

The Wnt Target Jagged-1 Mediates the Activation of Notch Signaling by Progastrin in Human Colorectal Cancer Cells

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Abstract

The Wnt and Notch signaling pathways are both abnormally activated in colorectal cancer (CRC). We recently showed that progastrin depletion inhibited Wnt signaling and increased goblet cell differentiation of CRC cells. Here, we show that progastrin down-regulation restores the expression by CRC cells of the early secretory lineage marker *Math-1/Hath-1* due to an inhibition of Notch signaling. This effect is mediated by a decreased transcription of the Notch ligand *Jagged-1*, downstream of β -catenin/Tcf-4. Accordingly, recombinant progastrin sequentially activated the transcription of Wnt and Notch target genes in progastrin-depleted cells. In addition, restoration of *Jagged-1* levels in these cells is sufficient to activate Tcf-4 activity, demonstrating the occurrence of a feedback regulation from Notch toward Wnt signaling. These results suggest that progastrin could be instrumental in maintaining the concomitant activation of Wnt and Notch pathways in CRC cells, further highlighting the interest of progastrin targeting for the clinical management of CRC. [Cancer Res 2009;69(15):6065–73]

Introduction

The intestinal epithelium displays a high turnover rate, and a tight balance between proliferation and differentiation is necessary to ensure homeostasis. Notch signaling plays an essential role in controlling this balance, as part of a complex regulatory network with Wnt and other signaling pathways. Abnormal activation of this network is responsible for disrupting tissue homeostasis in diseases, such as colorectal cancer (CRC; refs. 1, 2).

In mammals, four Notch receptors and five Notch ligands (Delta-1, Delta-like 3, Delta-like 4, Jagged-1, and Jagged-2) have been identified. Under physiologic conditions, ligands expressed at the plasma membrane of epithelial cells bind to Notch receptors located at the surface of their immediate neighbors. Binding triggers a cleavage of the Notch transmembrane receptor by γ -secretase, resulting in a nuclear translocation of the Notch intracellular domain. The Notch intracellular domain binds to the constitutive DNA-binding protein CSL/RBP-J/K, converting it from

a repressor to a transcriptional activator (2). Notch signaling is active in the stem/progenitor compartment of intestinal crypts, playing a role in stem cell maintenance, in cooperation with the Wnt pathway, and controlling cell fate decisions between the absorptive and secretory lineages during differentiation (1). Recent data showed that Notch signaling is also strongly activated in primary human CRC (3), as well as in the intestinal adenomas of APC^{Min/+} mice (4), suggesting that its activity is probably regulated by the Wnt pathway and that Notch pathway inhibitors may provide new treatment avenues for intestinal neoplasia (5).

Previous results from our and other laboratories showed that progastrin, a gastrin precursor secreted by a large proportion of colorectal tumors, is a potent activator of signaling pathways promoting cancer progression (6–8). The gene encoding progastrin is a target of the β -catenin/Tcf-4 pathway (9), and we recently showed that inhibition of endogenous progastrin synthesis in CRC cells down-regulated Tcf-4 activity, reduced tumor growth, and increased the proportion of goblet cells within the residual intestinal adenomas of mice carrying a heterozygous *Apc* mutation (10). Whereas this latter phenotype differs from that observed upon constitutive inactivation of the Wnt pathway, which drives cells toward the enterocyte lineage (11), a similar induction of goblet cell differentiation was recently observed by van Es and colleagues after Notch pathway inhibition through the conditional removal of CSL/RBP-J or treatment of APC^{Min/+} mice with a γ -secretase inhibitor (4). Therefore, we asked whether Muc-2 reexpression in progastrin-depleted CRC cells could be induced by a modulation of the Notch pathway and dissected the molecular events underlying the coordinated activation of the Wnt and Notch cascades by progastrin in CRC cells.

Materials and Methods

Materials and cell culture conditions. The polyclonal Hes-1 antibody was kindly provided by Dr. T. Sudo. The polyclonal Math-1 antibody was obtained from the Developmental Studies Hybridoma Bank (National Institute of Child Health and Human Resources/University of Iowa). LY411,575 was a gift from Dr. P.C. May (Lilly). SW480 cells were maintained as described in ref. (10).

Tissue collection, microdissection, and RNA preparation. Specimens of 26 colon tumors and matching healthy epithelium were obtained according to French government regulations and with ethical committee approval, frozen, and prepared as described (10).

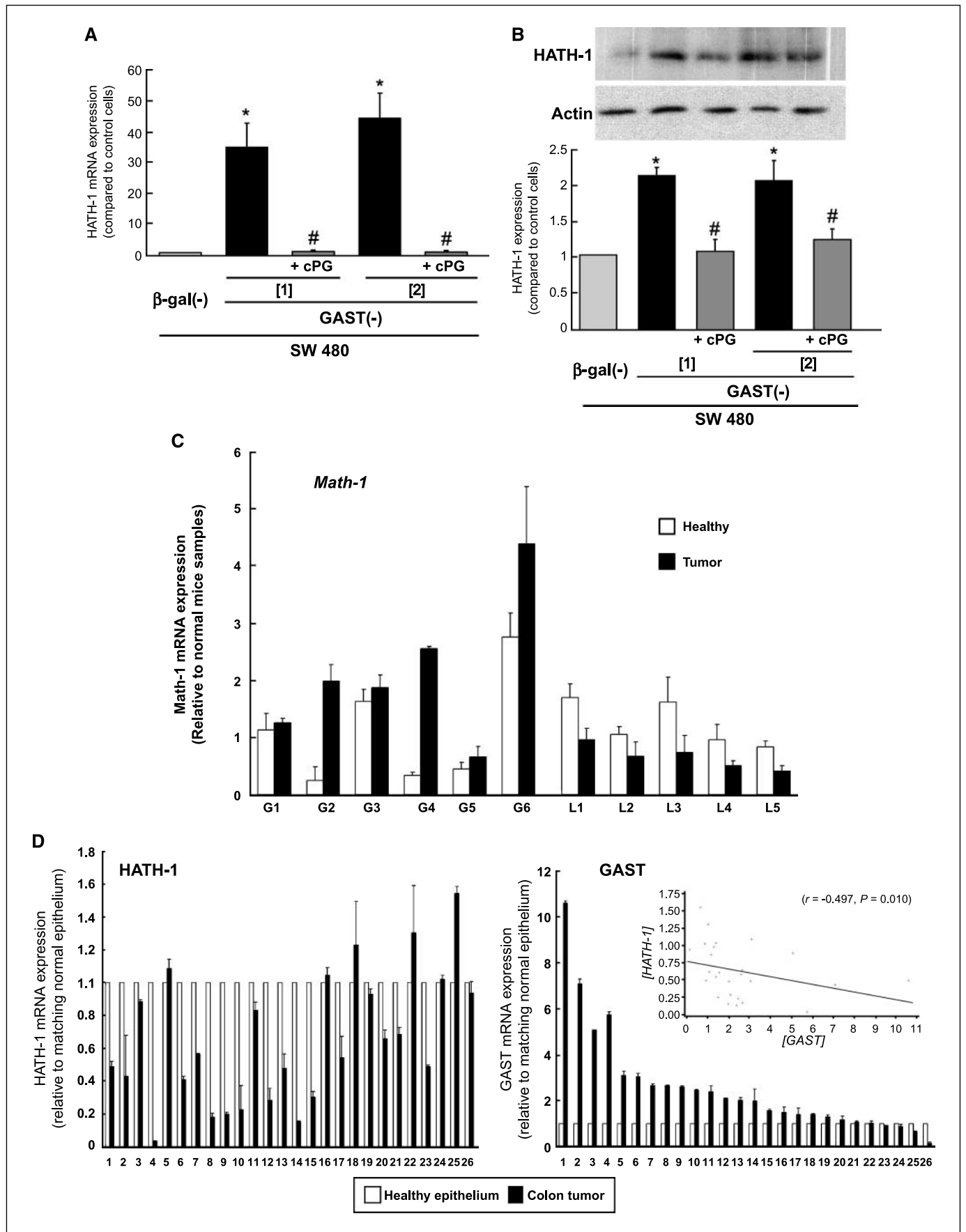
Quantitative reverse transcription-PCR. Total RNA (1 μ g) was pretreated with DNase and used for reverse transcription with M-MLV reverse transcriptase (Invitrogen). Primer sequences and quantitative PCR conditions are provided in Supplementary Table S1.

