Biology of Human Tumors

Clinical Cancer Research

Targeting the Wnt Pathway and Cancer Stem Cells with Anti-progastrin Humanized Antibodies as a Potential Treatment for K-RAS-Mutated Colorectal Cancer

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Abstract

Purpose: Patients with metastatic colorectal cancer suffer from disease relapse mainly due to cancer stem cells (CSC). Interestingly, they have an increased level of blood progastrin, a tumor-promoting peptide essential for the self-renewal of colon CSCs, which is also a direct β-catenin/TCF4 target gene. In this study, we aimed to develop a novel targeted therapy to neutralize secreted progastrin to inhibit Wnt signaling, CSCs, and reduce relapses.

Experimental Design: Antibodies (monoclonal and humanized) directed against progastrin were produced and selected for target specificity and affinity. After validation of their effectiveness on survival of colorectal cancer cell lines harboring B-RAF or K-RAS mutations, their efficacy was assessed *in vitro* and *in vivo*, alone or concomitantly with chemotherapy, on CSC self-renewal capacity, tumor recurrence, and Wnt signaling.

Results: We show that anti-progastrin antibodies decrease self-renewal of CSCs both *in vitro* and *in vivo*, either alone or in combination with chemotherapy. Furthermore, migration and invasion of colorectal cancer cells are diminished; chemosensitivity is prolonged in SW620 and HT29 cells and posttreatment relapse is significantly delayed in T84 cells, xenografted nude mice. Finally, we show that the Wnt signaling activity *in vitro* is decreased, and, in transgenic mice developing Wnt-driven intestinal neoplasia, the tumor burden is alleviated, with an amplification of cell differentiation in the remaining tumors.

Conclusions: Altogether, these data show that humanized anti-progastrin antibodies might represent a potential new treatment for K-RAS–mutated colorectal patients, for which there is a crucial unmet medical need. *Clin Cancer Res; 23(17); 5267–80. ©2017 AACR.*

Introduction

Survival rates for patients diagnosed with metastatic colorectal cancer are still very low, regardless of continual advances in the screening and therapeutic management of the disease (1), in particular with targeted therapies aiming to accompany and/or replace classical cytotoxic compounds (2). Even though antiangiogenic and anti-EGFR compounds are routinely used to treat colorectal cancer patients, and that anti-EGFR compound cetuximab in non–RAS-mutated colorectal cancers has been proven to delay recurrence, the majority of these targeted therapies have been ineffective in treating colorectal cancer and other cancers. These treatments are ineffective mainly due to a variety of mechanisms such as inducing selection of clones with prior resistance, cancer stem cell (CSC)-driven relapse, or the emergence of recurring tumors induced by cell plasticity (3). Currently, only RAS mutation status is used as a negative predictive marker to avoid treatment with anti-EGFR agents in patients with metastatic colorectal cancer (4). In addition, the efficacy of these therapies can be hindered by target-related and/or nonselective side effects as well, as targeted pathways are frequently paramount for function, development, or renewal of organs (5, 6). It has been extremely difficult to create molecules that are efficient at targeting tumor cells as well as have few/innocuous side effects on healthy organs. The advancement of such molecules depends on crucial parameters, such as their capacity to target a subset or all tumor cells, the dependency of the tumor cells on the targeted pathway,

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Translational Relevance

Up to now, there is no functional targeted therapy for patients bearing a K-RAS–mutated tumor. Nearly 50% of colorectal cancer tumors are K-RAS mutated and when a K-RAS wild-type tumor relapses, the majority of patients develop K-RAS mutations and do not respond to anti-EGFR therapy anymore. It is therefore very important that a new therapy is proposed for these patients. Here, we developed an innovative therapy using specific humanized mAb targeting progastrin, a peptide essential for survival of cancer stem cells that are responsible for tumor recurrence. We showed that neutralizing progastrin with our antibody robustly inhibits both cell proliferation and migration/invasion, significantly decreases CSC self-renewal, and increases chemosensitivity in colorectal cancer cells harboring K-RAS mutations. Given the absence of observable side effects, our data indicate that this humanized monoclonal anti-progastrin antibody is an extremely promising therapy to treat K-RAS–mutated colorectal cancer patients.

and the identification of therapeutic timeframes during which these molecules demonstrate a robust affinity for tumor cells versus healthy ones (7, 8).

Our primary hypothesis postulates that by neutralizing the 80-amino acid peptide progastrin using specific mAbs, we should meet most of these standards. The quantities of progastrin secreted by colorectal tumors are significant (9–11); however, normal gastric epithelium release very little progastrin, mainly because of partial maturation during digestion where progastrin is transformed into amidated gastrin (12). This peptide, whose encoding gene is a direct target of β-catenin/TCF4 (13), is also under the control of the RAS pathway (14, 15). Progastrin has been extensively studied concerning its intense tumor-promoting activity (16–18), whereas progastrin seems unnecessary for major organ development and homeostasis (18, 19). Moreover, we showed that Wnt (19) and Notch (20) pathway activities are upregulated by secreted progastrin from colorectal cancer cells, that progastrin acts as a powerful self-renewal promoter in cancer stem-like cells (21), cell subtypes responsible for driving treatment resistance and relapse after treatment in patients who received standard-of-care cytotoxic combinations (22).

In this article, we establish that the levels of secreted progastrin from colorectal cancer cells increase after tumors and/or tumor cells are exposed to cytotoxic and/or hypoxic conditions, and that mAbs of murine or humanized origin specific for full-length progastrin increase apoptosis, decrease proliferation, decrease migration/invasion of human colorectal cancer cells, and self-renewal of human CSCs, as well as Wnt-driven tumorigenesis in mice. Ultimately, we provide data showing that the antibodies may extend chemosensitivity and postpone recurrence of xenografted tumors in mice treated with multiple rounds of chemotherapy, all the while leaving healthy tissue unaffected, even after extended antibody treatment.

Materials and Methods

Animal experiments

Animal work was carried out in compliance with the ethical regulations approved by the Animal Experimentation Ethics Committee of the Languedoc-Roussillon region (CEEA-LR), France, in the A1 (for $Apc^{\Delta 14/+}$ mice) and A2 (for nude mice) animal facilities of the Cancer Research Institute of Montpellier (Montpellier, France; IRCM, agreement B34-172-27), Neurosciences Institute of Montpellier (Montpellier, France; INM, agreement B34-172-36), and Functional Genomics Institute (IGF, agreement D34-172-13).

Patient samples

Human plasma samples were obtained with full patient consent, and the trials were approved by the ethics board of the "Centre Hospitalier Régional Universitaire de Montpellier." The levels of progastrin were compared with the levels measured in the plasma of healthy volunteer donors from the "Etablissement Français du Sang."

Proliferation assay

T84 or SW620 single-cell suspensions were homogeneously seeded in 6-well plates (NUNC 055426), at 1×10^5 or 1.2×10^5 cells per well, respectively, in complete medium, and incubated for 7 hours at 37°C in 5% CO_2. After cell adherence, the medium was removed and the wells were refilled with serum-free culture medium for overnight incubation. The treatments then began by changing the medium for fresh serum-free medium containing the antibodies of interest at the indicated concentration. For the hypoxia experiments, the plates were transferred in a 37°C 5% CO_2 1% O_2 humidified incubator-hood (Sci-Tive-U, Ruskinn-Awel), at the beginning of the treatment. The treatment was renewed twice daily for 48 hours, before dissociating the cells and assaying the concentration of propidium-positive and -negative cells with a C6 Accuri flow cytometer (Becton Dickinson).

Colonosphere formation assay

Colonospheres were cultivated in ultralow adherence (ULA) polystyrene flasks or plates (Corning), in M11 medium: DMEM/F12 with GlutaMAX (Gibco 31331-028), 20 μg/mL insulin (Sigma I0908), 1% N2 supplement (Gibco 17502-048), 20 ng/mL EGF (R&D Systems 236-EG), 10 ng/mL bFGF (R&D Systems 233-FB/CF), 3 mg/mL D-glucose (Sigma 49139), and 1% penicillin–streptomycin (PanBiotech P06-07100).

Briefly, for the colonosphere formation assay, the dissociated T84 cells remaining after the proliferation assay were stained with 1 μg/mL 7-AAD (Sigma A9400) during 20 minutes at room temperature, before sorting and seeding 300 viable (7-AAD negative) single cells per well in 24-well ULA plates with a FACSAria (Becton Dickinson), in 500 μL M11 medium per well. After 11 days in a 37°C 5% CO_2, each well was photographed via bright-field microscopy (Nikon ECLIPSE TS100, image acquisition with NIS-Element F), the pictures were analyzed with ImageJ (NIH, Bethesda, MD), and the colonospheres with a mean diameter above 30 μm were counted.

