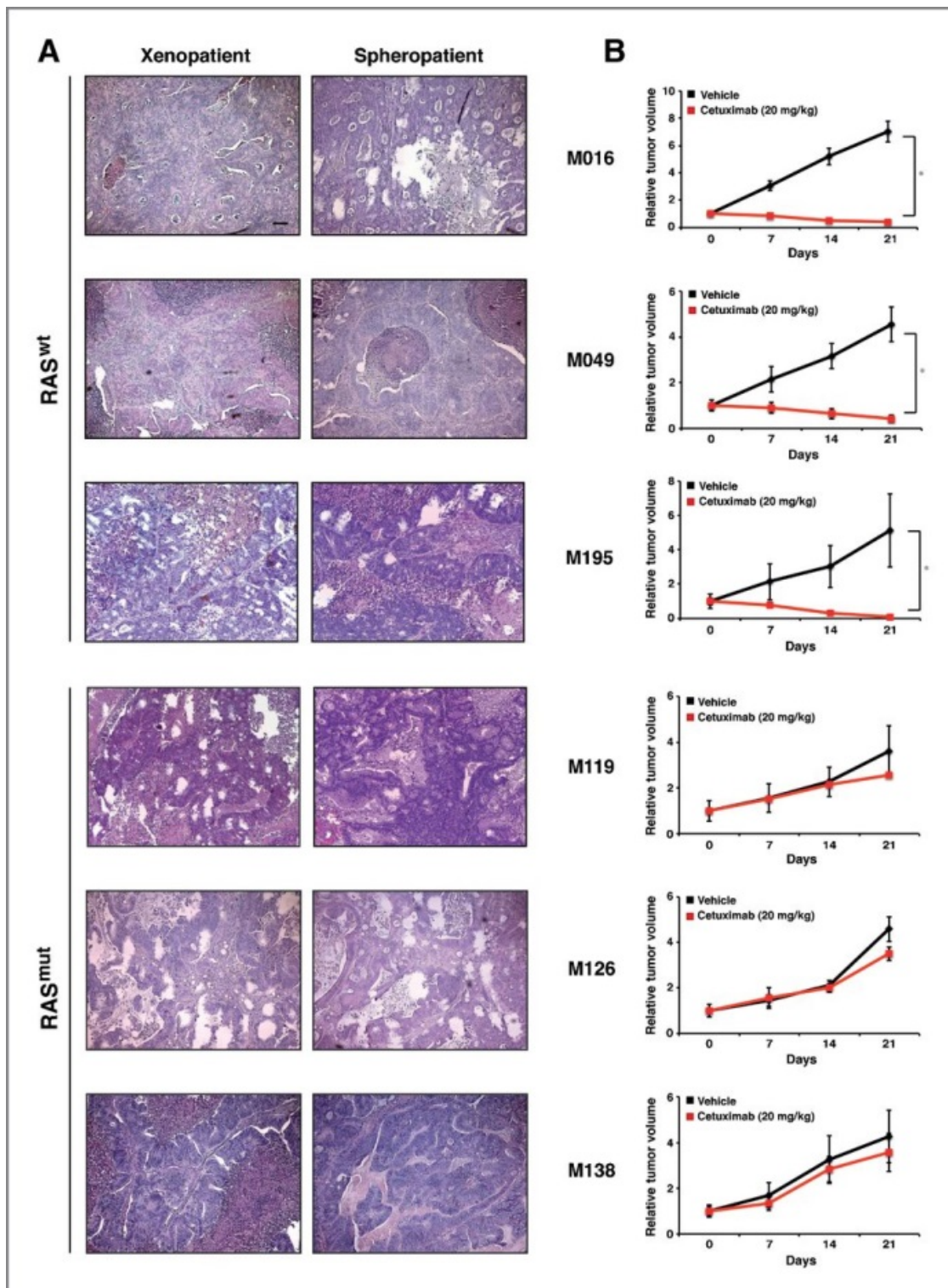
Figure 1.
Xenospheres generate tumors that recapitulate morphology and therapeutic sensitivity of the original xenopatients. A, tumor sections (hematoxylin and eosin staining). Scale bar, 100 μm. B, tumor growth curves. Graphs, tumor volume fold increase/decrease versus day 0 ± SEM ($n = 6$ for M016 and M049; $n = 3$ for M195, M119, M126, and M138; *, $P < 0.05$).