Protein lysates, SDS-PAGE, and Western immunoblotting. Protein lysates and immunoblotting were performed as described (6). Band intensities were quantified using ImageJ 1.32J (NIH).

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

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Immunofluorescent staining. Immunofluorescent staining was performed as described previously (6). Slides were observed using a laser scanning LEICA sp2 confocal microscope with a $\times 40$ oil immersion lens.

Transient transfections and luciferase reporter gene assays. The day before transfection, 5×10^4 SW480 cells per well were seeded in 24-well plates. Cells were transfected with Wnt or Notch reporter plasmid (250 ng) and 25 ng of *Renilla* luciferase, using 5 μ L/well Exgen 500 (Euromedex). At 48 h after transfection, luciferase activity was quantified using the dual-luciferase reporter assay system (Promega) and normalized relative to *Renilla* activity.

Statistical analyses. Statistical analyses were performed using SAS 9.1 for Windows. The Student's *t* test was used for *in vitro* experiments. The Spearman rank test was used to determine the correlation between the nonnormally distributed *GAST*, *MATH-1*, and *JAGGED-1* gene expressions in human tumors.

Results

Expression of the secretory lineage marker Math-1/Hath-1 is up-regulated after progastrin depletion in CRC cells. We recently described that inhibiting progastrin expression promoted terminal differentiation of intestinal tumor cells into goblet cells *in vitro* and *in vivo* (10), similar to the effect obtained after inhibition of the Notch signaling pathway *in vivo* (4, 12). To determine whether the detected increase in goblet cell numbers after progastrin down-regulation in CRC cells reflects an early-lineage differentiation choice, we quantified the expression of Math-1/Hath-1, a bHLH transcription factor considered as an early marker of secretory lineages in the intestine and known to be repressed by Notch signaling (2). Expression of Hath-1 (human atonal homologue-1) mRNA and protein was strongly increased in SW480 CRC cells, wherein endogenous progastrin expression was switched off using a short hairpin RNA (shRNA) directed against the *GAST* gene [SW480/*GAST*⁽⁻⁾; ref. 10], when compared with control cells expressing a β -galactosidase-specific shRNA [SW480/ β -gal⁽⁻⁾; Fig. 1A and B]. This increase was reversed when SW480/*GAST*⁽⁻⁾ cells were transfected with a codon-optimized, shRNA-insensitive, preprogastrin construct [SW480/*GAST*⁽⁻⁾ + cPG]. Corroborating this result, *HATH-1* mRNA and protein expressions were also up-regulated after transient progastrin down-regulation in HCT-116 and DLD-1 cells (Supplementary Fig. S1 online), two cell lines previously shown to secrete progastrin (6, 13), showing that the progastrin-mediated regulation of Hath-1 expression is consistent across several CRC cell lines. Similar to what we previously showed in SW480 cells (10), increased Hath-1 expression was correlated with an increased expression of the goblet cell-specific marker Muc-2 in HCT-116 and, to a lesser degree, DLD-1 cells (Supplementary Fig. S1 online), suggesting that these cells have retained a potential to differentiate when endogenous progastrin is down-regulated.

To verify these results *in vivo*, we quantified *Math-1* (mouse atonal homologue-1) mRNA expression in the intestinal adenomas

of APC Δ 14 mice (14), injected daily with small interfering RNA (siRNA) directed against either the progastrin-encoding gene (*GAST*) or the luciferase gene (*LUC*) for 15 days. As previously described, treatment of these mice with the *GAST*-selective siRNA led to an important decrease of tumor sizes and numbers when compared with controls and with a strong differentiation of the remaining adenomas along the goblet cell lineage, as shown by a strong increase in Muc-2 expression (10). Here, *Math-1* expression levels were similar in the healthy intestinal epithelium of APC Δ 14/*LUC* mice to those detected in wild-type animals and were strongly decreased in most intestinal adenomas of these mice (Fig. 1C) in line with their poor differentiation level. In contrast, *Math-1* expression was strongly increased in most residual adenomas of *GAST* siRNA-treated mice (Fig. 1C) in line with their previously described Muc-2 expression increase. No strict correlation was found between *Math-1* levels in the healthy epithelium and the adenomas after *GAST* siRNA treatment. Similar to SW480, HCT-116, and, to a lesser degree, DLD-1 cells, intestinal adenomas of these mice were previously shown to secrete progastrin but very little of the smaller progastrin-derived peptides (6, 10, 13), strongly suggesting that the observed effects are indeed due to the full-length prohormonal peptide.

Finally, to assess whether the observed regulation of *Math-1*/*Hath-1* levels by progastrin is reflected in human CRC *in situ*, *HATH-1* mRNA expression was analyzed in microdissected colorectal tumors and matching healthy samples from 26 patients. Results were analyzed in correlation with *GAST* gene expression in these samples, 21 of which having previously been published (10).

We observed that *HATH-1* mRNA expression was particularly repressed in those tumors previously shown to overexpress progastrin (with the exception of patients 3 and 5) but was higher in those displaying lower progastrin levels. A significant statistical correlation was found between increased *GAST* gene expression and repression of *HATH-1* (Spearman's correlation coefficient: $r = -0.497$, $P = 0.010$), corroborating the relevance of the progastrin-induced repression of *Math-1*/*Hath-1* *in vivo* (Fig. 1D).