ELDA

For the direct extreme limiting dilution analysis (ELDA) setup *in vitro*, untreated cells were dissociated and then stained with 1 μg/mL 7-AAD (Sigma A9400) for 20 minutes at room temperature, before sorting and seeding the viable (7-AAD negative) single cells in 96-well ULA plates with a FACSAria (Becton Dickinson), in 100 μL M11 medium per well containing 5 μg/mL of the antibody to test. Twenty replicates of each cellular density were FACS seeded. Every 72 hours, 2 μL of

250 μg/mL antibodies was added per well to renew the treatment. After 10 days for the HT29 and DLD1 cells, or 11 days for the T84 cells, each well was observed by two operators using brightfield microscopy (Nikon ECLIPSE TS100), and the number of wells containing at least one viable colonosphere with a diameter above 30 μm was counted.

For the indirect ELDA setup *in vitro*, the cells were seeded in T75 flasks as for maintenance passage and incubated for 24 hours. On the next day, the medium was removed and replaced by a combination of chemotherapy [100 μmol/L 5-fluorouracil (5-FU) and 1 μmol/L SN38] and antibody of interest (at 100 μg/mL) diluted in complete medium. 5-FU was provided by Sigma (F6627) and SN38, the active metabolite of irinotecan, by Tocris (2684). After 72 hours in a 5% CO_2 humidified incubator, the treatment medium was removed, the flasks were washed with PBS, and fresh complete medium without treatment was added, before replacing the flasks in the incubator for 72 hours. Finally, the remaining viable cells were sorted and seeded with a FACSAria in 96-well ULA plates without further treatment, as described above. After 11 days in a 37°C 5% CO_2 humidified incubator, the number of colonosphere-containing wells was determined as described in the direct ELDA setup.

For ELDA *in vivo*, xenografted tumors were treated either by the antibodies alone during 1 week, or in combination with chemotherapy during 3 weeks followed by 2 weeks of treatment by the antibodies alone. The treated tumors were then surgically extracted, dissociated into single cells as described above, and the concentration of the viable cells was assayed by flow cytometry with a C6 Accuri (Becton Dickinson) after propidium iodide (PI) or 7-AAD staining.

ELDA was performed on the pools of tumors of each experimental group; a pool consisted of the same number of viable human tumor cells of each tumor of the group. Viable single tumor cells from the pools were sorted with FACSAria (Becton Dickinson), serial diluted in serum-free/antibiotic-free medium: Matrigel (1:1), and subcutaneously transplanted into the right flank of 6- to 7-week-old female nude mice (Hsd:AthymicNude-Foxn1nu, Harlan Laboratories) at escalating doses: 10, 100, 300, or 1,000 cells per animal. For each experimental group, at least 10 animals per density were injected. The proportion of engrafted and nonengrafted animals was used to calculate the number of tumor-initiating cells within the original treated tumor sample. Tumor cells were considered as successfully engrafted when tumors reached a volume of 60 mm^3. Tumors were measured three times weekly using a digital caliper to determine longest and perpendicular tumor diameter, and tumor volumes were calculated as described above.

Treatment of colorectal cancer cell xenografts

Colorectal cancer cells (10^6) in 150 μL of 1:1 cold serum-free/antibiotic-free medium:Matrigel (BD Biosciences 354234) were subcutaneously injected into the right rear flank of 6- to 7-week-old female Swiss nude mice (Crl:NU(lco)-Foxn1nu, Charles River Laboratories). When tumors reached a mean volume of approximately 100 mm^3, mice were randomized with the EasyStat (AICOS) software into groups with equal number of animals according to age, body weight, and tumor size.

The treatments were started on the day of the randomization.

Antibodies diluted in 0.9% NaCl were administered twice weekly by intraperitoneal injection of 30 mg/kg. Treatments with the antibodies lasted until the end of the study.

When cotreating with chemotherapy, 22 mg/kg of irinotecan (Pfizer) diluted in 0.9% NaCl were injected intraperitoneally 1 hour prior to the antibodies. The mice were still treated with the antibodies during the washout steps.

Animals were observed and weighed twice weekly, at the same time as tumor volumes were measured using a digital caliper. Tumor size was calculated using the following formula: $V = \text{length} \times \text{width}^2/2$, where length represents the largest tumor diameter and width represents the perpendicular tumor diameter. Body weight change was calculated as $\Delta BW = [(BWi - BW0)/BW0] \times 100\%$; BWi was the body weight on the day of treatment and BW0 was the body weight on the first day of the treatment.

Animals were sacrificed either at the end of the study, or when tumors reached a volume of 1,500 mm^3, if tumor ulceration was observed, if body weight loss exceeded 20%, or if significant deteriorations were observed in mouse health. Euthanasia by cervical dislocation was done after gaseous anesthesia (isoflurane).

$Apc^{\Delta 14/+}$ mice treatment and tumor scoring

Five-week-old $Apc^{\Delta 14/+}$ mice were randomized into two groups of 6 animals. Randomization was performed according to age, gender, body weight, and progenitors. Mice were weighed and observed for clinical signs at least twice weekly.

After a 6-week treatment with 9 mg/kg of pAb antibody, the mice were euthanized by cervical dislocation after gaseous anesthesia (isoflurane) and the whole intestinal tracts were collected, washed in PBS, turned inside-out, and fixed in 10% neutral-buffered formalin at 4°C overnight. The intestine was imaged using brightfield microscopy (Nikon AZ100, image acquisition with NIS-Element F). All adenomas were counted using ImageJ software.

Results

Plasma and cellular progastrin levels increase upon exposure to chemotherapy- or hypoxia-inducing conditions in colorectal cancer

CSCs are thought to resist chemotherapy and drive posttreatment relapse (22). As autocrine secretion of progastrin is important for colon CSC survival and self-renewal (21), we analyzed whether progastrin was detectable in patients receiving FOLFIRI and Avastin, a common therapeutic combination in colorectal cancer (23). We developed an ELISA test and compared plasma levels of human progastrin before and 48 hours after treatment onset in 15 patients. Plasma progastrin was elevated upon treatment in most patients, with 5 patients exhibiting a $\geq$2-fold increase, suggesting that cytotoxic and antiangiogenic therapy induce progastrin secretion (Fig. 1A). We also quantified progastrin levels in plasma samples from 136 colorectal cancer patients, in comparison with 103 control samples obtained from blood transfusion centers, where median plasmatic progastrin levels fell below detection levels. Progastrin was detected in plasma samples from early- to late-stage colorectal cancer, with median levels increasing from 6 pmol/L for stage I/II to 18 pmol/L for stage III/ IV patients (Supplementary Fig. S1A). These results are in accordance with previous reports (10, 24) and confirm that progastrin is a relevant therapeutic target in colorectal cancer patients that are eligible for medical treatment.