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^wt spheropatients M016, M049, and M195 underwent tumor regression (Fig. 1B and Supplementary Table S2), whereas RAS^mut spheropatients M119, M126, and M138 experienced tumor progression similar to controls (Fig. 1B). Of note, CCICs of spheropatient M016 (as well as the bulk of the corresponding xenopatient) exhibited a *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.

Xenospheres display in vitro properties and markers of CCICs
CCICs 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 *in vitro* and to switch toward 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^mut survived longer than RAS^wt xenospheres (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 CCIC from primary colorectal cancer (6, 7), was very heterogeneous within and among the remaining xenospheres. This is not surprising, as in metastatic colorectal cancer both CD133+ and CD133− cells display tumor-initiating ability (26).
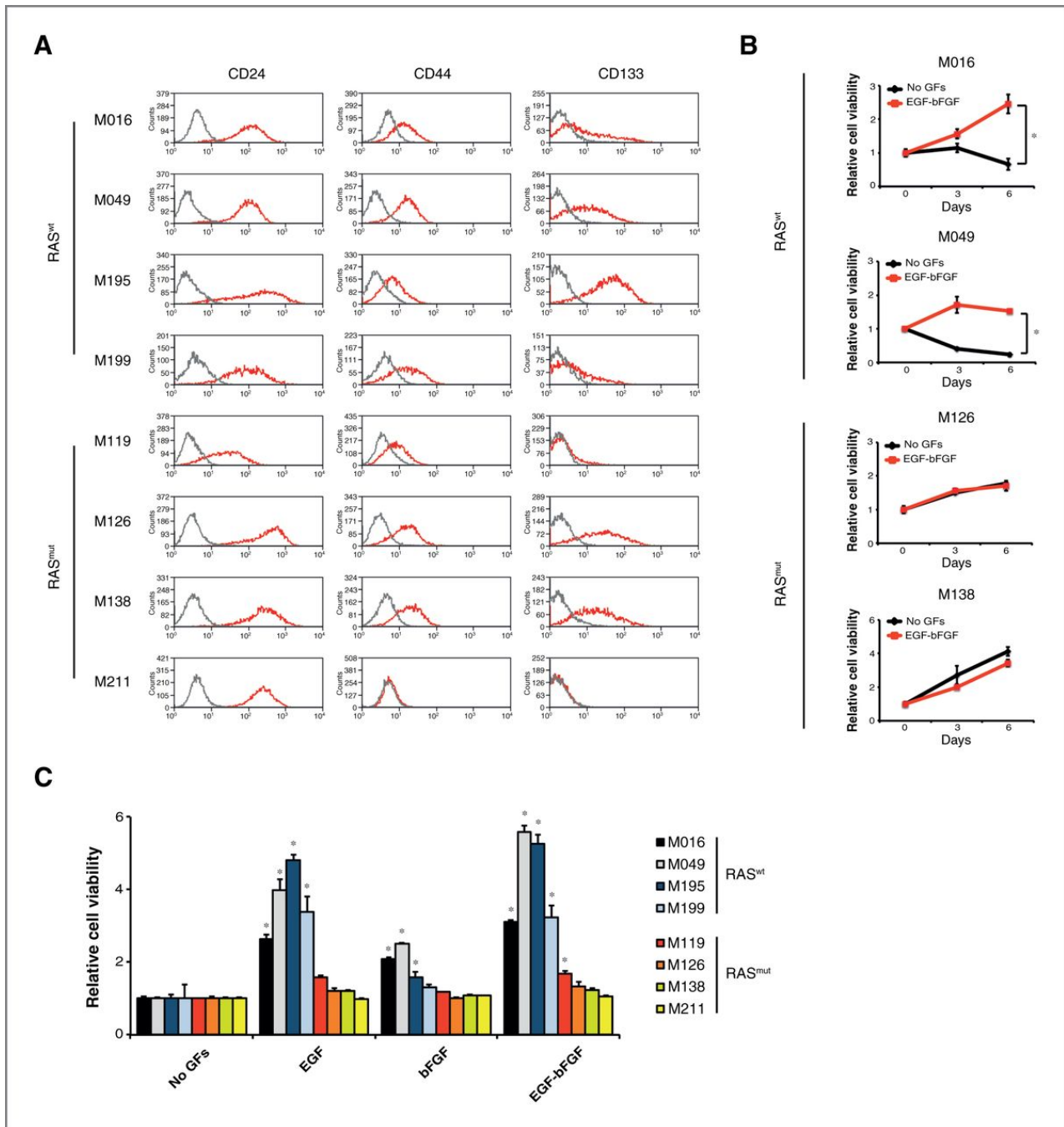
Figure 2.