Taken together, these results show that the effect of progastrin depletion on goblet cell differentiation in CRC cells is likely to reflect an ability to modulate early lineage choice and suggest that it may do so by regulating the Notch signaling pathway.

Progastrin depletion inhibits Notch transcriptional activity.

To assess whether progastrin depletion in CRC cells could decrease Notch transcriptional activity, we transiently transfected SW480/ β gal⁽⁻⁾, SW480/*GAST*⁽⁻⁾, and SW480/*GAST*⁽⁻⁾ + cPG cells with a reporter construct containing 12 RBP-JK binding sites upstream of the luciferase gene (15). Figure 2A shows that Notch transcriptional activity was significantly lower in SW480/*GAST*⁽⁻⁾ cells compared with SW480/ β gal⁽⁻⁾ cells and that this inhibition was reversed when progastrin expression was restored in these cells [SW480/*GAST*⁽⁻⁾ + cPG]. In agreement with this result, the mRNA (Fig. 2B)

Figure 1. Progastrin represses MATH-1/HATH-1 expression in colorectal tumors. Quantification of *HATH-1* mRNA (A) and protein (B) expression was performed on SW480 cells stably expressing β -galactosidase-specific [SW480/ β gal⁽⁻⁾; light gray columns] or *GAST* gene-specific [SW480/*GAST*⁽⁻⁾] shRNA (black columns), transfected or not with a shRNA-insensitive preprogastrin cDNA (cPG, dark gray columns). Results are expressed as fold induction of expression levels found in SW480/ β gal⁽⁻⁾ cells [* $P < 0.05$ compared with SW480/ β gal⁽⁻⁾; # $P < 0.05$ when compared with SW480/*GAST*⁽⁻⁾; Student's *t* test, $n = 3$] using reverse-transcription-quantitative PCR and Western blotting, respectively. C, 3-mo-old APC Δ 14 mice were treated for 2 wk with siRNA targeting either the murine *GAST* (G) or the luciferase (L) gene, and *Math-1* mRNA levels were quantified in samples collected from healthy epithelium or adenomas, as described in ref. 10. *Math-1* mRNA expressions in healthy (\square) and tumor (\blacksquare) mucosa are expressed relative to that quantified in the intestine of wild-type C57BL6 mice ($n = 6$ mice for *GAST* siRNA-treated mice, $n = 5$ for controls). D, *GAST* and *HATH-1* mRNA expressions were quantified using reverse transcription-quantitative PCR in microdissected tumors (\blacksquare) from 26 patients with CRC, relative to the amounts found in their respective matching healthy epithelium (\square), which were normalized to 1 for each patient. Columns, means of three independent experiments; bars, SE. Insert, values of *GAST* and *HATH-1* gene expression were plotted against each other, and the regression curve is shown. Spearman's correlation coefficient (r) is provided with its degree of significance (P).

