Cancer Research

# Autocrine Secretion of Progastrin Promotes the Survival and Self-Renewal of Colon Cancer Stem–like Cells

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## Abstract

Subpopulations of cancer stem–like cells (CSC) are thought to drive tumor progression and posttreatment recurrence in multiple solid tumors. However, the mechanisms that maintain stable proportions of self-renewing CSC within heterogeneous tumors under homeostatic conditions remain poorly understood. Progastrin is a secreted peptide that exhibits tumor-forming potential in colorectal cancer, where it regulates pathways known to modulate colon CSC behaviors. In this study, we investigated the role of progastrin in regulating CSC phenotype in advanced colorectal cancer. Progastrin expression and secretion were highly enriched in colon CSC isolated from human colorectal cancer cell lines and colon tumor biopsies. Progastrin expression promoted CSC self-renewal and survival, whereas its depletion by RNA interference–mediated or antibody-mediated strategies altered the homeostatic proportions of CSC cells within heterogeneous colorectal cancer tumors. Progastrin downregulation also decreased the frequency of ALDH$^{high}$ cells, impairing their tumor-initiating potential, and inhibited the high glycolytic activity of ALDH$^{high}$ CSC to limit their self-renewal capability. Taken together, our results show how colorectal CSC maintain their tumor-initiating and self-renewal capabilities by secreting progastrin, thereby contributing to the tumor microenvironment to support malignancy. *Cancer Res; 76(12); 3618–28. ©2016 AACR.*



## Introduction

Cancer stem cells (CSC) have been implicated in the initiation, maintenance, and propagation of hematologic and solid tumors (1, 2). They represent a source of phenotypic heterogeneity in several cancer types (3), where they play a role in metastasis development (4) and resistance to current therapies (5). The recent literature suggests that the CSC phenotype may be tightly regulated by extrinsic cues coming from the surrounding microenvironment (6), including other tumor cells (7), suggesting that the regulation of differentiation and cell plasticity by microenvironment-driven signals controls phenotypic heterogeneity within tumor cell populations. Thus, the demonstration that non–stem tumor cells are able to convert into self-renewing tumor-initiating cells (8) challenges the hierarchical concept that constitutes a pillar of the CSC paradigm, pointing towards the existence of a CSC state rather than fixed CSC entities (9). Yet, these findings also suggest that homeostatic proportions of identifiable tumor-initiating and nontumorigenic cell subpopulations exist in individual tumors and that drugs targeting stem-like populations only are likely to trigger plasticity until this homeostatic equilibrium is reattained, thus allowing posttreatment relapse. A better understanding of signals that maintain homeostatic proportions of CSCs and/or regulate phenotypic plasticity between non-stem and stem-like cells is therefore essential to inform future combination therapies aiming to minimize relapse.

Extrinsic cues controlling the phenotype of colorectal CSCs include soluble factors (secreted by stromal or tumor cells) that directly regulate key signaling pathways for the CSC phenotype, such as Wnt or Notch (6, 7). We hypothesized that progastrin, secreted by human colorectal tumors (10, 11) and exhibiting strong tumor-promoting functions in colorectal cancer (12, 13), may play a role in CSC regulation. We previously demonstrated that progastrin regulates the Wnt and Notch

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pathways in colorectal cancer (12, 14), suggesting that it could affect the phenotype of colon CSCs, which rely on these pathways for their survival (15). Progastrin was shown to promote the proliferation of progenitor cells in the mouse colonic epithelium (16) and a link has been suggested between progastrin expression and populations of cells expressing CD133 (17), DCAMKL1 (18), or CD44 (19) in mouse colonic crypts and human cancer cell lines. Yet, expression of these markers is not restricted to CSCs (20–23), and the functional role of progastrin on CSC self-renewal and tumor-initiating potential has not been documented.

Here we demonstrate that colorectal CSCs require this peptide to maintain their tumor-initiating and self-renewal ability and demonstrate that this regulation acts through the promotion by progastrin of a highly glycolytic phenotype in colon CSCs. We further establish that downregulation of progastrin expression or neutralization of its activity alters the long-term tumor-initiating capacity of these cells *in vitro* and *in vivo*.

## Materials and Methods

### Patient-derived tumor cell lines

Human colorectal cancer cell lines T84, SW620, HCT116, HT29, and RKO (obtained from the ATCC between 2005 and 2010) were cultured in a humidified atmosphere at 37°C and 5% $CO_2$ in complete DMEM. Cell line authenticity was last tested in December 2015 using STR profiling (LGC standards). Patient-derived colorectal cancer cells (CPP1-19-24-25) were prepared from fresh biopsies (Human ethics agreement #2011-A01141-40) as described under Supplementary Methods.

### Sphere formation assays

***Sphere formation.*** One hundred cells per well were seeded in ultra-low adherent 96-well plates (Corning) in 100 μL of M11 medium. After 7 days, photos of each sphere (>40 μm) were taken and sphere size was measured using the ImageJ software.

***Self-renewal.*** Cells were transiently transfected once, seeded, and resulting spheres were passaged every 7 days. For antibody treatment, cells were treated once a day with 3 μg/mL polyclonal anti-human IgG (Jackson ImmunoResearch, 309-005-008) or progastrin-selective antibodies. Counting was performed 7 days later. CSC frequency was determined using the Extreme Limiting Dilution Analysis (ELDA; ref. 24) 7 days after seeding in M11 medium (10 wells/condition).

### RNA quantitation

Total RNA was extracted using the RNeasy Mini/Micro Kit (Qiagen) and treated with DNAse-1. cDNA was synthetized using M-MLV or SuperScript II (when RNA < 250 ng; Invitrogen). Relative gene expression was measured by real-time PCR as described previously (12). GAPDH expression was used as an internal calibrator. Primer sequences are provided in Supplementary Methods.

RNA expression profiling of cell lines was performed using Affymetrix Human gene ST 2.0 DNA microarrays (see Supplementary Methods). Raw transcriptome data were deposited in the ArrayExpress repository (http://www.ebi.ac.uk/arrayexpress/, accession number: E-MTAB-3215).

### siRNA transfection

siRNA transfection was performed in suspension, using Lipofectamine RNAiMAX and siRNA duplexes (siβgal and siPG, Invitrogen), according to manufacturer's recommendation. Six hours after transfection, cells (1–6 cells/μL) were seeded in ultra-low adherent flasks or 96-well plates. siRNA sequences were described previously (12).

### Neutralizing progastrin antibodies

Polyclonal anti-progastrin antibodies were produced and purified by Eurogentec as detailed under Supplementary Methods. We used ELISA to confirm their lack of binding to other peptides in the gastrin family, and their ability to reverse the effects of progastrin (Supplementary Fig. S2D) was validated on p38 MAPK phosphorylation (see Supplementary Methods).

### *In vivo* xenograft experiments

*In vivo* experiments followed French guidelines for experimental animal studies (DSV agreement C34-172-27).