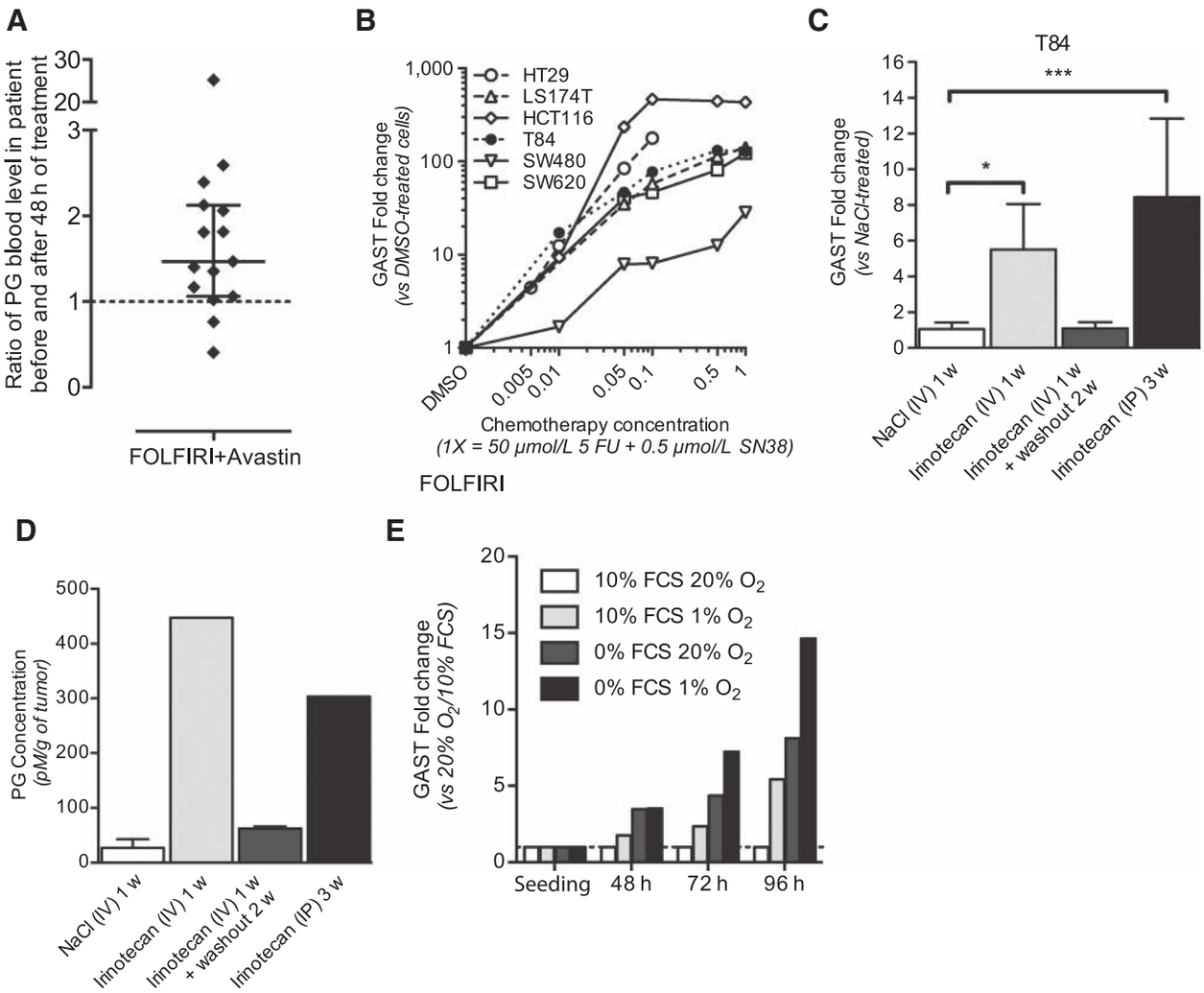

Figure 1.

Plasma and cellular progastrin levels increase upon exposure to chemotherapy- or hypoxia-inducing conditions in colorectal cancer. **A,** Progastrin levels in plasma from colorectal cancer patients 48 hours after FOLFIRI + Avastin treatment onset, expressed as a ratio of pretreatment levels for each patient were quantified using sandwich ELISA. Scatter plot showing the median and interquartile range. **B,** *GAST* transcript levels in colorectal cancer cells treated for 72 hours with increasing 5-FU + SN38 concentrations, expressed as the mean fold increase in comparison with vehicle-treated cells ($n \geq 3$ for each cell line). **C** and **D,** *GAST* transcript levels (**C**) and concentration of progastrin peptide (**D**) in SW620 xenografts treated with irinotecan or vehicle intravenously (IV) or intraperitoneally (IP) for 1 or 3 weeks (w), expressed as mean + SD from seven biological replicates (**C**) or expressed as mean + SD from one pool of seven biological replicates (**D**). **E,** *GAST* transcript expression in T84 cells grown ± 10% serum and either 1% or 20% oxygen at various time point, expressed as mean ($n = 3$). One-way ANOVA; *, $0.01 < P \leq 0.05$; ***, $P \leq 0.001$. *GAST* transcript levels are normalized to GAPDH.

Furthermore, we observed a dose-dependent induction of the progastrin-encoding gene in six colorectal cancer cell lines carrying different mutational profiles (Supplementary Tables S1 and S2) that remained alive after a 72-hour treatment of 5-FU and SN38 (Fig. 1B). Accordingly, expressions of *GAST* mRNA (Fig. 1C) and peptide (Fig. 1D) were significantly increased in xenografted tumors of nude mice treated with intraperitoneal or intravenous irinotecan.

We also quantified progastrin expression following nutrient and oxygen deprivation *in vitro*, conditions that commonly affect tumors over their lifetime, including after exposure to cytotoxic or antiangiogenic compounds (25). *GAST* mRNA expression increased over time in T84 cells following serum starvation (0% FCS) or placement under hypoxic conditions (1% O_2), compared with controls (10% FCS, 20% O_2; Fig. 1E). *GAST* gene expression was further increased in cells grown under 0% FCS/1% O_2 combined conditions, reaching a 15-fold induction (Fig. 1E). This result was corroborated using three other colorectal cancer cell lines (Supplementary Fig. S1B).

Engineering and characterization of a humanized therapeutic antibody targeting progastrin

Because progastrin is a circulating tumor-specific prooncogenic growth factor (10, 18, 19, 21, 26), we hypothesized that progastrin represents an ideal target for neutralizing antibody to treat colorectal cancer.

We generated 23 murine mAbs selectively binding progastrin but not other *GAST* gene products using direct ELISA (Supplementary Fig. S2A and S2B). Using direct ELISA and BIAcore, we showed that these mAbs exhibited high affinities for human progastrin (hPG), ranging K_D from 10^{-7} to 10^{-12} mol/L (Supplementary Fig. S2C; Supplementary Table S3). Targeted epitopes were characterized using AlaScan and SPOT techniques (Supplementary Table S3).

We next selected the best neutralizing mAb to humanize based on their capacity to inhibit the effect of progastrin on cell growth, which contributes to its tumor-promoting role (18, 19). We treated SW620 cells with each mAb for 48 hours and quantified the growth inhibition relative to control antibody-treated cells (Supplementary Fig. S3). Half of tested anti-progastrin mAbs reduced SW620 cell growth by $\geq$25%, with 5 of them reducing growth by $\geq$50% compared with control.

These biochemical and biological characteristics were used to select five mAbs to be humanized (Supplementary Table S4).

Using BIAcore, we found that three of the 10 Hz mAbs maintained their affinity for progastrin compared with their murine "ancestor," with K_D values in the low nanomolar range (Supplementary Table S4). The strong affinity and the specificity of these humanized antibodies for full-length progastrin only was confirmed using ELISA (Fig. 2A and B).

The bioactivity of anti-progastrin humanized antibodies was again tested on colorectal cancer cell growth *in vitro*. Two of the three anti-progastrin humanized antibodies significantly reduced SW620 cell growth (Fig. 2C). On the basis of biochemical characteristics and biological activity profiles, we selected Hz8CV2 as our lead therapeutic antibody. Details about humanization are provided in Fig. 2D and E.

To ensure that Hz8CV2 not only recognized recombinant but also native progastrin, including O-sulfated and phosphorylated forms (27), we stably overexpressed progastrin in HCT116 cells and showed that these cells secrete post-translationally modified progastrin (Supplementary Fig. S4). Importantly, Hz8CV2 was able to detect native progastrin purified from HCT116-progastrin supernatants by gel filtration with a 2-fold increased affinity compared with recombinant progastrin, as demonstrated using direct ELISA (Fig. 2F).

Anti-progastrin therapeutic antibody decreases cell proliferation and increases cell death.

Hz8CV2 induced a dose-dependent decrease of cell growth in two independent colorectal cancer cell lines, down by 90% at the optimal Hz8CV2 dose tested, namely 5 μg/mL for SW620 and 20 μg/mL for T84 (Fig. 3A and B). Decreased cell growth was correlated with a significant increase of PI-positive cells in SW620 (from 9% to 14%) and T84 (from 6.6% to 10.5%; Fig. 3C), suggestive of increased cell death. Furthermore, the proportion of T84 cells positive for both PI and Annexin V (markers of apoptosis) was 20.1% after treatment with Hz8CV2, compared with 7.4% in cells treated with control antibodies (Supplementary Fig. S5).

Finally, Hz8CV2 decreased cell growth by 80% and increased the proportion of PI-positive T84 cells (10%–14%) when used under conditions shown above to increase *GAST* gene expression (0% FCS/1% O_2; Fig. 3D).

Anti-progastrin therapeutic antibody decreases stem cell frequency in colorectal cancer cell lines

Because of the similarities between CSCs and normal stem cells in their primary characteristics (self-renewal and multipotent differentiation), methods developed originally for analysis and characterization of adult stem cells have been transferred to CSCs. The first one is to study known CSC cell surface marker. The second is the use of functional assays in which we exploit the ability of CSCs to initiate colonospheres formation *in vitro* in nonadherent plates and tumor formation *in vivo*, the last being by far the best gold standard for CSC analysis.