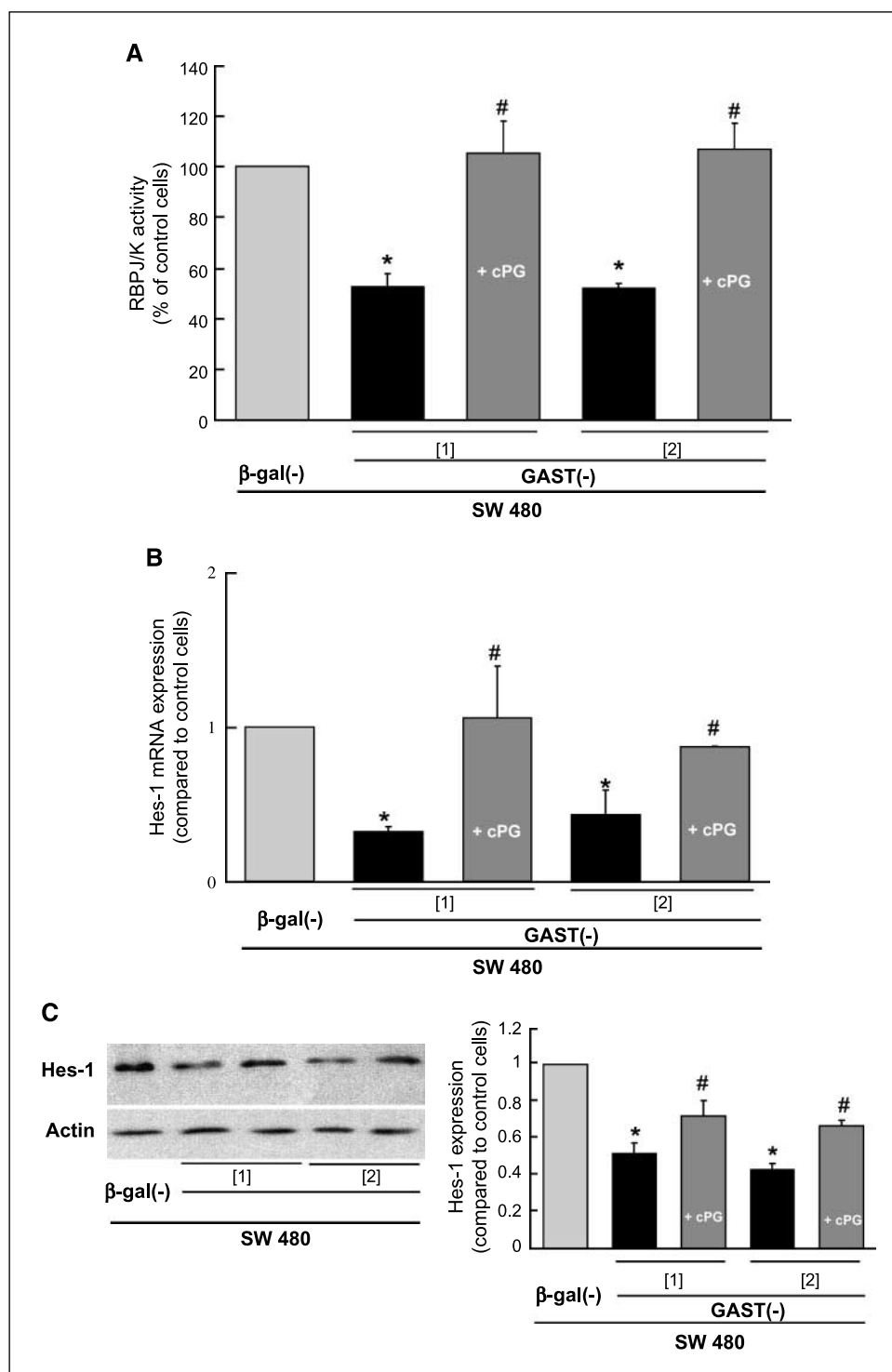


Figure 2. Notch pathway inhibition in human CRC cells after depletion of endogenous progastrin. **A**, Notch transcriptional activity was quantified using the RBPJ/K luciferase reporter gene assay in SW480/ β gal⁽⁻⁾ (light gray columns) and SW480/GAST⁽⁻⁾ (black columns), transfected or not with a shRNA-insensitive preprogastrin cDNA (cPG, dark gray columns). Results are expressed as a percentage of expression levels found in SW480/ β gal⁽⁻⁾ cells [**P* < 0.05 compared with SW480/ β gal⁽⁻⁾; #, *P* < 0.05 when compared with SW480/GAST⁽⁻⁾; *n* = 3]. **B** and **C**, mRNA (**B**) and protein (**C**) expression of the Notch target gene *HES-1* was quantified in SW480/ β gal⁽⁻⁾ and SW480/GAST⁽⁻⁾ cells (clones [1] and [2]; black columns) or in the same cells transfected with an shRNA-insensitive preprogastrin cDNA (cPG, dark gray columns) using reverse transcription-quantitative PCR and Western blotting, respectively. Results are expressed as fold induction of levels found in SW480/ β gal⁽⁻⁾ [light gray columns; **P* < 0.05 compared with SW480/ β gal⁽⁻⁾ cells; #, *P* < 0.05 when compared with SW480/GAST⁽⁻⁾ cells; Student's *t* test, *n* = 3] for mRNA and protein expression, respectively.

and protein (Fig. 2C) expression of Hes-1, one of the best-defined Notch target genes, was significantly lower after progastrin depletion in SW480 cells but was restored in SW480/GAS⁽⁻⁾ + cPG cells.

Notch pathway inhibition by progastrin depletion is mediated by the down-regulation of Jagged-1. To determine whether a transcriptional regulation of Notch ligands or receptors was involved in the modulation of Notch transcriptional activity

by progastrin, we first measured the mRNA expression of those isoforms expressed in the colon (Jagged-1, Jagged-2, Delta-1, Notch-1, and Notch-3 receptors) before and after progastrin depletion in SW480 CRC cells. Progastrin depletion significantly inhibited Jagged-1 expression, whereas leaving mRNA expression of Notch-1, Notch-3, Delta-1, and Jagged-2 unchanged (Fig. 3A). Jagged-1 was also significantly reduced after siRNA-mediated, transient *GAST* gene down-regulation in HCT116 and DLD-1 cells