T84 shCT/shPG cells were pretreated with doxycycline (1 μg/mL) 48 hours before injection. $ALDH^{high}$ cells were resuspended in DMEM/Matrigel (v/v) and injected subcutaneously into the right flank of female balb/C nude CD1 mice (Charles River Laboratories). For secondary injection, tumors were dissociated as described for human tumors, and a Ficoll gradient was used to remove dead cells/debris. Doxycycline (2 mg/mL) was provided in the drinking water 96 hours before injection and throughout the experiment. Tumors were measured twice a week using a caliper [volume = (length × width × thickness)/2]. Tumor samples were fixed in 4% paraformaldehyde and paraffin-embedded.

SW480 and CPP19 cells transfected with shPG or full-length human progastrin (as in ref. 12) were grown as monolayers in FBS-containing medium. $ALDH^{high}$ cells were injected subcutaneously into the right flank of female balb/C nude CD1 mice, and tumors were measured as above.

### ALDEFLUOR assay and flow cytometry

Staining with ALDEFLUOR (STEMCELL Technologies), with CD44 (Miltenyi Biotec, 130-098-110), EpCAM (Miltenyi Biotec, 130-080-301), CD133 (Miltenyi Biotec, 130-098-829), LGR5 (BD, #562913), and Annexin V (Fluoroprobe) antibodies was performed according to manufacturer's instructions. Negative cells were respectively defined using the ALDH inhibitor diethylaminobenzaldehyde (DEAB) or isotype controls. Dead cells were excluded on the basis of 7-aminoactinomycin D (7-AAD, Invitrogen) exclusion.

### Mitochondrial metabolism

Cellular bioenergetics [including oxygen consumption (OCR) and extracellular acidification rates (ECAR)] were determined on an XF24 Extracellular Flux Bioanalyzer (Seahorse Bioscience). Cells per well ($n$ = 50,000) were seeded into 24-well XF24 cell culture microplates and grown overnight. Three wells containing medium alone were used as background. One hour before the assay, growth medium was replaced with unbuffered assay medium (pH = 7.4, Seahorse Bioscience) containing 25 mmol/L glucose (Sigma-Aldrich) and placed in a non-$CO_2$ incubator for $CO_2$ and temperature stabilization. ECAR and OCR were analyzed during a mitochondrial stress test using consecutive administration of oligomycin (2 μmol/L), FCCP (1.5 μmol/L),

and rotenone/antimycin A (2 μmol/L). To determine the impact of glycolysis on progastrin-regulated self-renewal, ALDH$^{high/neg}$ T84 cells were transfected with GAST- or βgal-selective siRNA, and their stem cell frequency was quantified using ELDA in the presence of DMSO (0.01%) or 0.05 μmol/L antimycin A.

### Statistical analysis

Statistical tests were performed using GraphPad Prism Version 6, using Mann–Whitney, ANOVA, or Student *t* test for *in vitro* studies, with appropriate *post hoc* tests for multiple comparisons. *In vivo* survival curves were derived from Kaplan–Meier estimates, and the curves were compared by log-rank tests. A 5% cutoff was used to validate result significance.

## Results

### Progastrin expression is elevated in conditions that promote CSC self-renewal

We first used colorectal cancer cell lines and cells isolated from patients with colorectal cancer to determine whether progastrin expression was detected in conditions that promote CSC function *in vitro*. While expression of the progastrin-encoding gene (*GAST*; not shown) as well as progastrin secretion (Fig. 1B) were detectable when cells were grown as monolayers in serum-supplemented medium (2D), both were strongly elevated in cells grown as colonospheres (3D; Fig. 1A and B).

As an alternative, we enriched CSCs based on their elevated aldehyde dehydrogenase (ALDH) activity. ALDH is a detoxifying enzyme involved in the resistance to alkylating enzymes. Similar to other cancers (25), selection based on its activity leads to enrichment of functional CSCs in colorectal cancer, as ALDH$^{high}$ colorectal cancer cells repeatedly generate colonospheres *in vitro* and tumors in immunocompromised animals (26, 27). ALDH$^{high}$ colorectal cancer cell lines and patient-derived colorectal cancer cells displayed enriched ALDH1A1 mRNA levels (Supplementary Fig. S1A and S1B) and higher levels of GAST mRNA (Fig. 1C) than ALDH$^{neg}$ cells, although the latter clearly expressed detectable levels of *GAST* mRNA compared with human fibroblasts and glioblastoma cells, where no specific GAST signal was detected using RT-qPCR (Supplementary Fig. S1C).

To further confirm that CSCs expressed elevated levels of progastrin, we quantified GAST mRNA in colorectal cancer cells sorted using other proposed CSC markers. Cells enriched using CD44/EpCAM (28) or LGR5 (29) expressed significantly higher GAST mRNA compared with marker-negative populations (Supplementary Fig. S1D–S1G). GAST expression was not consistently higher in cells enriched using CD133/1 (Supplementary Fig. S1H–S1K), a more controversial CSC marker in cultured colorectal cancer cells (21, 22). The percentage of CD133/1–positive cells was higher in colorectal cancer cell lines than in most patient-derived cells (Supplementary Fig. S1I), and GAST mRNA was elevated in CD133$^{high}$ cells only in samples where CD133 expression enriched a small cell subpopulation (CPP1, Supplementary Fig. S1I–S1K). These results strongly suggest that progastrin