Xenospheres display *in vitro* markers of CCICs and different growth factor (GF) requirements depending on *RAS* mutational status. A, flow-cytometric analysis of CD24, CD44, and CD133 in xenospheres. B, growth curves of xenospheres. Graphs, relative viability increase versus day 0 ± SEM ($n = 3$; *, $P < 0.05$). C, cell viability of xenospheres kept in basal stem-cell medium either alone (no growth factors) or with the indicated growth factors for 6 days. Columns, relative cell viability versus no growth factors ± SEM ($n = 3$; *, $P < 0.05$).

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 RAS^wt but not RAS^mut xenospheres requires exogenous growth factors

In growth curve assays, representative RAS^wt xenospheres (M016 and M049) proliferated only in the presence of exogenous growth factors, that is, EGF-bFGF, whereas representative RAS^mut 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, RAS^{wt} cells formed spheres only in the presence of exogenous growth factors, whereas RAS^{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^{wt} 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 RAS^{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 CCICs *in vitro*. As expected, in representative RAS^{mut} 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 RAS^{wt} xeno- and spheropatients (see above; Fig. 1B; ref. 4), it did not significantly affect proliferation or apoptosis of the corresponding RAS^{wt} 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 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^{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^{wt} xenospheres (Fig. 3E), in accordance with the *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^{wt} xenospheres from apoptosis induced by cetuximab (Fig. 3F and G).
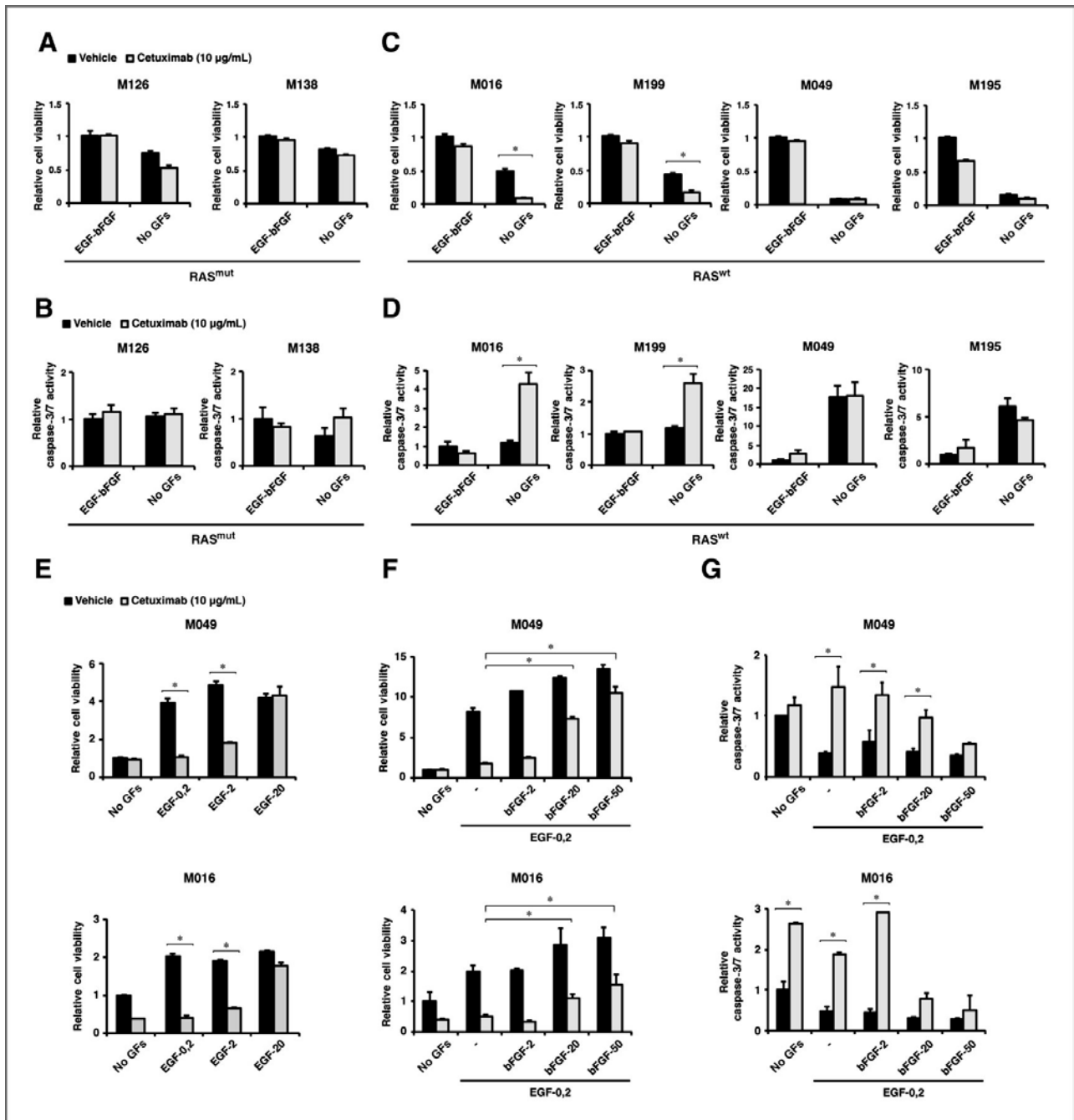
Figure 3.
Xenosphere response to EGFR inhibition hinges on *RAS* mutation and on paracrine/autocrine EGF signaling. A–D, cell viability (A and C) and caspase-3/7 activity (B and D) assays of xenospheres kept for 4 days in standard (EGF-bFGF) or basal [no growth factors (GF)] stem-cell medium. Caspase 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 ($n = 3$; *, $P < 0.05$).