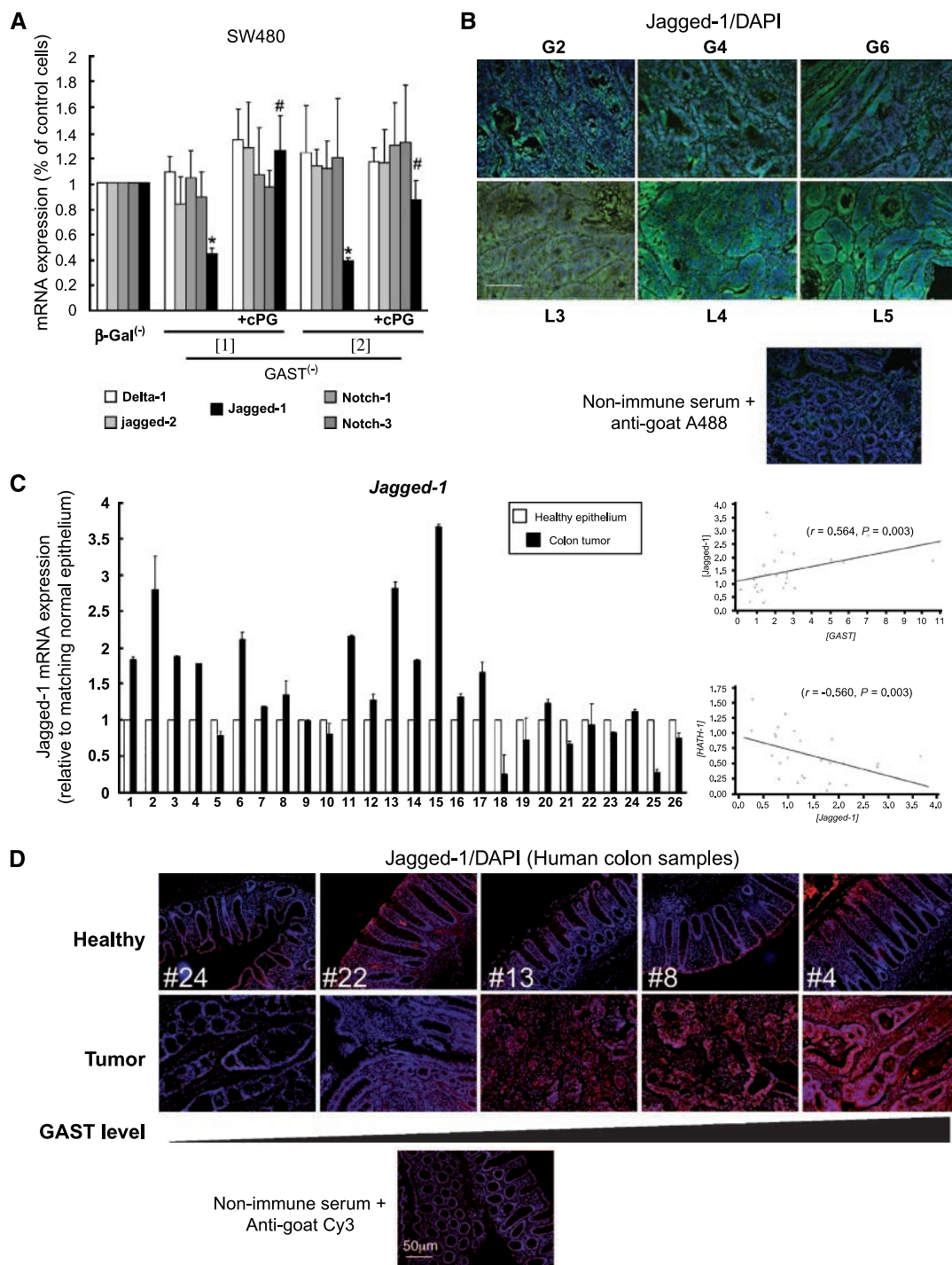


Figure 3. Progastrin enhances Jagged-1 expression in intestinal tumor cells *in vitro* and *in vivo*. **A**, expression of Notch ligands and receptors present in the colon (Delta-1, Jagged-2, Notch-3, and Jagged-1) was quantified using reverse transcription-quantitative PCR on SW480/ β gal⁻ and SW480GAST⁻ cells, with or without reexpression of an shRNA-insensitive preprogastrin cDNA (cPG). Results are expressed as fold induction of levels found in SW480/ β gal⁻ [**P* < 0.05 compared with SW480/ β gal⁻; #, *P* < 0.05 compared with SW480/GAST⁻ cells; Student's *t* test, *n* = 3]. **B**, 3-mo-old APC Δ 14 mice were treated for 2 wk with siRNA targeting either the murine *GAST* gene or the luciferase gene, as in Fig. 1. Intestinal adenomas were paraffin-embedded and processed for Jagged-1 (Alexa 488, green) and 4',6-diamidino-2-phenylindole (DAPI, blue) immunofluorescent staining. Representative images are provided from GAST⁻ (mice G2, G4, and G6) and Luc⁻ (mice L3–L5) adenomas collected in the ileum. Bar, 100 μ m. Additional higher magnification images are provided in Supplementary Fig. S3. **C**, Jagged-1 mRNA expression was quantified using reverse transcription-quantitative PCR in microdissected tumors (■) from 26 patients with CRC, relative to the amounts found in their respective matching healthy epithelium (□), which were normalized to 1 for each patient. Columns, means of three independent experiments; bars, SE. Insert, values of *GAST* and *Jagged-1* gene expression and *HATH-1* and *Jagged-1* gene expression were plotted against each other, and the regression curves are shown. Spearman's correlation coefficients (*r*) are provided with their degree of significance (*P*). **D**, representative immunofluorescent staining for Jagged-1 (Cy3, red) and DAPI (blue) on colorectal tumor samples from patients with increasing progastrin expression. Patient numbers correspond to those shown in Fig. 1. Bar, 50 μ m. Additional higher magnification images are provided in Supplementary Fig. S3.

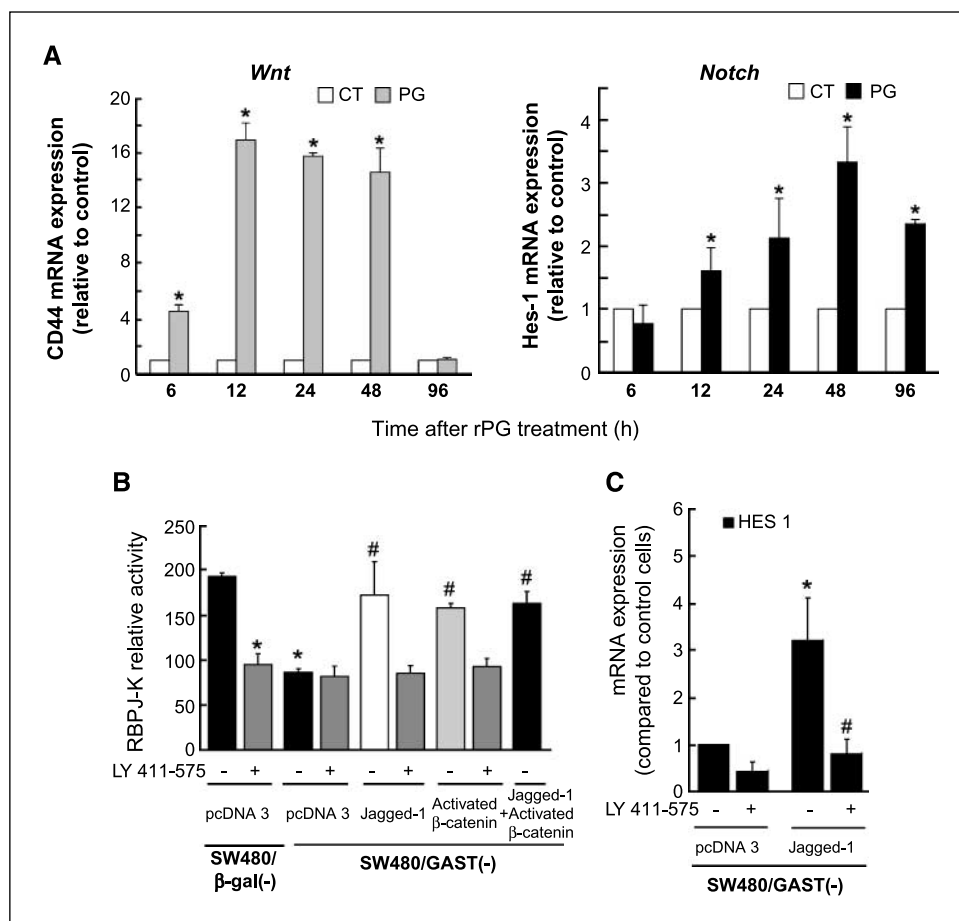


Figure 4. Sequential activation of β -catenin/Tcf-4 and Notch signaling by progastrin in CRC cells. **A**, activation of the Tcf-4 transcriptional program by progastrin precedes that of Notch signaling. Expression of mRNAs for *CD44* and *HES-1* was quantified on SW480/GAST⁽⁻⁾ cells treated (black columns) or not (white columns) with 10 nmol/L progastrin for the indicated time. *, $P < 0.05$ relative to untreated SW480/GAST⁽⁻⁾ cells; $n = 3$. **B**, Notch transcriptional activity was quantified using the RBPJ/K luciferase reporter gene assay in SW480/ β gal⁽⁺⁾ (black columns) or SW480/GAST⁽⁻⁾ cells transfected with either pcDNA3 (black columns), Jagged-1 (white columns), activated β -catenin (light gray columns), or Jagged-1 and activated β -catenin (black columns) and then treated or not with the γ -secretase inhibitor LY 411-575 (dark gray columns). *, $P < 0.05$ compared with SW480/ β gal⁽⁻⁾ cells; #, $P < 0.05$ compared with SW480/GAST⁽⁻⁾ cells transfected with pcDNA3; $n = 3$. **C**, expression of mRNAs for *HES-1* was quantified on SW480/GAST⁽⁻⁾ cells transfected or not with Jagged-1 and treated or not with the γ -secretase inhibitor LY 411-575 as indicated. *, $P < 0.05$ compared with SW480/GAST⁽⁻⁾ cells transfected with pcDNA3; #, $P < 0.05$ compared with SW480/GAST⁽⁻⁾ cells transfected with Jagged-1; $n = 3$.

(Supplementary Fig. S2), and its expression was restored after reexpression of a codon-optimized, shRNA-insensitive progastrin construct in SW480 GAST⁽⁻⁾ cells (Fig. 3A). In addition, Jagged-1 up-regulation was detected after transfection of an activated β -catenin mutant ($\Delta 87\beta$ cat) or after expression of a shRNA directed against the β -catenin/Tcf-4 inhibitor ICAT in progastrin-depleted SW480 GAST⁽⁻⁾ cells (Supplementary Fig. S2), confirming that Jagged-1 was indeed under the control of the Wnt pathway downstream of progastrin in CRC cells.