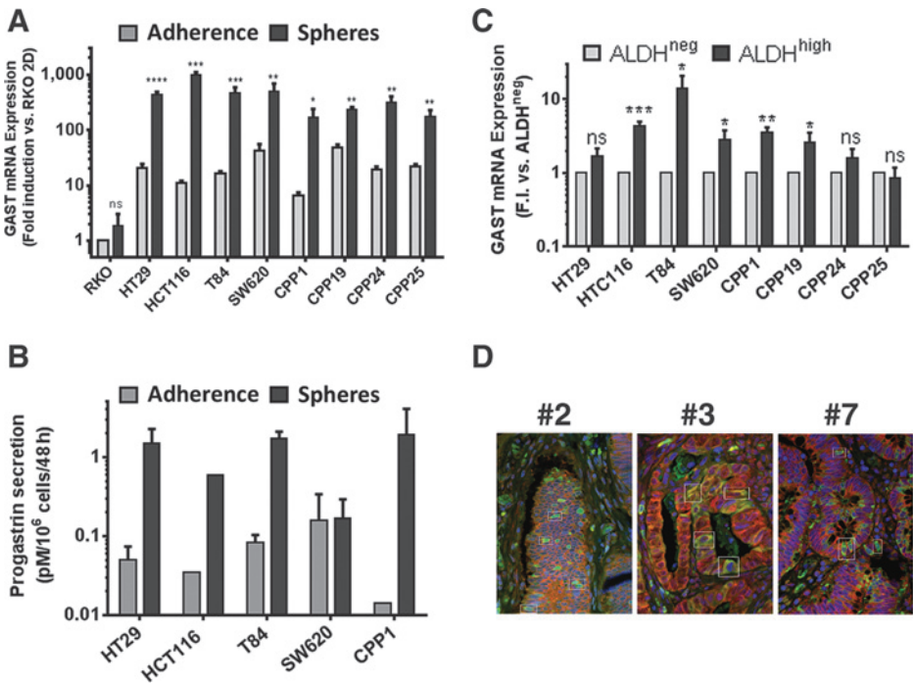


**Figure 1.**

Progastrin is enriched in conditions that promote CSC self-renewal. A, *GAST* mRNA quantification in colorectal cancer cell line monolayers (Adherence, light bars) or colonospheres (Spheres, dark bars). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, nonsignificant, Mann–Whitney test compared with matched 2D samples. Note that SW620 grew as small clusters under "sphere" conditions. B, progastrin secretion quantified by ELISA ($n = 2$ per sample unless otherwise indicated; #, $n = 1$) in the conditioned medium of colorectal cancer cell line monolayers (light bars, 2D) or colonospheres (dark bars, 3D). Data expressed as pmol/L/10$^6$ cells/48 hours. C, *GAST* mRNA quantification in ALDH-negative cells (ALDH$^{neg}$, light bars) and ALDH-high cells (ALDH$^{high}$, dark bars) purified from colorectal cancers. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant, Mann–Whitney test compared with matched ALDH$^{neg}$ population. D, progastrin (red) and ALDH1 (green) staining of human colorectal cancer samples from three different patients (#2, #3, #7). Nuclei are stained with DAPI (blue). Scale bars, 20 μm. A–C, mean ± SEM from at least three independent experiments unless stated otherwise.

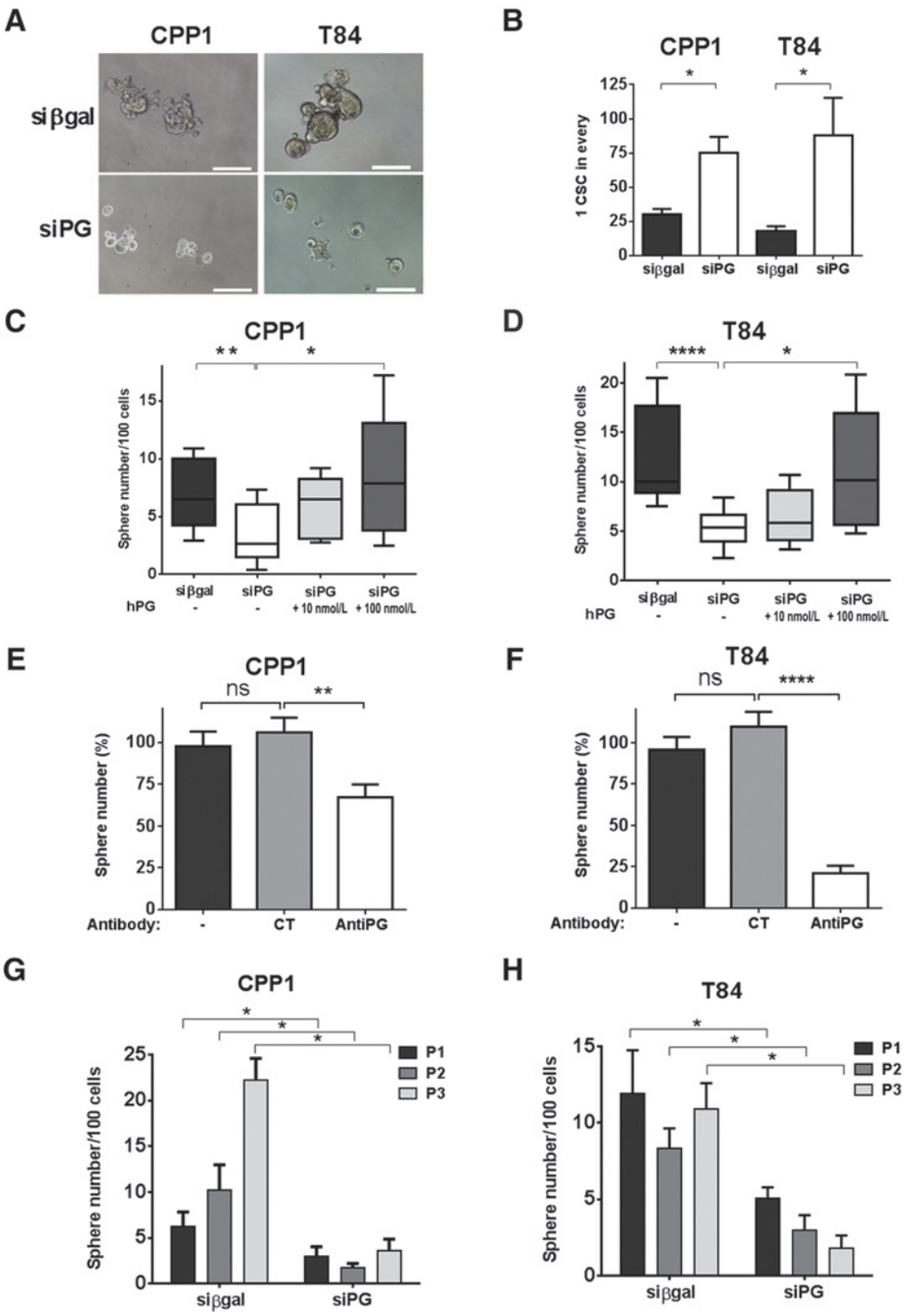


**Figure 2.**
Progastrin depletion impairs CSC survival and self-renewal *in vitro*. Representative images of spheres (A), CSC frequency (B), and sphere number quantification (C and D) in CPP1 and T84 cells 7 days after transfection of a *GAST*-specific (siPG, white plot) or a control siRNA (siβgal, dark plot). Cells were supplemented or not daily with 10 or 100 nmol/L recombinant human progastrin (hPG). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$, Mann–Whitney test. E and F, impact of a daily treatment with 3 μg/mL progastrin-selective (antiPG, white bars) or control (CT, gray bars) polyclonal antibody on the ability of CPP1 (E) and T84 (F) cells to form colonospheres after 7 days. **, $P < 0.01$; ****, $P < 0.0001$; ns, nonsignificant, one-way ANOVA test. G and H, self-renewal potential of CPP1 (G) or T84 (H) over three consecutive passages (7-day growth between passages) following siβgal or siPG transfection at passage 1. *, $P < 0.05$, Mann–Whitney test. Mean ± SEM from at least three independent experiments.

expression is increased in colorectal CSCs, since growing cells as colonospheres or selecting ALDH$^{high}$ (Supplementary Fig. S1L and S1M; refs. 27, 30), CD44$^{high}$/EpCAM$^{high}$ (28) or LGR5$^{high}$ (29) tumor cells enriches cells with functional CSC characteristics. Accordingly, progastrin was highly expressed in ALDH$^{high}$ cells and in areas surrounding them within colorectal cancer samples (Fig. 1D). These findings suggest that progastrin secretion is not an exclusive characteristic of colon CSCs but is nevertheless higher in these cells than in their non-CSC counterparts.