Interestingly, when cultured without growth factors (Fig. 3C and D), RAS^wt 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 RAS[wt] 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 RAS[wt] 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 colorectal cancer sample and two human fibroblast cell lines (MRC5 and hTERT; ref. 18) were used to produce FCM. These were analyzed by multiplex cytokine arrays, revealing high amounts of interleukin (IL)-6, IL-8, Rantes, Gro-α, and HGF, but negligible quantities of EGF and FGFs (Fig. 4A).
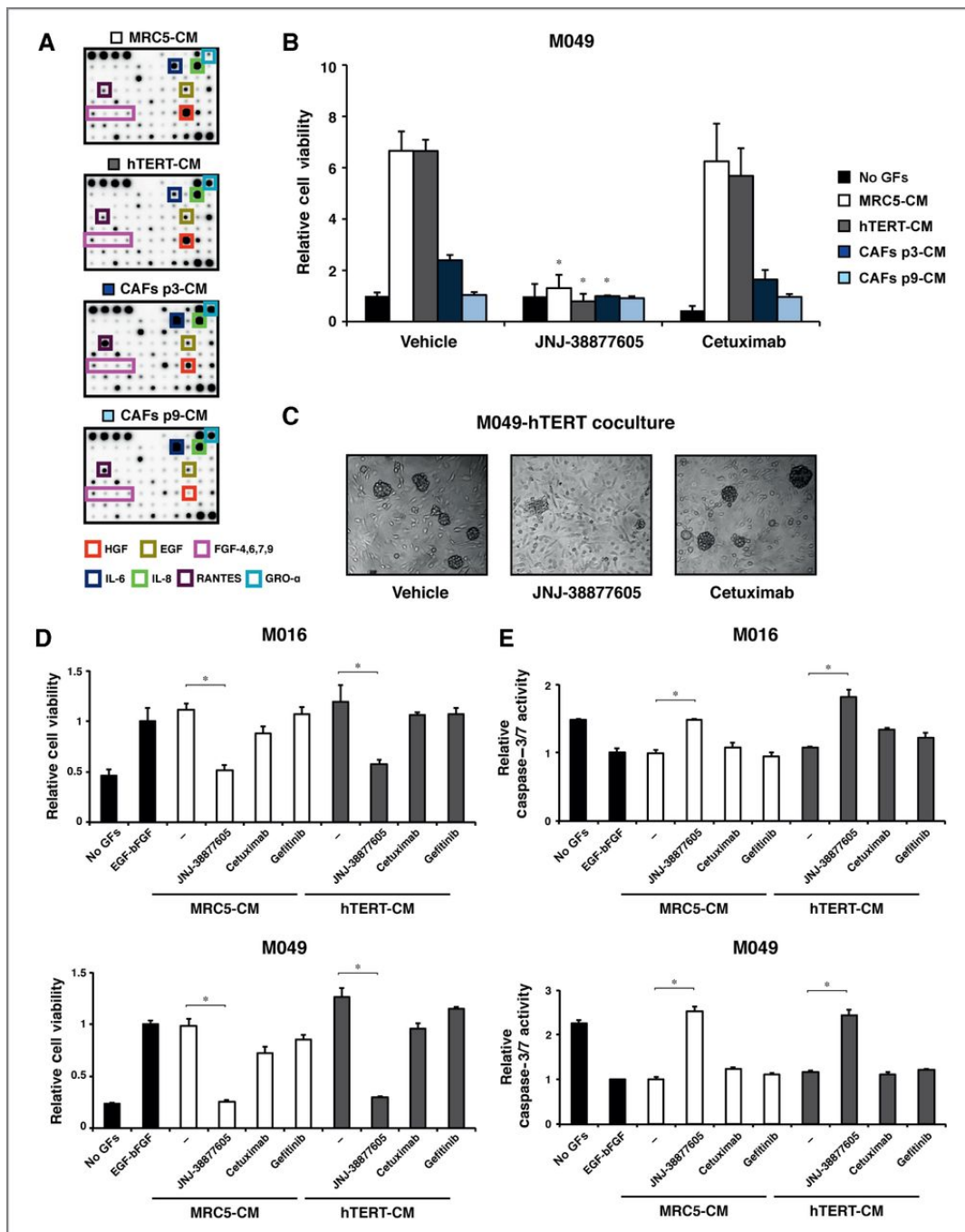
Figure 4.
Xenosphere proliferation and cetuximab resistance are sustained by fibroblast-derived HGF. A, cytokine array of conditioned media (CM), produced from human fibroblast cell lines MRC5 and hTERT or freshly isolated CAFs at passage 3 (p3) or 9 (p9). B, cell viability assay of xenospheres cultured 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. 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 ($n = 3$; *, $P < 0.05$).