In vivo we then found that Jagged-1 immunoreactivity was strongly reduced in tumor sections from APC $\Delta 14$ mice treated with siRNA directed against the progastrin-encoding gene (*GAST*) when compared with those treated with siRNA directed against the luciferase gene (*LUC*; Fig. 3B; Supplementary Fig. S3). In accordance with this result, Jagged-1 mRNA (Fig. 3C) and protein (Fig. 3D; Supplementary Fig. S3) were highly expressed in human colorectal tumors expressing high progastrin levels, whereas Jagged-1 expressed was weaker in tumors with low progastrin levels. Weak Jagged-1 staining was detected in colonic crypts within the macroscopically healthy mucosa of these patients, irrespective of levels found in the matching tumor sample. Staining was found throughout the crypt and was marginally higher in the brush border area in the healthy epithelium, whereas membrane and/or cytoplasmic staining was prominent in tumor samples displaying elevated levels (Supplementary Fig. S3). Elevated *Jagged-1* mRNA expression in 26 microdissected human colorectal tumor samples was statistically correlated with increased *GAST* gene expression

(Spearman's correlation coefficient: $r = 0.564$, $P = 0.003$) and with *HATH-1* down-regulation ($r = -0.560$, $P = 0.003$; Fig. 3D), strongly suggesting that the expression of these genes is not independent in CRC.

These results suggest that activation of the β -catenin/Tcf-4 complex by progastrin is involved in the up-regulation of Jagged-1 detected in CRC cells and show that progastrin down-regulation in these cells is sufficient to significantly reduce Jagged-1 expression.

Crosstalk between β -catenin/Tcf-4 and Notch pathways is modulated by progastrin in colorectal tumor cells. Because the data presented above suggested that regulation of the Wnt pathway could be instrumental for the modulation of Notch by progastrin, we analyzed the time course of Wnt and Notch pathway reactivation by recombinant progastrin in SW480 GAST⁽⁻⁾ cells. In agreement with results presented above, we found that the expression of Wnt target genes, such as CD44, preceded that of Notch-specific target genes like Hes-1 (Fig. 4A). Indeed, transcriptional activation of Wnt target genes such as CD44 was significantly increased by 6 hours of treatment, reaching a maximum after 12 hours and returning to control levels by 96 hours. In contrast, increased expression of the Notch target gene Hes-1 was only detectable from 12 hours after treatment and was maximal at 48 hours. To directly assess whether the detected down-regulation of Jagged-1 expression was indeed responsible for the inhibition of Notch signaling in progastrin-depleted cells, we also analyzed the transcriptional activity of RBPJ/K (Fig. 4B), as well as Hes-1 mRNA expression (Fig. 4C), after restoration of Jagged-1 expression in

SW480 GAST⁽⁻⁾ cells. Jagged-1 reexpression in progastrin-depleted cells strongly increased Notch transcriptional activity and Hes-1 mRNA expression, and both effects were reversed by the γ -secretase inhibitor LY-411-575, confirming that Jagged-1 acts by activating the canonical Notch pathway in these cells. In addition, RBPJ/K transcriptional activity was also activated after transfection of SW480 GAST⁽⁻⁾ cells with the activated β -catenin mutant, and cotransfection of Δ 87 β cat with Jagged-1 did not show any additive effect, confirming that Jagged-1 mediates the Wnt-mediated activation of Notch signaling in these cells (Fig. 4B).

Next, we wanted to establish whether the progastrin-mediated modulation of Jagged-1 expression and Notch signaling could, in turn, affect β -catenin/Tcf-4 transcriptional activity. Indeed, restoration of Jagged-1 expression in progastrin-depleted cells significantly increased the transcriptional activity of Tcf-4 (Fig. 5A), as well as the expression of the Tcf-4 target genes CD44, MYC, CCND1, and SOX9 (Fig. 5B; Supplementary Fig. S4).

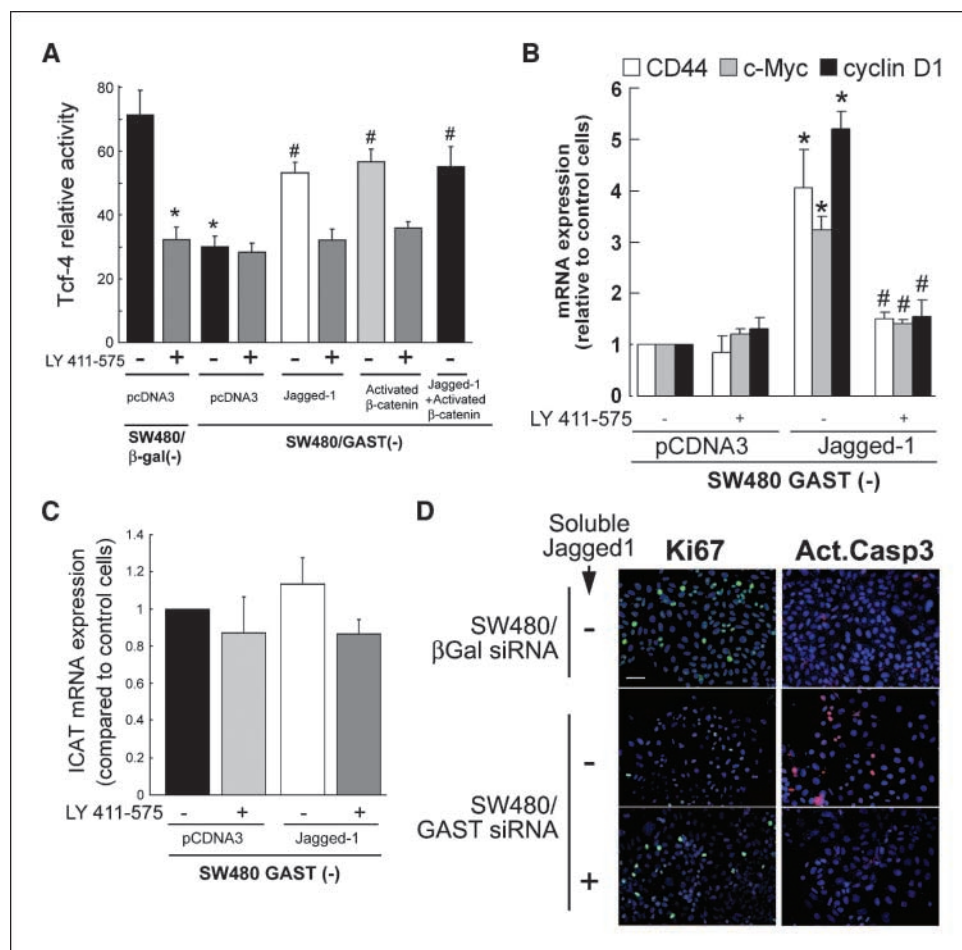
In addition, we found that these rescuing effects of Jagged-1 restoration in progastrin-depleted cells were prevented when cells were preincubated with the γ -secretase inhibitor LY-411-575, showing that they rely on the activation of canonical Notch signaling (Fig. 5A). Treatment with this inhibitor also decreased Tcf-4 activity in control SW480/ β Gal⁽⁻⁾ cells, indicating that Notch signaling promotes Tcf-4 activity in these cells. As expected, Tcf-4 activity was stimulated by transfection with the activated β -catenin mutant described above, and no additive effect was

found after cotransfection of the Jagged-1 and Δ 87 β cat constructs (Fig. 5A).