**Progastrin promotes the self-renewal of colorectal cells *in vitro***

To determine whether CSCs could respond to progastrin as part of an autocrine and/or paracrine loop, we determined the impact of progastrin on the colonosphere formation ability of T84 and CPP1 cells. Colonosphere formation was strongly impaired following transfection with a GAST gene–specific siRNA (Fig. 2A and Supplementary Fig. S2A and S2B), with a 3- to 4-fold reduction of CSC frequency quantified using ELDA (Fig. 2B)), and this effect was reversed when GAST siRNA-treated cells were incubated with recombinant human

progastrin (Fig. 2C and D and Supplementary Fig. S2C and S2D). The size of remaining colonospheres was smaller in progastrin-depleted cells than in controls (Supplementary Fig. S2B), corroborating other studies demonstrating proproliferative and antiapoptotic effects of progastrin (19, 31). Similar results were obtained upon neutralization of progastrin using selective polyclonal antibodies (Fig. 2E and F and Supplementary Fig. S2D and S2E) and upon inducible shRNA-mediated progastrin downregulation (Supplementary Fig. S2F and S2G), the former indicating that this effect is linked to an autocrine/paracrine function of the peptide.

Serial sphere passaging demonstrated that siRNA-mediated progastrin depletion performed during the initial passage induced a long-term impairment of the sphere-forming potential of colorectal cancer cells when compared with controls (Fig. 2G and H), indicating that self-renewal of sphere-forming colorectal cancer cells is severely altered in the absence of progastrin.

### Progastrin regulates the maintenance of the ALDH$^{high}$ tumor-initiating cell pool

As our results and those of others point to ALDH activity as a functional marker to enrich stem-like cells in colorectal cancer, we assessed whether the effect of progastrin on self-renewal was due to a direct regulation of the ALDH$^{high}$ cell subpopulation. siRNA-mediated progastrin downregulation significantly decreased ALDH$^{high}$ cell proportions within colorectal cancer cell lines and patient-derived cells (Fig. 3A and B), suggesting that progastrin is essential to stabilize the proportion of CSC-like cells under homeostatic conditions.

The recent literature suggests that stochastic transitions between non-stem and stem-like cells contribute to the maintenance of phenotypic equilibrium within cancer cell populations (8, 32). To gain further insight concerning the effect of progastrin on ALDH$^{high}$ CSC maintenance, we analyzed the individual capacity of ALDH$^{high}$ and ALDH$^{neg}$ cells to regenerate population heterogeneity when grown under nonchallenging adherent

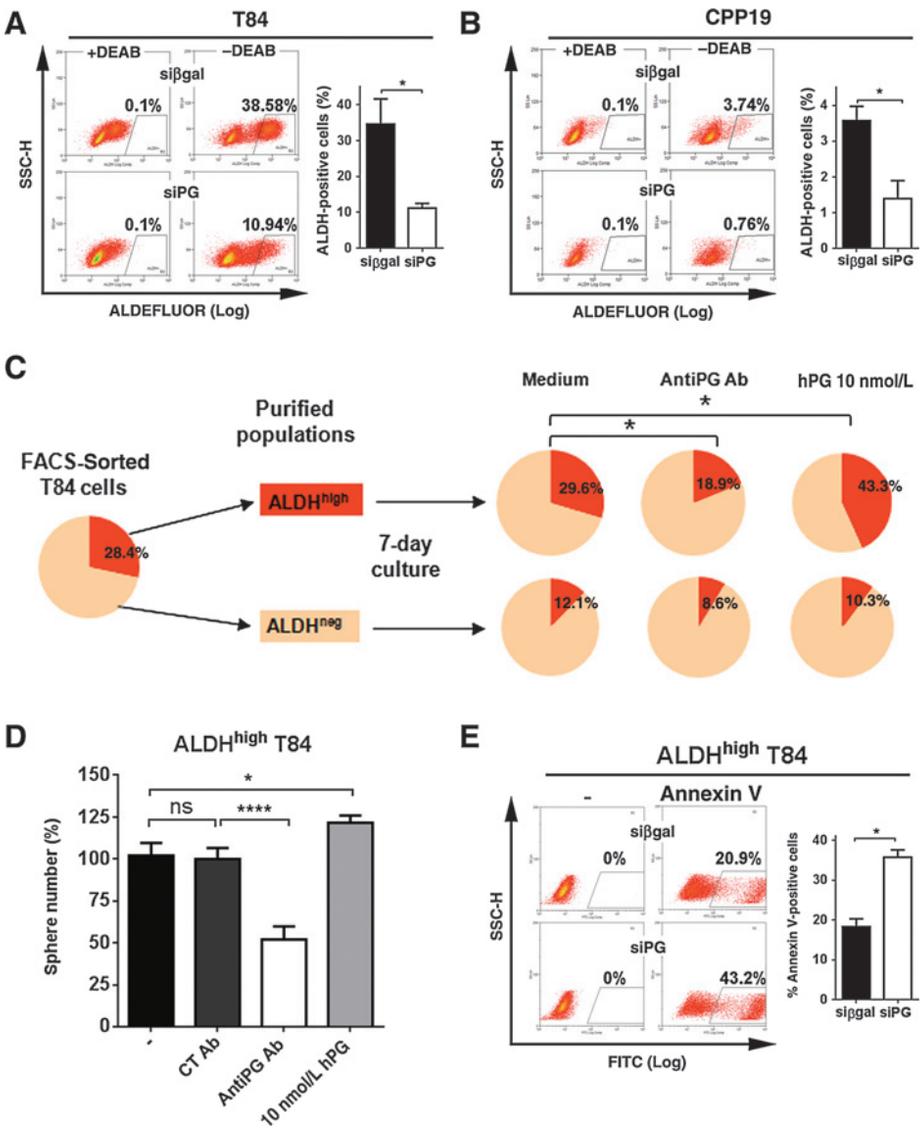


**Figure 3.**
Progastrin regulates the maintenance of the ALDH$^{high}$ cancer stem cell pool. A and B, representative FACS profiles (left) and quantification (right) of ALDH$^{high}$ cells (%) in T84 (A) and CPP19 (B) under colonosphere conditions 4 days after siβgal or siPG transfection. *, $P < 0.05$, Mann–Whitney test. C, percentage of ALDH$^{high}$ cells in parental T84 cells and 7 days after seeding purified populations of ALDH$^{high}$ (top) or ALDH$^{neg}$ (bottom) cells as monolayers in medium supplemented or not (daily) with 10 nmol/L hPG or 1.6 μg/mL of anti-PG antibodies. *, $P < 0.05$, Mann–Whitney test. D, colonosphere formation 7 days after ALDH$^{high}$ cell purification and growth in suspension in the presence of antibodies (3 μg/mL daily, anti-PG antibody, white bar; control antibody, dark gray bar) or recombinant progastrin (hPG 10 nmol/L, clear gray bar), compared with untreated controls (-, black bar). *, $P < 0.05$; ****, $P < 0.0001$; ns, nonsignificant, one-way ANOVA test. E, representative FACS profiles (left) and quantification (right) of apoptotic cell percentages 72 hours after ALDH$^{high}$ cell transfection with siβgal or siPG. All data are presented as the mean ± SEM of three to four independent experiments.