Using the representative RAS^wt 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 and 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 and B). Moreover, FCM-induced proliferation was completely blocked by JNJ-38877605, a specific inhibitor of the HGF receptor, MET (Fig. 4B; ref. 17), or by the anti-MET monovalent antibody DN30-FAB (Supplementary Fig. S3A; ref. 31). Importantly, FCM not only sustained the growth of xenospheres, but it also protected them from cetuximab (Fig. 4B). Similarly, coculture with hTERT fibroblasts also sustained xenosphere growth and viability, which were impaired after the 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 RAS^wt 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 and E and Supplementary Fig. S3B and S3C), attesting that HGF mediates the protective activity of FCM. Interestingly, in RAS^wt M199, FCM promoted viability, but inhibition by JNJ-38877605 was attenuated (Supplementary Fig. S3B and S3C) likely by the expression of a strong EGF autocrine loop (Supplementary Fig. S2B). Consistently, this xenosphere maintained sensitivity to EGFR inhibitors (Supplementary Fig. S3B and S3C).

The role of HGF in sustaining CCIC proliferation was further confirmed by supplying purified HGF as a single growth factor (Fig. 5A). RAS^wt 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 and C). Protection was likely mediated by activation of mitogen—activated protein kinase (MAPK) and AKT pathways, which