We previously showed that *de novo* expression of the β -catenin/Tcf-4 inhibitor ICAT was responsible for the inhibition of β -catenin/Tcf-4 transcriptional activity after progastrin depletion (10). Here, we found that the expression of the Notch target gene Hes-1 was elevated in progastrin-depleted cells where ICAT up-regulation was prevented by expression of a selective ICAT shRNA (Supplementary Fig. S2), confirming that the regulation of Tcf-4 activity induced by progastrin-mediated ICAT regulation also extends to a modulation of Notch signaling. In contrast, no significant modifications in the level of ICAT mRNAs were detected after transfection of Jagged-1 into SW480/GAST⁽⁻⁾ cells (Fig. 5C), suggesting that ICAT is not involved in the feedback modulation of Tcf-4 activity by Notch signaling detected here. Our data also shows that restoration of Jagged-1 levels in progastrin-depleted cells did not affect the expression and localization of Tcf-4 and β -catenin (not shown) or the expression of the Tcf-4 inhibitors TLE(1-5) (not shown).

Finally, to assess the potential role of progastrin-regulated Notch signaling on tumor cell proliferation and apoptosis, we respectively analyzed the expression of Ki67 and activated caspase-3 in SW480 cells transfected or not with β Gal or GAST-selective siRNA and treated or not with soluble recombinant Jagged-1 (Fig. 5D). In agreement with the effect of the Notch pathway on differentiation, incubation with soluble Jagged-1 was found to reverse the effects of

Figure 5. Crosstalk between APC/ β -catenin/Tcf-4 and Notch in progastrin-depleted cells. **A**, Tcf-4 transcriptional activity was quantified using the TOP/FOP luciferase reporter gene assay in SW480/ β gal⁽⁻⁾ (black columns) or SW480/GAST⁽⁻⁾ cells transfected with either pcDNA3 (black columns), Jagged-1 (white columns), activated β -catenin (light gray columns), or Jagged-1 and activated β -catenin (black columns) and then treated or not with the γ -secretase inhibitor LY 411-575 (dark gray columns). *, $P < 0.05$ compared with SW480/ β gal⁽⁻⁾ cells; #, $P < 0.05$ when compared with SW480/GAST⁽⁻⁾ cells transfected with pcDNA3; $n = 3$. **B**, expression of mRNAs for CD44, c-Myc, and cyclin D1 was quantified on SW480/GAST⁽⁻⁾ cells transfected or not with Jagged-1 and treated or not with the γ -secretase inhibitor LY 411-575 as indicated. *, $P < 0.05$ compared with SW480/GAST⁽⁻⁾ cells transfected with pcDNA3; #, $P < 0.05$ compared with SW480/GAST⁽⁻⁾ cells transfected with Jagged-1; $n = 3$. **C**, expression of ICAT mRNA was quantified on SW480/GAST⁽⁻⁾ cells transfected (white columns) or not (black columns) with Jagged-1 and treated or not (light and dark gray columns) with the γ -secretase inhibitor LY 411-575 as indicated. *, $P < 0.05$ compared with SW480/GAST⁽⁻⁾ cells transfected with pcDNA3; #, $P < 0.05$ compared with SW480/GAST⁽⁻⁾ cells transfected with Jagged-1; $n = 3$. **D**, immunofluorescent staining for the proliferation marker Ki67 and for the apoptosis marker-activated caspase-3 was performed on SW480 cells, transiently transfected with β gal⁽⁻⁾ or GAST-selective siRNA oligonucleotides, and treated or not with 1 μ g/mL soluble Jagged-1 recombinant peptide for 48 h, as indicated. Bar, 50 μ m.



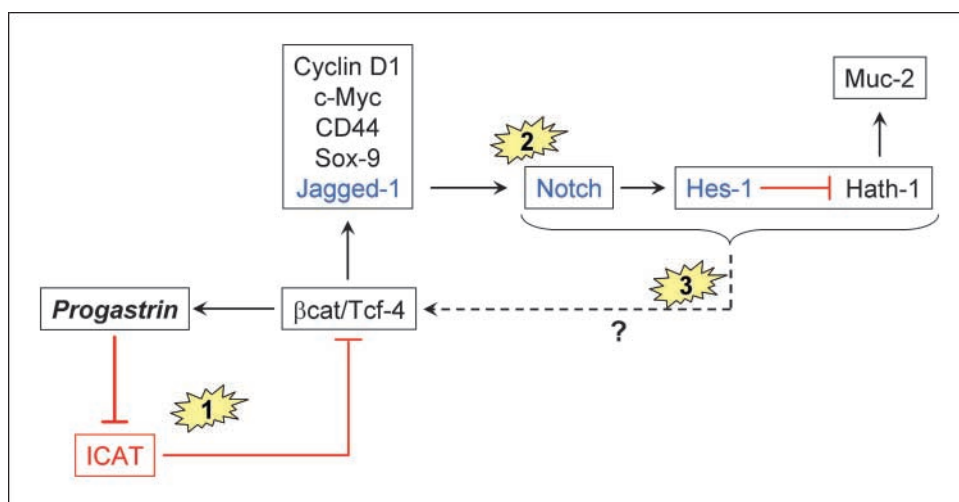


Figure 6. Schematic representation of progastrin-driven Wnt and Notch signaling in CRC cells. Progastrin stimulates Wnt signaling by repressing the endogenous inhibitor ICAT (⊖), leading to enhanced transcription of Tcf-4 target genes. Among those, increased expression of Jagged-1 promotes Notch signaling and represses the expression of the secretory lineage (⊖). In turn, increased Notch signaling seems necessary to maintain elevated β -catenin/Tcf-4 activity via an as yet unknown mechanism (⊖).

progastrin down-regulation on apoptosis activation. In addition, soluble Jagged-1 partially rescued the proliferative potential of SW480 cells where progastrin had been down-regulated. However, it is unclear at this stage whether this result is directly related to a role of the Notch pathway on proliferation or if it is due to the positive feedback on Wnt activity described above.