Progastrin has been shown to promote colorectal cancer cell proliferation and colon CSC survival and self-renewal (18, 21). Accordingly, siRNA-mediated progastrin depletion in T84 cells decreased growth (Supplementary Fig. S6A and S6B) and reduced the proportion of cells expressing CD44 (34.5%–19%), ALDH (40.8%–18.3%) or both (38.9%–12.2%; Supplementary Fig. S6C–S6E). We therefore tested the hypothesis that humanized Hz8CV2 antibody should reduce colorectal cancer cell self-renewal *in vitro* and *in vivo*.

First, adherent T84 cells that survived Hz8CV2 treatment *in vitro* were isolated (7-AAD negative) using flow cytometry and seeded in ULA plates to grow colonospheres without further treatment. Colonospheres grown from Hz8CV2-treated cells were fewer by more than 50% compared with those from control antibody-treated cells (Fig. 4A).

Next, we performed an ELDA assay on colorectal cancer cells treated for 9 to 11 days with control antibody or with Hz8CV2. Consistent inhibition of stem cell frequency was induced by Hz8CV2 treatment, ranging from 41.8% to 61.7% inhibition across all cell lines (Fig. 4B).

We then analyzed the effect of 3-day treatment with a combination of 5-FU + SN38 and Hz8CV2 on CSC frequency. Under these conditions, Hz8CV2 induced a significant 57.9% decrease of CSC frequency in K-RAS–mutated T84 cells (Fig. 4C).

We adapted the ELDA setting *in vivo* to the BALBc/nude mouse subcutaneous xenograft model and treated animals with Hz8CV2 or control antibodies, alone or in combination with irinotecan. CSC frequency was statistically significantly reduced by 74.5% in T84 tumors and nonsignificantly by 37.7% in SW620 tumors treated with Hz8CV2 alone (Fig. 4D). Importantly, combining Hz8CV2 with irinotecan significantly decreased CSC frequency both in T84 and SW620 tumors (55.9% and 70.1% decrease, respectively; Fig. 4E). These results indicate that humanized anti-progastrin antibodies significantly decrease *in vivo* self-renewal, a key CSC characteristic, particularly when combined with chemotherapy.

Because CSCs also play a role in metastatic development (22, 28), we analyzed the effect of progastrin-selective antibodies on metastasis initiation. Following intrasplenic xenografting of SW620 cells, BALBc/nude mice were treated with the murine monoclonal mAb 3N or with control antibodies for 6 weeks, and the metastatic burden was thereafter quantified in the liver. The number of liver metastases was reduced after treatment with mAb 3N compared with controls, with mean tumor numbers per liver decreasing from 7.6 to 4.3 (Supplementary Fig. S7A). Then, tumor cells from all liver metastases were dissociated and grown under ULA conditions without further treatment. The number of colonospheres was

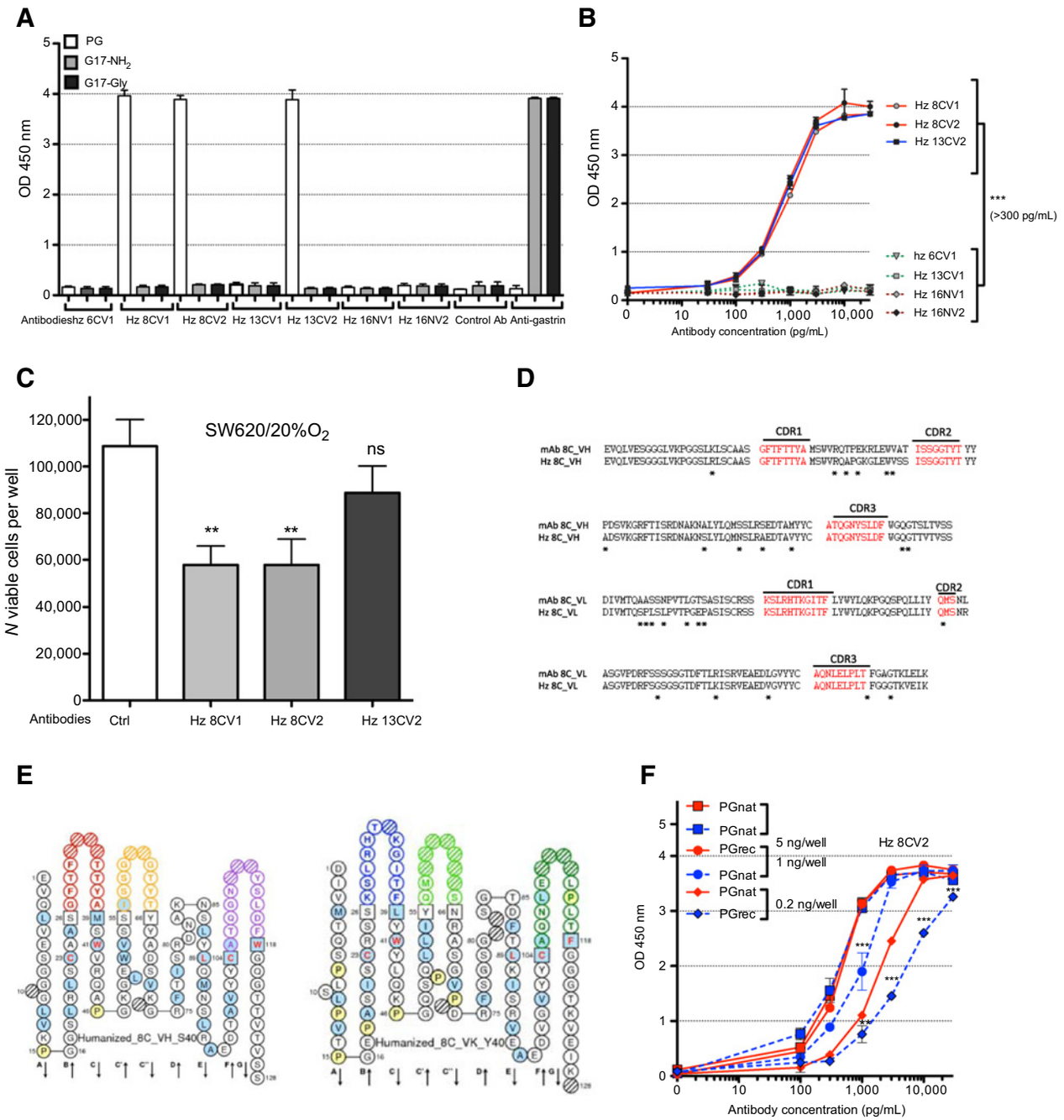

Figure 2.

Engineering and characterization of a humanized therapeutic antibody targeting progastrin. **A** and **B,** Ability to bind full-length recombinant human progastrin or processed gastrin-17 forms (**A**) and relative affinity for full-length recombinant human progastrin (**B**) was quantified using direct ELISA for seven candidate humanized antibodies, and expressed as mean + SD ($n \geq 2$ for each anti-progastrin antibody). **C,** SW620 cell counts after treatment with control or Hz anti-progastrin in serum-free medium with 20% O_2, expressed as mean ± SD ($n = 3$). **D,** CDR sequence alignments of murine mAb8C and humanized Hz8C. Stars, amino acids modifications. **E,** Collier de Perles representation of Hz8C V_H and V_K regions according to IMGT. **F,** Relative affinity of Hz8CV2 for full-length human progastrin produced in *E. coli* (PGrec) or in HCT116-progastrin cells (PGnat) was quantified using direct ELISA, expressed as mean ± SD ($n = 3$). Two-way ANOVA; ***, $P \leq 0.001$ for (**B** and **F**). One-way ANOVA; **, $0.001 < P \leq 0.01$ for **C**.

significantly reduced by over 3-fold in samples isolated from mAb3N–treated mice, suggesting that this treatment reduced the pool of CSCs *in vivo* (Supplementary Fig. S7B). Finally, colonospheres grown from these liver metastases were pooled, dissociated, and subcutaneously injected to a small number of second-generation animals, again without further treatment.