remained high when HGF was supplied together with cetuximab, and was extinguished when the MET inhibitor was also added (Fig. 5D). Similar to our observations with FCM, protection against cetuximab by purified HGF was dose-dependent (Supplementary Fig. S4A).
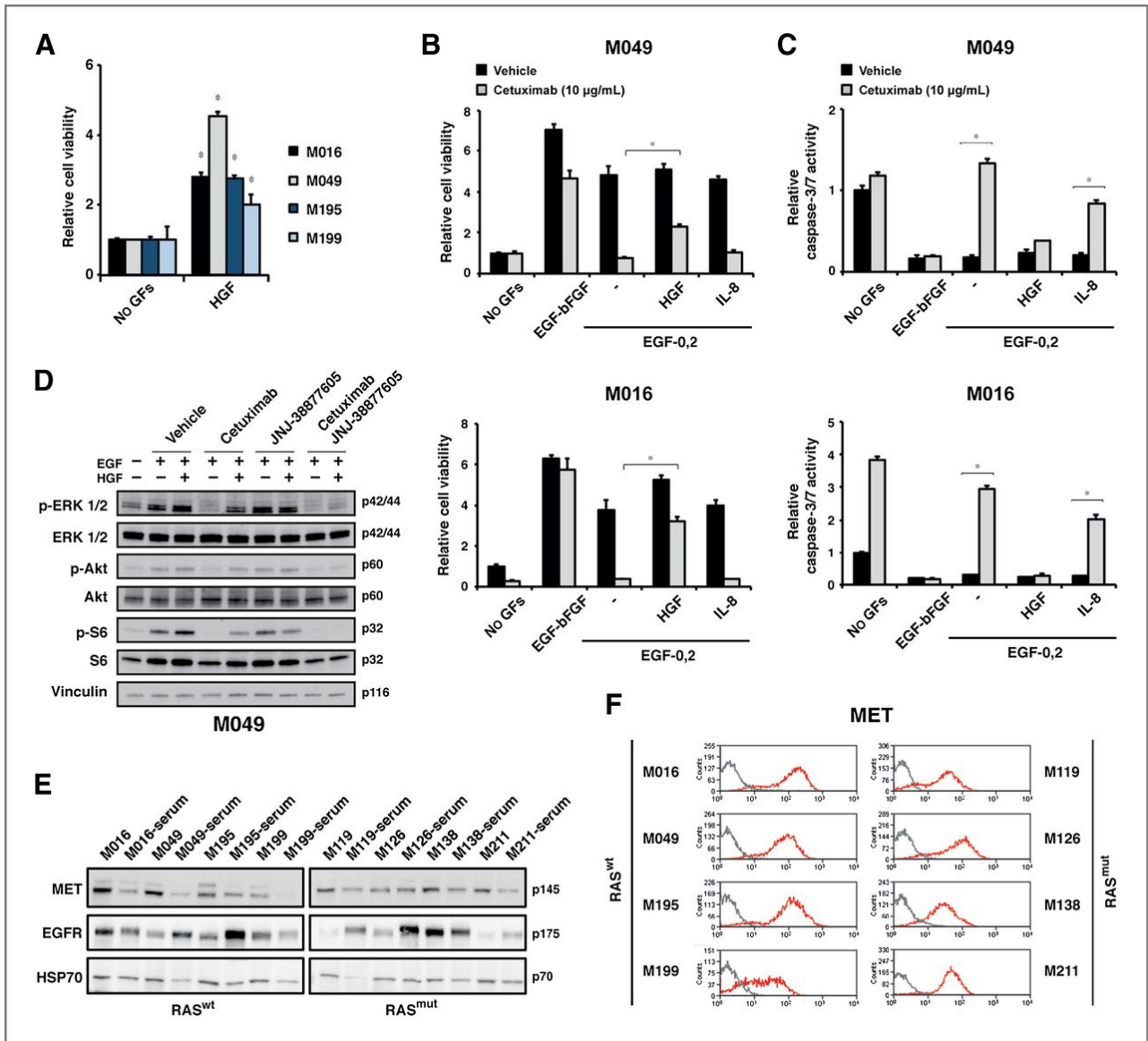
Figure 5.
HGF activity and MET expression in xenospheres. A, cell viability of xenospheres kept in basal stem-cell medium either alone [no growth factors (GF)] or with HGF (20 ng/mL) for 6 days. Columns, the relative viability versus no growth factors ± SEM ($n = 3$; *, $P < 0.05$). B and C, cell viability (B) and caspase-3/7 activity (C) assays of M016 and M049 xenospheres kept for 4 days in basal stem-cell medium alone (no growth factors) 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, the relative viability and caspase activity versus no growth factors and vehicle of a representative experiment ± SEM ($n = 3$; *, $P < 0.05$). D and 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 μmol/L) as indicated, or from the indicated xenospheres or their differentiated derivatives (serum; E). F, flow-cytometric analysis of MET.

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[wt] 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[mut] 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[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, and M138) and increased levels in the remaining five (M049, M195, M119, M126, and M211; Fig. 5E).