Taken together, these results indicate that, downstream from progastrin, β -catenin/Tcf-4 activity can stimulate Notch signaling via the modulation of Jagged-1 and that this activation of Notch is itself involved in maintaining a positive feedback on Wnt signaling via an as yet unknown mechanism. Activation of both pathways by progastrin seems essential to drive the protumorigenic effects of this peptide by disrupting the balance between proliferation and differentiation/apoptosis.

Discussion

In the present work, we have established that progastrin regulates the Notch pathway via the transcriptional modulation of Jagged-1 downstream of β -catenin/Tcf-4, that the Wnt and Notch pathways are sequentially activated by progastrin, that Notch and Wnt influence each other's activity, and that progastrin is a major regulator of this crosstalk in CRC cells. An insight into this regulatory network is provided in Fig. 6.

First, we showed that progastrin depletion promotes goblet cell differentiation via a modulation of early lineage determination, as evidenced by the up-regulation of the early secretory lineage marker Math-1. We also show that increased *MATH-1* expression resulted from a down-regulation of the canonical Notch pathway and of its target gene *HES-1*, which are known repressors of *MATH-1* expression (2). Whereas estrogens have been shown to increase Notch-1 and Jagged-1 expression in MCF-7 cells (16), this is, to our knowledge, the first report of Notch pathway regulation by a secreted factor in CRC cells. The mechanism involved in progastrin-mediated Notch activity regulation by progastrin involves a transcriptional modulation of Jagged-1 expression. This result implies that the regulation of Notch activity in CRC extends beyond the simple ability of neighboring cells to communicate via direct Notch ligand/receptor interaction and that progastrin-mediated signaling could provide some degree of tumor-wide coordination to Notch pathway activity, affecting all progastrin-sensitive cells within a tumor. It should be noted here that other

shorter gastrin forms resulting from the posttranslational maturation of progastrin could also play a part in the regulation of Wnt and Notch signaling *in vivo*. However, because full-length progastrin is the predominant *GAST* gene-derived peptide secreted by SW480 cells and intestinal adenomas of APC Δ 14 mice (10), our results clearly show that this prohormonal peptide is a likely regulator of these pathways in human colorectal tumors, where it is strongly overexpressed (13, 17).

In a previous study, we showed that progastrin was capable of promoting Wnt signaling via the repression of its endogenous inhibitor ICAT (10). Because we found in the present work that Jagged-1 was instrumental for Notch activation by progastrin and because Jagged-1 was recently described as a β -catenin/Tcf-4 target gene in hair follicles (18), it was of particular importance to decipher the kinetics of activation of the Wnt and Notch pathways in CRC cells in the presence of progastrin. We unraveled that the activation of Tcf-4 transcriptional targets precedes by several hours that of the Notch target gene *Hes-1* after stimulation of SW480/*GAST*⁽⁻⁾ cells with recombinant progastrin, indicating that Notch regulation by progastrin occurs downstream of its effect on the Wnt pathway. In agreement with these results, Jagged-1 and *Hes-1* expression were significantly increased when the β -catenin/Tcf-4 inhibition normally mediated by ICAT upon progastrin down-regulation was blocked using *ICAT*-selective siRNAs.

The decreased Notch activity induced by progastrin down-regulation suggests that chronic progastrin secretion is involved in sustaining high levels of Notch activity in tumor cells. Because Notch signaling has also been implicated in the regulation of proliferation in colon adenocarcinomas (19), it is conceivable that activation of this pathway may play a part in the shown proliferative effects of progastrin in CRC cells and mice tumor models (7, 8, 10). This hypothesis is corroborated by our results showing that the decreased proliferation detected after transfection of SW480 cells with *GAST* gene-selective siRNA was partially restored by incubation with a soluble recombinant Jagged-1 peptide. Whether this phenotypic rescue is due to a direct effect of the Notch pathway or related to the positive feedback of Notch on Wnt activity is currently unknown, but the present study nevertheless indicates that this mechanism of coordinated Wnt and Notch signaling activation by progastrin plays a significant role in promoting proliferation while slowing down the differentiation/apoptosis cascade.

The present study also brings the first experimental confirmation that Jagged-1 is regulated by Wnt in CRC cells, which was previously suggested through an *in silico* approach (20). Interestingly, Jagged-1 was shown to promote the maintenance of immature hematopoietic stem/progenitor cells (21) and induce epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin in breast cancer cells (22). These biological functions of Jagged-1 could provide a partial explanation for the shown ability of progastrin to promote tumor invasion and maintain dedifferentiation within tumor cells (6). In addition, we found that restoration of Jagged-1 expression in progastrin-depleted cells not only reinstated Notch signaling but was also capable of stimulating β -catenin/Tcf-4 activity in CRC cells. This effect was inhibited when cells were pretreated with a γ -secretase inhibitor, strongly suggesting that it reflected the ability of canonical Notch signaling to promote β -catenin/Tcf-4 activity. Because van Es and colleagues showed that γ -secretase inhibitor treatment signaling did not decrease the nuclear localization of β -catenin in APC^{Min/+} mice adenomas (4), it is possible that Notch signaling modulates the Wnt pathway by affecting the capacity of β -catenin/Tcf-4 to bind to the promoter of target genes or to efficiently mediate transcription once bound. Yet, our results showed that the stimulation of Wnt signaling downstream of Jagged-1-induced Notch activation was not due to a decreased expression of the endogenous inhibitors TLE(1–5) (data not shown) or of ICAT, and further studies will be necessary to unravel the molecular mechanism underlying this effect.

Although previous results obtained in other models suggested that Notch activation represses Wnt signaling (23), the Wnt and Notch pathways are jointly activated in neural and intestinal stem cells (1), mouse intestinal adenomas (4), and human CRCs (3). Because progastrin was able to enhance the transcriptional activity of APC/ β -catenin/Tcf-4 in CRC cells (10), we speculate that

progastrin could promote tumor expansion by maintaining high levels of Wnt and Notch activities and that blocking the activity of progastrin could provide an efficient way to down-regulate their activity toward a more physiologic level, thereby promoting differentiation and homeostasis. The data presented here suggest that progastrin could be instrumental to maintain this concomitant activation in CRC cells, thus further highlighting the interest of progastrin-targeting approaches for the clinical management of this disease.

Note Added in Proof

Since the present article was accepted, another study (24) also demonstrated experimentally that the link between Wnt and Notch in colon cancer cells goes through Jagged-1.

Disclosure of Potential Conflicts of Interest

J. Pannequin, N. Delaunay, J-F. Bourgaux, D. Joubert, and F. Hollande are co-authors on a patent application that relates to other aspects of progastrin biology. D. Joubert and F. Hollande: Expert testimony. The other authors disclosed no potential conflicts of interest.

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