culture conditions. Purified ALDH$^{high}$ cells generated populations comprising similar homeostatic proportions of ALDH$^{high}$ and ALDH$^{neg}$ cells to those found in their parental cells within 7 days of culture (Fig. 3C). The proportion of each subpopulation once equilibrium had been reached was regulated by progastrin, with proportions of ALDH$^{high}$ cells significantly increased in cells incubated with recombinant progastrin and decreased upon treatment with a progastrin-neutralizing antibody (Fig. 3C). This result suggests that progastrin hampers or slows down the differentiation of self-renewing ALDH$^{high}$ cells toward ALDH$^{neg}$ colorectal cancer cell phenotypes.

Purified ALDH$^{neg}$ cells gave rise to ALDH$^{high}$ subpopulations, although without reaching the initial equilibrium proportions by 7 days (Fig. 3C). This result suggests that ALDH$^{high}$ cells can be generated from ALDH$^{neg}$ colorectal cancer cells grown under conditions that facilitate cell survival, albeit at significantly slower rate than the ALDH$^{high}$ to ALDH$^{neg}$ conversion. In any case, the rate of transition from ALDH$^{neg}$ to ALDH$^{high}$ within 7 days of FACS-mediated purification was unaffected by recombinant progastrin or by progastrin-neutralizing antibodies (Fig. 3C), suggesting that progastrin did not regulate the ability of ALDH$^{neg}$ cells to generate ALDH$^{high}$ cells within this timeframe.

To assess whether this lack of effect on ALDH$^{neg}$ cells was paralleled by a differential ability of progastrin to engage signaling in these cells, we analyzed the ability of progastrin to regulate the phosphorylation of p38MAPK, which plays a role in regulating CSC characteristics such as drug resistance (33) and is phosphorylated in response to progastrin (34, 35). p38MAPK phosphorylation was promoted by recombinant progastrin and inhibited by progastrin-neutralizing antibodies in ALDH$^{high}$ T84 and CPP19 cells but remained unaffected in ALDH$^{neg}$ cells (Supplementary Fig. S3A), suggesting that ALDH$^{neg}$ colorectal cancer cells do not respond or differentially respond to progastrin compared with ALDH$^{high}$ cells. Taken together, these results suggest that progastrin strongly promotes the maintenance of the ALDH$^{high}$ tumor-initiating phenotype without promoting the transition of ALDH$^{neg}$ tumor cells into stem-like cells.

### Progastrin promotes the self-renewal and survival of ALDH$^{high}$ CSCs

As progastrin regulated homeostatic proportions of ALDH$^{high}$ stem-like cells without affecting the ability of ALDH$^{neg}$ cells to revert to an ALDH$^{high}$ phenotype, we focused on ALDH$^{high}$ cells to determine whether progastrin could directly regulate their self-renewal and/or survival.

Endogenous progastrin depletion in ALDH$^{high}$ cells or treatment with recombinant progastrin, respectively, decreased or increased their sphere-forming ability (Fig. 3D and Supplementary Fig. S3B), suggesting that progastrin promotes the self-renewal of these cells. In contrast, incubation with recombinant human progastrin or with medium conditioned by ALDH$^{high}$ cells was insufficient to allow colonosphere formation by ALDH$^{neg}$ cells, again suggesting that progastrin alone is insufficient to drive their phenotype toward functional CSCs or that ALDH$^{neg}$ cells are altogether not sensitive to progastrin.

We then investigated whether progastrin also promoted the survival of ALDH$^{high}$ colorectal CSCs. Since the proportion of ALDH$^{high}$ cells was already strongly decreased 4 days after transfection of colorectal cancer cells with progastrin-selective siRNA (see Fig. 3A and B), we quantified the proportion of ALDH$^{high}$ cells undergoing apoptosis 72 hours after transfection with control or progastrin-selective siRNA. The proportion of Annexin V–positive cells was significantly increased following transfection with progastrin siRNA compared with controls ($35.7 \pm 1.2\%$ vs. $18.3 \pm 1.5\%$ respectively, $n = 4$; Fig. 3E), suggesting that progastrin is instrumental to maintain ALDH$^{high}$ stem-like cell survival.

### Progastrin downregulation decreases tumor initiation by ALDH$^{high}$ cells *in vivo*

To determine whether, beyond decreasing the proportion of ALDH$^{high}$ cells in colorectal cancer cell populations, progastrin downregulation also inhibited their self-renewal *in vivo*, immunocompromised mice were xenografted with ALDH$^{high}$ T84 cells expressing doxycycline-inducible control (shCT) or progastrin-targeting (shPG) shRNA constructs. Efficiency of progastrin-selective shRNAs to downregulate GAST mRNA expression (Supplementary Fig. S2D), TIC frequency (Supplementary Fig. S2E), and ALDH$^{high}$ cell proportions (Supplementary Fig. S4A and S4B) was confirmed *in vitro*. Live cells that remained ALDH$^{high}$ following a 48-hour doxycycline induction of progastrin-selective shRNA were injected (250 or 25 cells/100 μL) into the flank of balb/C nude CD1 mice (Fig. 4A). Tumors appeared later in mice injected with 250 ALDH$^{high}$ shPG cells compared with controls (median tumor-free survival: shPG, 31 days; shCT, 23 days) but tumor incidence was similar (6 of 6) in both groups 50 days postinjection (Fig. 4B). In contrast, tumor incidence was lower in mice injected with 25 ALDH$^{high}$ shPG cells (1 of 6 mice) than in control ALDH$^{high}$ cells (4 of 6 mice; Fig. 4B), and tumor growth was reduced by progastrin depletion (Fig. 4D). GAST mRNA levels were lower in tumors collected from the shPG group at day 50, compared with controls (shCT; Supplementary Fig. S4C). To assess whether the diminished tumor initiation potential of ALDH$^{high}$ cells following progastrin downregulation leads to long-term decreased self-renewal, xenografted tumors from mice injected with 250 shCT or shPG ALDH$^{high}$ cells were collected at day 50 and enzymatically dissociated. Following Ficoll gradient–based live cell isolation, 25 or 250 unsorted cells were reinjected in new balb/C nude CD1 mice (Fig. 4A). Second-generation xenografts appeared later than their first-generation counterparts that were generated from ALDH$^{high}$ cells (median tumor-free survival times: 60 and 39 days in 25 shCT and 250 shCT second-generation groups - Fig. 4C, compared with 31 and 23 in first-generation controls; Fig. 4B). Zero of 6 and 2 of 6 tumors were detected up to 98 days after reinjection of 25 and 250 shPG cells, respectively, in comparison with 5 of 6 and 6 of 6 tumors in animals injected with 25 and 250 shCT cells (Fig. 4C). The tumor-initiating potential remained severely impaired in shPG cells by 100 days after secondary injection (i.e., before mice from the shCT group had to be culled on ethical grounds), with a greater than 47-fold reduction of estimated TIC frequency in the shPG group (1 in every 693) compared with controls (1 in every 14.5; $P = 8.46E^{-07}$). These results indicate that progastrin downregulation leads to a robust and long-term reduction of tumor-initiating frequency in human colorectal cancer cells and suggest that an impairment of ALDH$^{high}$ cell CSC potential is instrumental to this process. To confirm that progastrin indeed promotes tumor-initiating properties of colorectal cancer cells, we performed additional experiments using the SW480 colorectal cancer cell line and CPP19 patient-derived cells made to overexpress full-length human progastrin. Although these cell lines were less tumorigenic than T84 cells at low concentrations, tumor