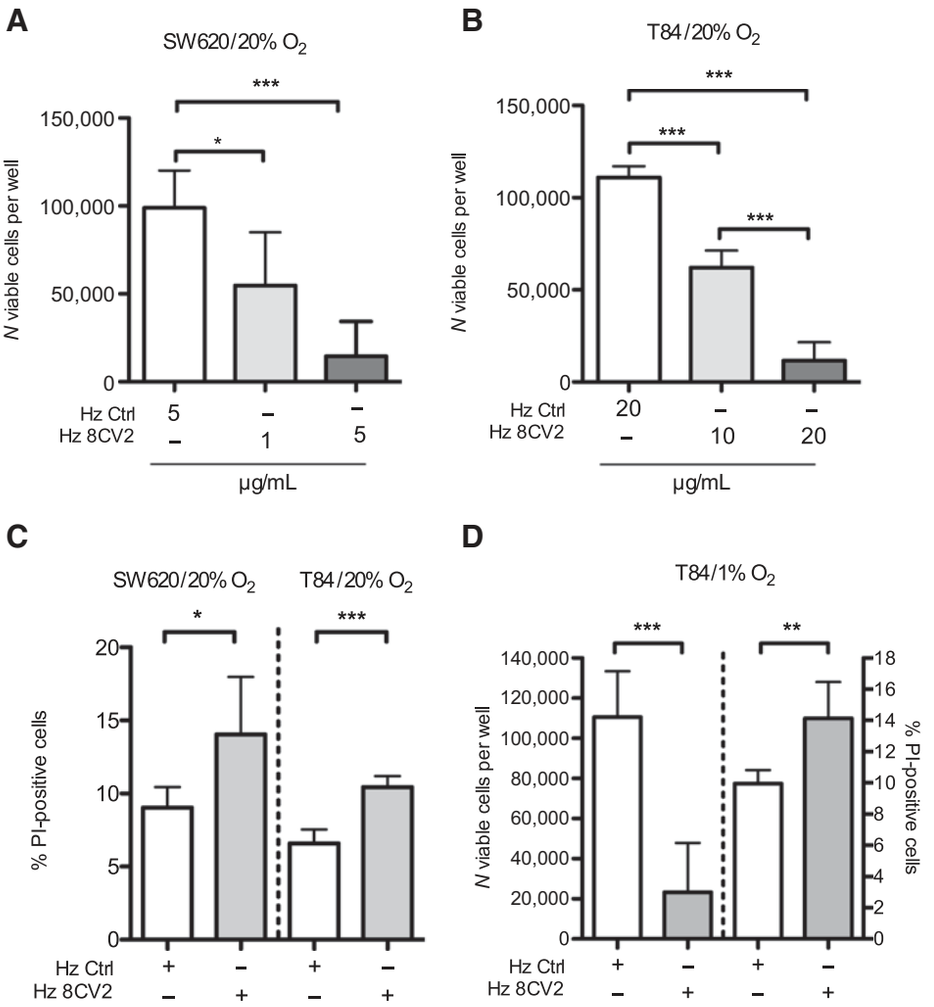

Figure 3.
Anti-progastrin therapeutic antibody decreases cell proliferation and increases cell death. **A–C,** Cell counts for SW620 (**A**) and T84 (**B**), and percentage of PI-positive SW620 and T84 cells (**C**) after treatment with control or Hz8CV2 at the indicated concentrations under 20% O_2 conditions, expressed as mean ± SD ($n > 10$). **D,** Cell counts and percentage of PI-positive T84 cells after treatment with Hz8CV2 under 1% O_2 conditions, expressed as mean ± SD ($n = 3$). All treatments were in serum-free medium. One-way ANOVA; *, $0.01 < P \leq 0.05$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$.

Tumorigenicity of cells stemming from mAb3N–treated first-generation animals was much lower than those from animals treated with control antibodies, suggesting that progastrin-neutralizing antibodies caused long-term impairment of tumor-initiation ability in metastatic SW620 cells (Supplementary Fig. S7C).

Anti-progastrin therapeutic antibody inhibits migratory/invasive properties of colorectal cancer cells

In view of the reported link between CSCs and epithelial-to-mesenchymal transition (EMT; ref. 28), we investigated the effect of Hz8CV2 on the migratory/invasive properties of colorectal cancer cells. We treated cells with Hz8CV2 or HzCtrl inside Boyden chambers for 48 hours, and a chemoattractant (10% FCS) was then added in the lower chamber for 8 hours. Hz8CV2 treatment decreased HCT116 cell migration and invasion by 42% and 40%, respectively (Fig. 5A and B). To determine whether this effect was reproduced *in vivo*, we treated BALBc/nude mice bearing subcutaneous T84 cell xenografts with Hz8CV2, dissociated cells from residual tumors after treatment, and performed *ex vivo* Boyden chamber assays as described above. The migration and invasion abilities of tumor cells isolated from Hz8CV2-treated xenografts were reduced by 30% and 41%, respectively, compared with those isolated from control mice (Fig. 5C and D). These results were corroborated using the B-RAF-mutated HT29 cell line, for which posttreatment *ex vivo* migration was decreased by 30% (Fig. 5E).

Anti-progastrin therapeutic antibody reduces the chemoresistance of colorectal cancer cell lines

To this day, 5-FU–based chemotherapy remains the cornerstone of treatment for patients with advanced colorectal cancer (23). Because conventional treatments enrich the CSC potential of colorectal and other tumors (22), and as anti-progastrin antibodies decreased CSC self-renewal, we quantified the impact of progastrin antibody treatment in combination with sequential chemotherapy cycles *in vivo*.

We selected three cell lines with different degrees of chemosensitivity toward irinotecan (Supplementary Fig. S8). For each cell line, 20 mice were xenografted and treated with irinotecan, from which two groups of 8 to 10 mice were randomized to also receive mAb8C or mAbCtrl. Individual treatment cycles with irinotecan (22 mg/kg, every 3 days) lasted for 18 days, while antibody treatment (30 mg/kg, every 3 days) was continued throughout the experiments. Chemosensitivity was defined as the stabilization of tumor growth or the decrease

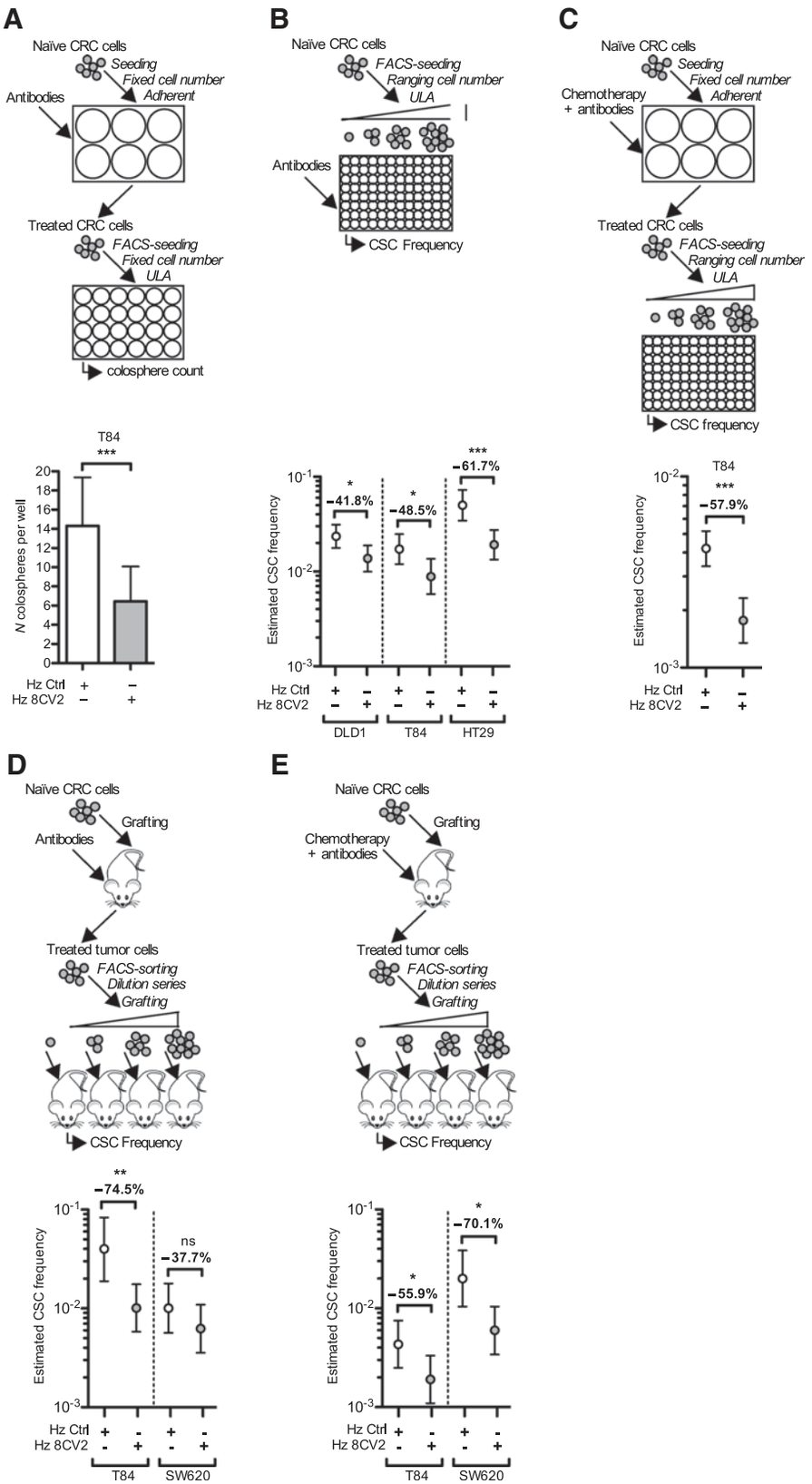

Figure 4.