RASwt 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 KRASwt metastatic colorectal cancers annotated for response to cetuximab monotherapy (Fig. 6A; ref. 20).
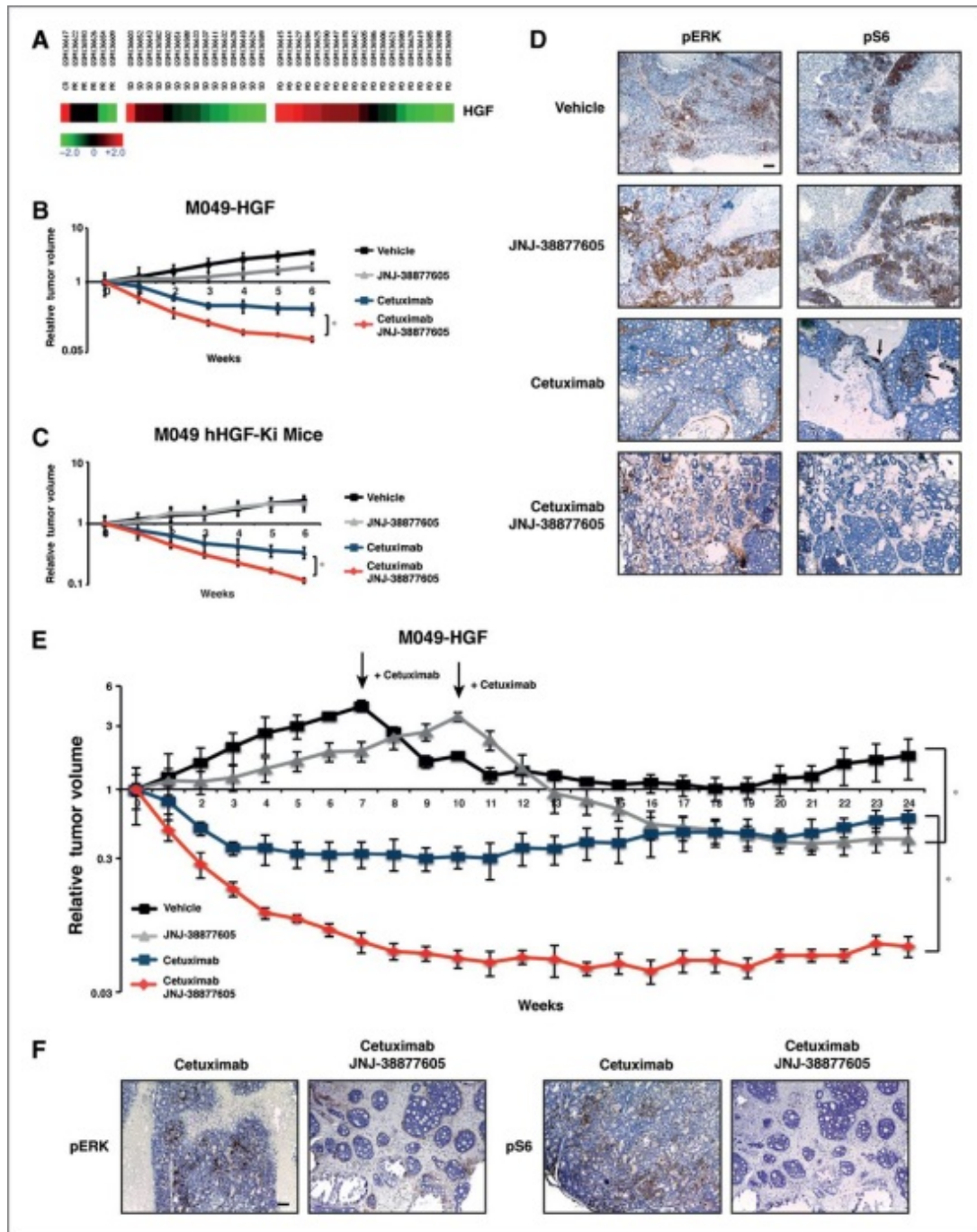
Figure 6.

RAS^{wt} 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. 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 (h*HGF*Ki; 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.

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 a single agent (as previously observed in RAS^{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. 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% tumor 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 (h*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 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. 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 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 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 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^{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 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[wt] 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 relevant in human patients, as suggested by our analysis of data from patients with metastatic colorectal cancer who received cetuximab as monotherapy (20); indeed, high HGF expression in KRAS[wt] tumors associates with poor response to cetuximab.

The ability of MET to support CCIC growth *in vitro* prompted us to reconsider previous results showing that, in xenopatients, MET inhibition failed to block metastatic colorectal cancer featuring wild-type *MET* (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 CCIC tumorigenicity, we generated two RAS[wt] 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, *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.

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