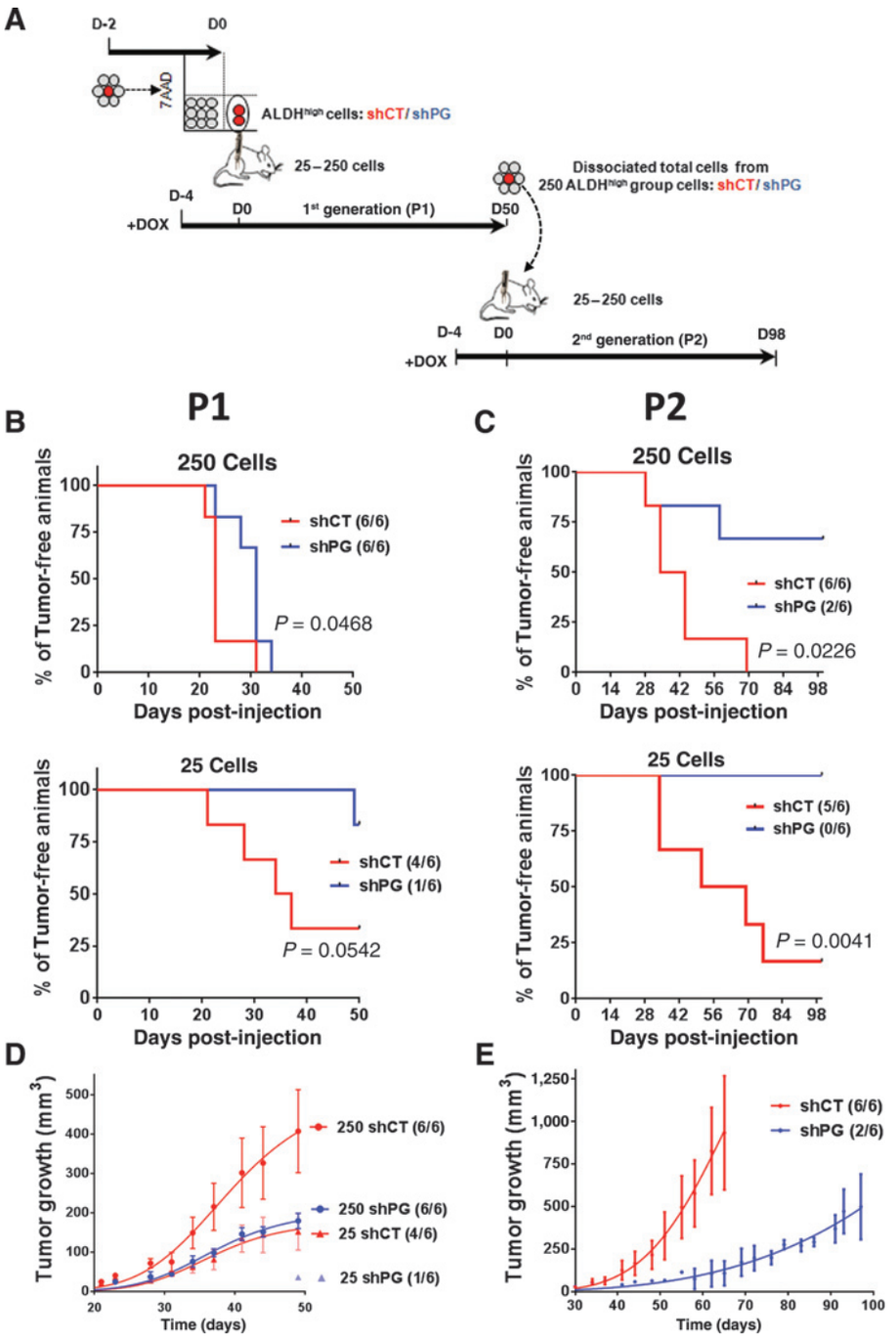


**Figure 4.**
Progastrin downregulation delays ALDH$^{high}$ cell–driven tumor initiation and decreases CSC self-renewal *in vivo*. A, *in vivo* protocol to evaluate the impact of progastrin downregulation on the CSC frequency of ALDH$^{high}$ T84 cells over two generations of xenografts in immunocompromised mice. T84 ALDH$^{high}$ cells were purified by FACS 48 hours after doxycycline-induced expression of control (shCT) or progastrin-selective (shPG) shRNA. Twenty-five or 250 purified ALDH$^{high}$ cells were subcutaneously injected into the right flank of nude CD1 mice. Viable dissociated cells from tumors initiated by 250 ALDH$^{high}$ cells were reinjected into a second generation of mice at similar concentrations to those used in generation 1, and tumor incidence and growth were monitored. B and C, Kaplan–Meier survival curves for first- (P1, B) or second-generation (P2, C) xenografts generated from 250 (top) or 25 (bottom) shCT (red) or shPG (blue) cells. *P* values were calculated using the log-rank test; $n = 6$ mice per group. D and E, tumor growth of shCT (red) or shPG (blue) xenografts following the initial injection of ALDH$^{high}$ cell (P1, D) or the second injection of unsorted cells (P2, E). $n = 6$ mice per groups.

incidence was increased for both cell types in progastrin-overexpressing cells compared with controls (Supplementary Fig. S4).

### Progastrin promotes the maintenance of a stem-like metabolic pathway in ALDH$^{high}$ CSCs

To gain mechanistic insight into the regulation of ALDH$^{high}$ cells by progastrin, we compared the mRNA expression profile of ALDH$^{high}$ cell–derived T84 colonospheres collected 72 hours after transfection with GAST or β-galactosidase–selective siRNA. The expression of 140 genes was significantly altered by the experimental downregulation of progastrin (92 upregulated and 48 downregulated, Supplementary Table S1). Clustering of all samples strongly separated GAST-siRNA from control siRNA samples and delineated an expression gradient along the three time points (6, 24, and 72 hours) for each type of siRNA, indirectly validating the robustness and biologic significance of this gene list (Supplementary Fig. S5A). Ontology analysis of this gene list using the DAVID functional annotation tool indicated that progastrin

regulates many genes involved in metabolic processes (including reduction/oxidation and fatty acid metabolism) and genes, notably histones, involved in chromatin organization (Supplementary Table S2).