Anti-progastrin therapeutic antibody decreases stem cell frequency in colorectal cancer cell lines. **A,** Number of T84 colonospheres formed following treatment with control or anti-progastrin humanized antibody under ULA conditions, expressed as mean ± SD ($n = 3$). **B–E,** Stem cell frequencies quantified using the ELDA web tool for DLD1, T84, HT29, or SW620 cells or xenografts as indicated. Data are expressed as estimated CSC frequency (median with range from 20 replicates of 6 cellular concentrations *in vitro* and 10 replicates of 4 cellular concentrations *in vivo)*. **B,** ELDA assay was run in the presence of control or anti-progastrin humanized antibody (DLD1 $n = 4$, T84 $n = 8$, HT29 $n = 6$). **C,** Cells were cotreated with 5-FU + SN38 and control or anti-progastrin humanized antibody under adherent conditions prior to ELDA seeding (T84 $n = 5$). **D** and **E,** ELDA quantification was performed in untreated second-generation mice after treatment of first-generation animals with control or anti-progastrin mAb (**D,** T84 $n = 2$, SW620 $n = 2$) or with irinotecan + control or anti-progastrin mAb (**E,** T84 $n = 1$, SW620 $n = 1$). Two-tailed t test and χ^2 test for ELDAs; ns, $P > 0.05$; *, $0.01 < P \leq 0.05$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$.

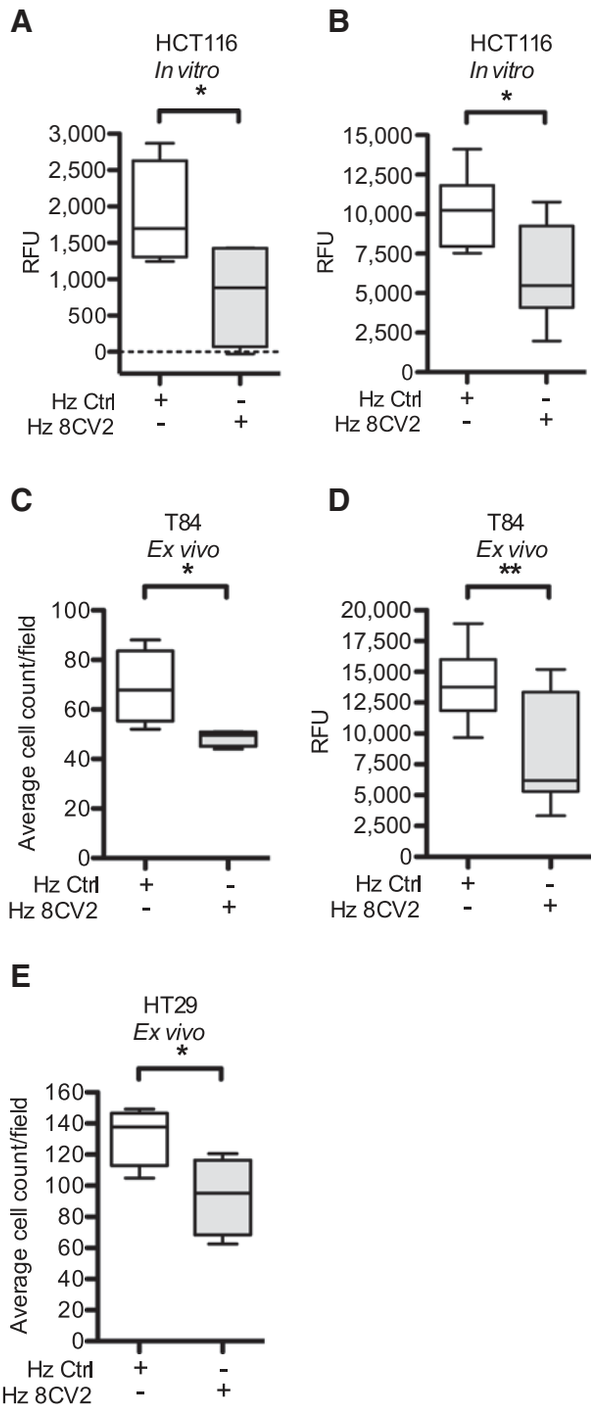

Figure 5.

Anti-progastrin therapeutic antibody inhibits migratory/invasive properties of colorectal cancer cells. **A** and **B,** HCT116 migration (**A**) and invasion (**B**) abilities after *in vitro* treatment with control or Hz8CV2 directly in the Boyden chambers (in **A**, 16 fields measured per replicate, $n = 4$; in **B**, 25 fields measured per replicate, $n = 2$). **C** and **D,** *Ex vivo* migration (**C**) and invasion abilities (**D**) were quantified following *in vivo* treatment of T84 xenografts with control or Hz8CV2. Cells from 4 treated tumors were collected, and pooled, then dispatched in four replicate chambers for migration (**C**, 4 fields counted for each) or 8 replicate chambers for invasion (**D**, 25 fields counted for each; $n = 2$). **E,** Migration was quantified after *in vivo* treatment of HT-29 xenografts with control or Hz8CV2, as described under **C**. Results are expressed as median (line), interquartile range (box), and range (whiskers). Two-tailed *t* test; *, $0.01 < P \leq 0.05$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$.

of tumor volume for at least three consecutive measurements under cotreatment.

Subcutaneous SW620 xenografts were maintained for up to four cotreatment cycles (Fig. 6A and B). Although only 38% of tumors from the control group remained chemosensitive by treatment cycle #4, all tumors remained chemosensitive across four cycles when cotreated with mAb8C and irinotecan (Fig. 6C).

For HT29-derived tumors treated with irinotecan and control antibody, 67% reached the ethical endpoint around day 55 to 62, before the third cotreatment cycle (Fig. 6D). In contrast, delayed growth was observed in 67% tumors treated with irinotecan and mAb8C, due to a robust increase of chemosensitivity during treatment cycle #2. Growth of these tumors resumed after irinotecan-induced selective pressure was removed in the wash-up period, although most remaining tumors showed marginal treatment response during treatment cycle #3 (Fig. 6D–F). Median survival was not statistically different between the two groups.

Finally, whereas all T84-derived tumors had reached the ethical endpoint after the second cotreatment cycle in the control group, most mice cotreated with mAb8C survived until after the third cycle (Supplementary Fig. S9A and S9B). Overall, tumor growth was delayed in the presence of mAb8C, and the median survival time increased significantly by 54% (74 vs. 48 days, $P = 0.0095$; Supplementary Fig. S9C).

As expected in a xenografted model with an antibody that mostly targets self-renewal, no effect on tumor volume was observed in response to treatment with Hz8CV2 alone (Supplementary Fig. S10). Importantly, we did not observe any adverse effects on body weight or general behavior in response to progastrin neutralization regardless of the chosen protocol (Supplementary Fig. S11).

Anti-progastrin therapeutic antibody inhibits Wnt signaling and APC mutation–driven tumorigenesis

We have previously shown that siRNA-mediated progastrin targeting decreases Wnt/β-catenin activity (19). Interestingly, treatment of SW480 cells with Hz8CV2 for 96 hours decreased the expression of survivin, a known direct Wnt pathway targets gene (29) by 40.7% (Fig. 7A; Supplementary Fig. S12A). We also found that treatment with Hz8CV2 significantly decreased transcriptional activity by 35.7% in T84-derived colonospheres (Fig. 7B).

Wnt inhibitors have attracted recent interest as potential therapeutic tools against colorectal cancer (30). Progastrin neutralization by Hz8CV2 had a similar effect on T84 cells' CSC frequency compared with the Wnt signaling inhibitor ICG-001, in accordance with the similar extent of Wnt/β-catenin activity inhibition induced by ICG-001 and progastrin-selective siRNA in LS174T cells (Supplementary Fig. S12B–S12D).

We quantified progastrin levels in blood samples from 40 adenomatous polyposis patients and observed an increase in circulating progastrin with a median concentration of

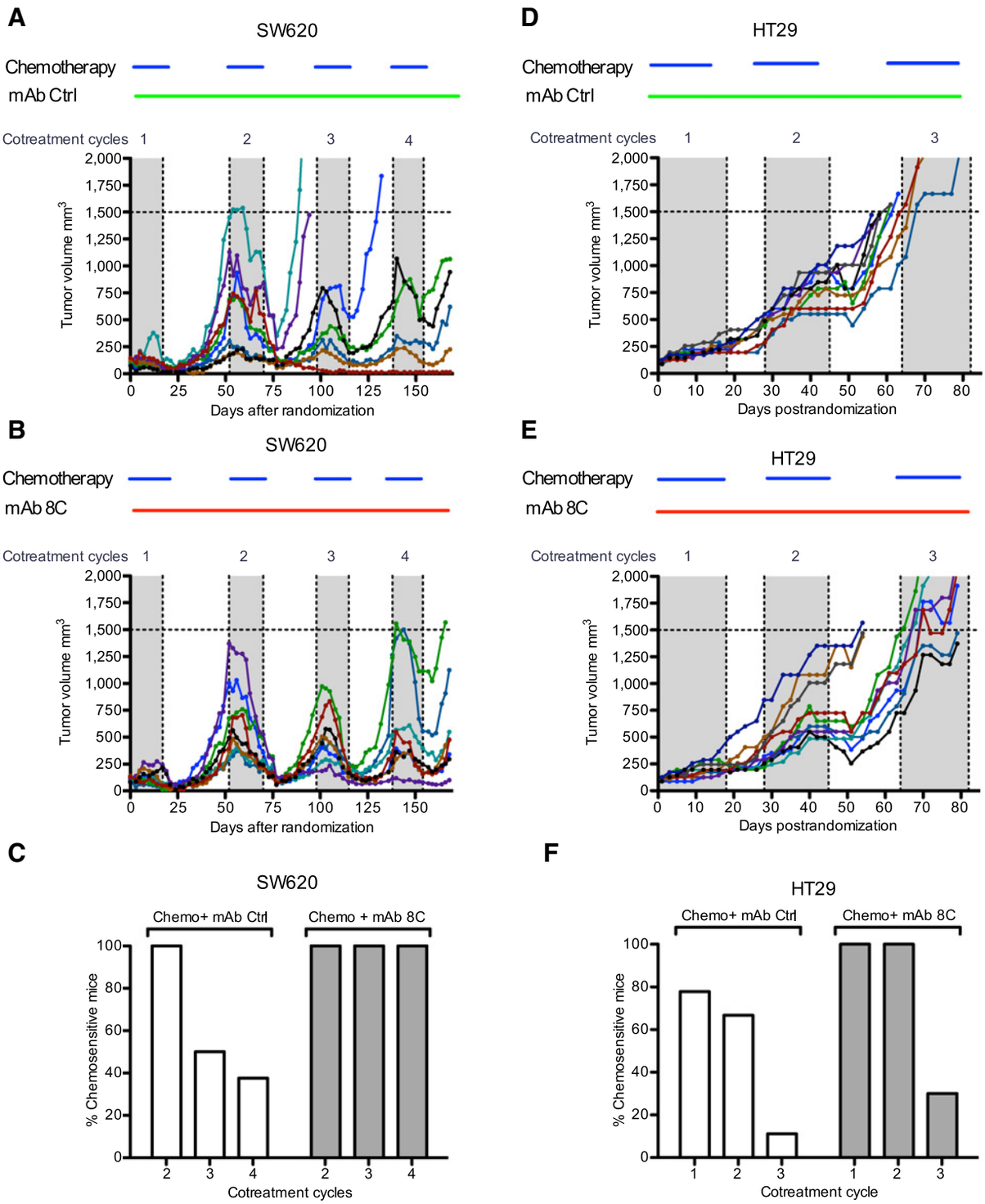

Figure 6.
Anti-progastrin therapeutic antibody reduces the chemoresistance of colorectal cancer cell lines. **A** and **B,** Individual tumor volumes in mice bearing SW620 xenografts treated with several cycles of irinotecan exposure (gray frames, 22 mg/kg) + control antibody (**A**) or mAb8C (**B**; 30 mg/kg; $n = 2$). **C,** Ratio of chemosensitive SW620 tumors during each chemotherapy cycle ($n = 2$). **D** and **E,** Individual tumor volumes in mice bearing HT29 xenografts treated with several cycles of irinotecan exposure + control antibody (**D**) or mAb8C (**E**; $n = 1$). **F,** Ratio of chemosensitive HT29 tumors during each chemotherapy cycle ($n = 1$). Chemosensitivity was defined as the stabilization of tumor growth or the decrease of tumor volume for at least three consecutive measurements under cotreatment.

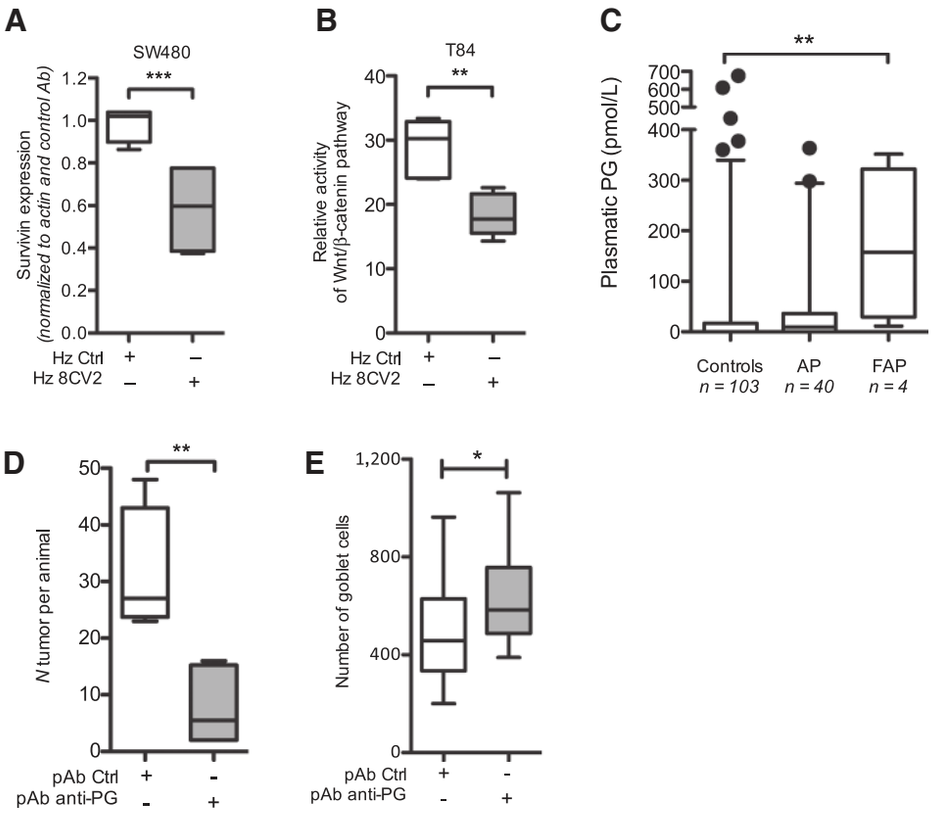

Figure 7.

Anti-progastrin therapeutic antibody inhibits Wnt signaling and APC mutation–driven tumorigenesis. **A,** Survivin expression in SW480 cells treated with control or Hz8CV2. **B,** Transcriptional activity of the β-catenin/Tcf complex measured using TOP-FOP in T84 colonospheres treated with control or Hz8CV2 ($n = 6$). **C,** Progastrin levels in plasma from patients with either adenomatous polyps (AP) or familial adenomatous polyposis (FAP) versus controls (collected from the general population). **D,** Number of tumors in the intestinal tract of $Apc^{\Delta 14/+}$ mice treated with control (4 mice) or anti-progastrin antibody (6 mice), measured and scored independently by 2 blinded operators ($n = 2$). **E,** Number of goblet cells across all $Apc^{\Delta 14/+}$ mice tumors after treatment with control or anti-progastrin mAb ($n = 2$). All results are expressed as median (line), interquartile range (box), and range (whiskers), except in **C** [median (line), interquartile range (box), 95th percentile (whiskers), and outliers (dots)]. All tests were two tailed. Two-way ANOVA (**A**), one-way ANOVA (**C**), or t-test (**B**, **D**, and **E**); *, $0.01 < P \leq 0.05$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$.

10 pmol/L. Furthermore, we tested 4 patients with familial adenomatous polyposis, an inherited syndrome characterized by hundreds of adenomatous colorectal polyps and triggered by germinal mutations of the Wnt regulator *APC* (31) and observed a median progastrin plasma concentration of 158 pmol/L (Fig. 7C).