In view of the demonstrated role of metabolic processes in the regulation of stem cell and CSC self-renewal (36), we further explored consequences of GAST downregulation on the metabolic activity of ALDH$^{high}$ CSCs. Using transmission electron microscopy or a selective antibody directed against a human mitochondrial protein, we found that the number of mitochondria was increased in colonospheres treated with GAST siRNA (Fig. 5A), as well as in residual tumor xenografts generated from shPG cells (Fig. 5B) compared with controls. We used a Seahorse XF bioanalyzer to establish whether this resulted in modifications of cellular bioenergetics within enriched ALDH$^{high}$ or ALDH$^{neg}$ cells. ALDH$^{high}$ cells displayed significantly higher basal and maximal glycolysis (as measured via ECAR) than ALDH$^{neg}$ cells (Fig. 5C and Supplementary Fig. S5B), whereas their basal mitochondrial OCR were similar. Taken together these results demonstrate a cellular metabolic switch toward glycolysis in ALDH$^{high}$ cells. Interestingly, while there was no difference in basal mitochondrial oxygen consumption, ALDH$^{neg}$ cells exhibited a significantly greater oxygen reserve, as demonstrated via a two-fold increase in maximal oxygen consumption (Fig. 5C and Supplementary Fig. S5B).

siRNA-mediated progastrin downregulation strongly affected the glycolytic profile of ALDH$^{high}$ cells, decreasing both basal and maximal glycolysis to levels equivalent to that observed in ALDH$^{neg}$ cells. In contrast to glycolysis, siPG treatment of ALDH$^{high}$ cells had no effect on overall mitochondrial activity (Fig. 5D and Supplementary Fig. S5B). Incubation with progastrin-neutralizing antibodies also significantly inhibited ALDH$^{high}$ cell glycolysis in the HT-29 cell line, and a similar trend was observed in HCT116 cells (Supplementary Fig. S5C and S5D). The expression of mRNA encoding lactate dehydrogenase B (*LDH-B*), hexokinase 2 (*HK2*) and to a lesser degree the ubiquitous glucose

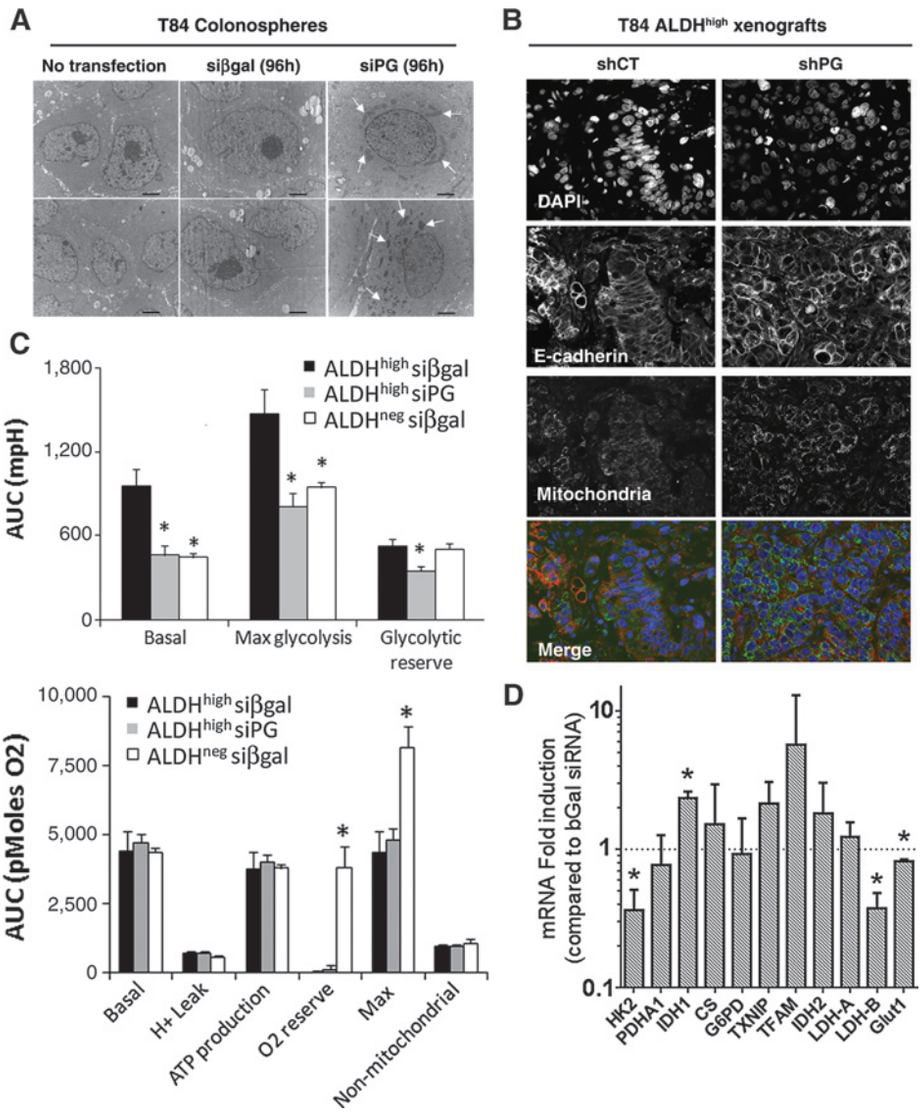


**Figure 5.**
Progastrin promotes the maintenance of a stem-like metabolic pathway in ALDH$^{high}$ CSCs. A, representative electron microphotographs of T84 colonospheres, 96 hours after transfection with siβgal or siPG. Note the enhanced number of mitochondria (white arrowheads) and the small nucleo/cytoplasmic ratio in siPG cells compared with controls. Scale bars, 2 μm. B, E-cadherin (red) and mitochondria (green) staining of xenograft tumors initiated by shCT or shPG ALDH$^{high}$ cells. Nuclei are stained with DAPI (blue). C, quantification of glycolysis (ECAR, top) and oxygen consumption (OCR, bottom) in ALDH$^{high}$ cells transfected with siβgal (black bars) or siPG (white bars) for 96 hours, compared with siβgal-transfected ALDH$^{neg}$ cells (gray bars). Mean ± SEM from 6 replicates of a representative experiment ($n = 3$ experiments). D, expression of genes involved in mitochondrial metabolism in ALDH$^{high}$ T84 cells transfected with siPG for 96 hours, expressed as fold ratio compared with siβgal-transfected ALDH$^{high}$ cells (dashed line). Mean ± SD from three experiments. *, $P < 0.02$, Mann–Whitney test.

transporter GLUT1 were significantly decreased in ALDH$^{high}$ cells. In contrast, the tricarboxylic acid (TCA) cycle regulator isocitrate dehydrogenase 1 (*IDH1*) was significantly increased (Fig. 5D). Finally we used the oxidative phosphorylation inhibitor antimycin A to prevent the decrease in glycolysis and the increase in oxidative phosphorylation induced by the PG siRNA in ALDH$^{high}$ cells. Antimycin A (0.05 μmol/L) prevented the inhibition of sphere-forming ability induced by progastrin downregulation in these cells (Supplementary Fig. S5E), suggesting that regulating mitochondrial metabolism was instrumental to the promoting role of progastrin on ALDH$^{high}$ CSC self-renewal. Taken together these results indicate that progastrin promotes the ability of ALDH$^{high}$ cells to maintain a pattern of mitochondrial metabolism that is reminiscent of that characterizing CSCs (36).