We then analyzed the effect of progastrin-selective antibodies in the $Apc^{\Delta 14/+}$ mouse model of Wnt-driven intestinal tumorigenesis (32). We treated mice for 6 weeks with anti-progastrin or control antibody from the age of 3 months, when they already bear colorectal polyps. We observed a 75% decrease in the number of tumors in animals treated with the anti-progastrin antibody compared with controls (Fig. 7D). The number of goblet cells, used as a reflection of tumor differentiation, was significantly higher in remaining adenomas of mice treated with anti-progastrin antibodies compared with those in control tumors (627 ± 35 vs. 495 ± 36 goblet cells per mice, respectively; Fig. 7E).

Importantly, treatment with anti-progastrin antibodies did not induce any detectable sign of toxicity. Mice treated with anti-progastrin or control antibodies were healthy and did not exhibit any statistical differences in weight or in renewal of their intestinal epithelium (Supplementary Fig. S13).

Discussion

The work presented herein focuses on the generation of humanized mAbs with a high affinity for native progastrin and that demonstrate therapeutic properties. First, we document the fact that exposure to chemotherapy or a hypoxic environment promotes the release of progastrin in patient plasma and its production by colorectal tumor cells, underlining the clinical importance that this target has in stage II–IV colorectal cancer patients who are receiving therapy. Second, we show that humanized antibodies against progastrin are capable of robustly hindering both cell proliferation and migration/invasion in colorectal cancer cells.

In addition, these antibodies significantly decrease CSC self-renewal *in vitro* and *in vivo* as well as impair Wnt-driven tumor initiation. Finally, the therapeutic potential of the anti-progastrin antibodies is further highlighted with the demonstration that chemotherapy and the antibodies combined provoke an increase in chemosensitivity and delays the recurrence of *in vivo* tumors.

The fact that progastrin is overexpressed and detectable in plasma from colorectal cancer patients has been known for a while now (10, 33). Currently, the progastrin receptor at the surface of tumor cells remains elusive; however, the peptide has a wide range of tumor-promoting effects, such as stimulating proliferation and angiogenesis (17, 34), inhibiting differentiation and apoptosis (19, 20), and also promoting CSC self-renewal (21). Interestingly, Beck and colleagues showed that skin CSCs secrete a large amount of VEGFA that will lead to the growth of the tumor by not only attracting endothelial cells but also by stimulating the expression of genes that are enriched in CSCs such as cyclin D1 and sox2 (35). Therefore, we can hypothesize that the effect of our anti-progastrin antibody on CSCs might be related, at least in part, to the blockage of the proangiogenic activity of progastrin that causes the pool of CSCs to shrink and the tumor to regress.

When chemotherapy (FOLFIRI) and antiangiogenic antibodies (Avastin) were combined during treatment of colorectal cancer patients, a swift increase of systemic progastrin was detected after treatment onset. As progastrin in patients is generated directly from the tumors (33), it seems very likely that treatment itself induces cytotoxic and hypoxic stresses, leading to a tumor response, in turn increasing the levels of progastrin. We were able to demonstrate *in vitro* that colorectal tumor cells secreted higher levels of progastrin when under cytotoxic treatment or hypoxic conditions, but also when the cells were deprived of serum mimicking reduced nutrient access. Although it has been previously reported in multiple species including humans that hypoxia increases the expression of the gene encoding progastrin (36, 37), our results demonstrate for the first time, to the best of our knowledge, a clear link between an increase of progastrin secretion and cytotoxic treatment of colorectal cancer.

We have previously brought to light that progastrin stimulates CSC survival and self-renewal (21), which aligns well with previous data showing that chemotherapy (22) and hypoxic conditions (38) select for cancer cells with stem cell properties. For these reasons, we naturally investigated the idea that capturing progastrin via specific antibodies would reduce the capacity of colorectal cancer stem cells to resist cytotoxic treatment and prove beneficial for patient treatment when combined with chemotherapy and selected a humanized antibody (Hz8CV2) as a potential drug candidate.

With this new tool in hand, we wanted to verify whether Hz8CV2 could impair self-renewal, a hallmark of CSCs. Indeed, targeting these cells has become a prominent strategy for obtaining durable antitumor effects in a multitude of cancers, including colorectal (39). Current methods involve inhibiting developmental signaling pathways (Wnt, Notch) or increasing the activity of pathways like PKA leading to a decrease in tumor initiation (40). BMI1, a small-molecule inhibitor of self-renewal, has been identified as a tool of interest in treating colorectal cancer (39). Another new field of interest is to target membrane proteins or extracellular ligands involved in the regulation of CSC survival or self-renewal. Auspicious examples are anti-IL4–neutralizing antibodies, which prevent the autocrine prosurvival signals driven by IL4 on $CD133^+$ cells, thereby increasing the efficiency of chemotherapy (41), and CD44-specific antibodies that target CSCs and bulk tumors as well as decrease the recurrence after radiotherapy in pancreatic tumor models (42). Our work here shows that anti-progastrin antibodies are capable of strongly downregulating colorectal cancer stem cell self-renewal, decreasing Wnt signaling and Wnt-driven tumorigenesis. The latter was expected as it has already been shown that knocking out the GAST gene in an APC^{min} mouse model induces a reduction in the number of adenomas. However, the effect we show here is more important than in the knockout model. This could be due to the different models used, APC^{min} mice and $Apc^{\Delta 14/+}$ mice, or to the fact that the anti-progastrin antibody treatment was initiated once the tumors were already present. Furthermore, we demonstrated that the anti-progastrin antibodies are capable of robustly diminishing the motility and invasive potential of colorectal cancer cells, traits that are correlated with CSCs via activation of the transcriptional program driving the EMT (43). Taken together, our results show that the function and/or survival of colon CSCs is directly affected by anti-progastrin antibodies, confirming previous results that established the stimulating role of progastrin on colorectal cancer stem cells (21).

Consequently, when we performed consecutive chemotherapy cycles combined with the mAb8C murine anti-progastrin antibody on K-RAS- or B-RAF–mutated tumor xenografts, we found an increase in the chemosensitivity for SW620 and HT29 tumors and a significant delayed relapse for T84 ones, underlining the therapeutic potential of anti-progastrin antibodies. In accordance with recent results revealing the emergence of K-RAS mutations and acquired resistance after cancer therapy (40), we could use our anti-progastrin antibody in combination with chemotherapy either on patients with preexisting K-RAS or B-RAF mutations, or on patients with K-RAS wild type to avoid acquisition of this mutation during treatment, or to treat patients after *de novo* acquisition of this mutation. In addition, although the most common target proteins for which therapeutic antibodies are currently being developed are found on the extracellular membrane of tumor cells (44), novel antibodies are now emerging that target secreted ligands (45), and the majority of them involve tumor angiogenesis pathways. So far, the most commercially successful of these has been the anti-VEGF antibody bevacizumab (2), but other antibodies, such as anti-angiopoietins 1 and 2, or anti-FGF2 in renal cell carcinoma (46), have demonstrated good results in preclinical models. Although humanized anti-angiopoietin-2 showed promising early results, this was not the case during phase III trials (47).

It would seem that for the foreseeable future, 5-FU–based chemotherapeutic combinations are likely to represent the bulk of colorectal cancer treatment; therefore, combining 5-FU with anti-progastrin antibodies presents an encouraging therapeutic treatment for minimizing or preventing postchemotherapy relapse. A critical aspect and advantage of humanized anti-progastrin antibodies is that they did not provoke any adverse effects during the 5 months of twice-weekly treatment of xenografted mice with up to 30 mg/kg of antibody, contrary to several other approaches (5, 6). These data correlate directly with data showing that gastric and pancreatic changes observed in progastrin-encoding gene KO mice were not the cause of progastrin's absence, but instead of its processing product, amidated gastrin (48). This points to the idea that progastrin itself does not have a crucial role in the survival or function of healthy adult organs, and therefore, the induction of target-specific side effects by therapeutic anti-progastrin antibodies should be extremely limited.

Altogether, the work presented here describes the generation and humanization of very specific anti-progastrin antibodies possessing the capacity to improve tumor chemosensitivity and survival after treatment in combination with chemotherapy in preclinical mouse models, to reduce CSC self-renewal, and to decrease Wnt signaling activity and Wnt-driven tumorigenesis. Given the absence of observable side effects, even after relatively long treatment periods, our data indicate that humanized monoclonal anti-progastrin antibodies are an extremely promising therapy representing at last a breakthrough for K-RAS–mutated colorectal cancer patients.

Disclosure of Potential Conflicts of Interest

A. Prieur holds ownership interest (including patents) in Accompagnement Pharma. F. Bibeau reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Amgen, Bristol-Myers Squibb, MSD, Merck, and Sanofi. No potential conflicts of interest were disclosed by the other authors.

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