## Discussion

This study demonstrates that progastrin, secreted by human colorectal tumors (10, 11), promotes the survival and self-renewal of cancer stem cells. It also shows that progastrin inhibition in ALDH$^{high}$ CSCs decreases their glycolytic profile down to levels found in non-CSCs, strongly reducing their tumor-initiating ability *in vitro* and *in vivo*.

CSCs are partly responsible for the phenotypic and functional intratumor heterogeneity in several human cancers including colorectal carcinoma (37). The presence of distinct cell subpopulations with different levels of tumorigenicity within colorectal tumors was first reported a few years ago (38, 39), and the involvement of CSCs in colorectal tumor progression and recurrence has been documented since in murine and human models (7, 40, 41). Recent studies indicate that functional characteristics of these cells are not solely driven by intrinsic cues but are also under the regulation of signals coming from the microenvironment (6), including from tumor cells themselves (7). The current study highlights the important role of progastrin in this process.

Progastrin overexpression and its proliferative and tumor-promoting effects in colorectal cancer have been well-documented (12, 42, 43) and previous studies linked progastrin production with the expression of CD133, DCAMKL1, or CD44 (17–19), markers that enrich but are not specific for CSCs (20–23). Importantly, these studies did not provide functional data demonstrating a role for progastrin in regulating CSCs hallmarks such as self-renewal. Our study is therefore the first to provide a functional demonstration that the tumor-initiating potential of self-renewing CSCs in advanced colorectal cancer falls under the regulation of progastrin *in vitro* and *in vivo*, including in colorectal cancer cells directly isolated from patient samples.

Our results indicate that colorectal CSCs and, to a lesser degree, other tumor cells can secrete progastrin. In turn, progastrin promotes the self-renewal and tumor-initiating ability of CSCs contained within the ALDH$^{high}$ cell populations. Progastrin secretion may thus form an integral part of the microenvironmental niche that fosters promoting conditions for tumor initiation and self-renewal at stages of the carcinogenic process when stem-like capabilities provide a selective advantage to tumor cells. This hypothesis is corroborated by recent results demonstrating that progastrin overexpression increases the number of Lgr5$^{+}$ cells in an early carcinogenesis mouse model (13) and promotes the proliferation of colonic progenitors by promoting IGF2 secretion by myofibroblasts (44). Similarly, secretion by tumor cells of factors that promote their own proliferation or CSC self-renewal has been reported in the case of IL4 (7) and TGFβ (45), raising interest in these factors as potential therapeutic targets or prognostic biomarkers. Accordingly, in view of the reported role of CSCs during early stages of tumor initiation, the promotion of self-renewal identified here provides a potential rationale for the proposed role of progastrin overexpression as a risk marker of neoplastic transformation in patients with hyperplastic colonic polyps (46).

Homeostatic equilibrium within heterogeneous tumor cell populations involves proliferation and cell death rates of CSCs and non-CSCs, as well as rates of phenotypic transitions between the CSC and non-CSC states (8, 32, 47). Using ALDH activity as a marker to enrich functional CSCs, our results indicate that progastrin promotes ALDH$^{high}$ CSC survival and self-renewal. In contrast, although we found that ALDH$^{neg}$ cells were able to generate ALDH$^{high}$ cells when grown under non-challenging conditions, progastrin did not control this phenotypic plasticity or confer any CSC-like characteristics to ALDH$^{neg}$ cells within the timeframe of our experiments (7–10 days). The lack of detectable effect on ALDH$^{neg}$ cells suggests that these cells are likely to be poorly or not sensitive to progastrin. Alternatively, it is also possible that progastrin induces differential signaling events leading to other biologic functions, unexplored in the current study, in ALDH$^{neg}$ cells.

The receptor mediating these effects of progastrin on CSCs remains to be characterized. Previous studies suggested that the ubiquitous protein Annexin II may represent a receptor for progastrin (48). Although our results do not rule out this possibility, we found that expression of Annexin II was decreased or stable under culture conditions where self-renewal is enhanced, such as in colonosphere assays or in ALDH$^{high}$ subpopulations (Supplementary Fig. S1M and S1N) when compared with controls. In addition, convincing data from Jin and colleagues suggested that progastrin can act at least partly *in vivo* via activation of CCK2 receptors (16). Yet, we were not able to detect CCK2 receptor mRNA in the colorectal cancer cells used to characterize the effects of progastrin in this study (data not shown), suggesting that progastrin may display both direct and indirect effects and/or that the progastrin signaling networks in mice and human are somewhat different.

Finally, we found that progastrin inhibition using siRNA or neutralizing antibodies induces a metabolic shift in ALDH$^{high}$ cells, driving them away from their highly glycolytic profile and reinstating mitochondrial oxidative phosphorylation. This shift was not only concomitant with but also essential for the decrease in their self-renewal ability driven by progastrin inhibition. Several studies suggested that glycolytic pathways are enhanced within CSCs (49, 50), leading to the recently coined concept of "metabostemness" (51). Targeting metabolic pathways to restore the predominance of oxidative phosphorylation may thus be a useful therapeutic strategy against CSCs. Indeed, a recent study (52) convincingly demonstrated that metabolic reprogramming (defined as a shift toward glycolysis and a decrease in mitochondrial numbers) is essential to the self-renewal of nasopharyngeal carcinoma CSCs, and our results demonstrate that similar reprogramming may be controlled in colon CSCs by autocrine/paracrine progastrin secretion.

In summary, this study suggests that inhibiting progastrin decreases the proportion of CSCs within colorectal cancer cell

populations and reduces the tumor-initiating potential of residual CSCs. Considering the enrichment of these cells upon exposure of colorectal cancer cells to chemotherapy and their role in posttreatment recurrence (40, 53), these result suggest that targeting progastrin could provide significant therapeutic benefit to colorectal cancer patients undergoing antiproliferative therapy.

## Disclosure of Potential Conflicts of Interest

F. Hollande reports receiving commercial research grant and was a consultant/advisory board member of Servier Pharma and had ownership interest (including patents) in Biorealites sas. No potential conflicts of interest were disclosed by the other authors.

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