R-Spondin1/LGR5 Activates TGFβ Signaling and Suppresses Colon Cancer Metastasis

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Abstract

Leucine-rich repeat containing G-protein–coupled receptor 5 (LGR5), an intestinal stem cell marker, is known to exhibit tumor suppressor activity in colon cancer, the mechanism of which is not understood. Here we show that R-spondin 1 (RSPO1)/LGR5 directly activates TGFβ signaling cooperatively with TGFβ type II receptor in colon cancer cells, enhancing TGFβ-mediated growth inhibition and stress-induced apoptosis. Knockdown of LGR5 attenuated downstream TGFβ signaling and increased cell proliferation, survival, and metastasis in an orthotopic model of colon cancer in vivo. Upon RSPO1 stimulation, LGR5 formed complexes with TGFβ receptors. Studies of patient specimens indicate that LGR5 expression was reduced in advanced stages and positively correlated with markers of TGFβ activation in colon cancer. Our study uncovers a novel cross-talk between LGR5 and TGFβ signaling in colon cancer and identifies LGR5 as a new modulator of TGFβ signaling able to suppress colon cancer metastasis.

Introduction

LGR5, a leucine-rich repeat-containing G-protein–coupled receptor (GPCR; refs. 1, 2), was found to be a marker for proliferating adult stem cells in small intestine (3). LGR5 and its homologs LGR4 and LGR6 are receptors of R-spondins (RSPO; refs. 4, 5), which are secreted agonists of canonical Wnt signaling (6, 7). Unlike other GPCRs, LGR4-6 are not coupled to either G proteins or β-arrestin after stimulation by RSPOs (4). Instead, they have been shown to regulate Wnt canonical signaling, with both activation and inhibition reported in different cell contexts (5, 8). LGR5 itself is also a transcriptional target of Wnt signaling (9).

The physiological role of LGR5 during normal intestinal development and tumor initiation has been extensively studied. LGR5 deficiency leads to premature Paneth cell differentiation, associated with Wnt activation, whereas it has no detectable effects on differentiation of other cell lineages, nor on epithelial cell proliferation or migration (10). The role of LGR5 in intestinal tumor development is controversial. Conditional deletion of APC in LGR5-positive but not LGR5-negative cells led to a rapid growth of large adenomas in small intestine in a mouse model (11), providing evidence that intestinal tumors arise from LGR5-positive cells. However, ablation of LGR5-positive stem cells in the crypts had no effect on APC loss–induced crypt hyperplasia (12), indicating that the absence of LGR5 does not impair intestinal tumor initiation. Furthermore, there are other conflicting reports regarding the functional role of LGR5 in colon cancer tumorigenesis. Some studies reported that RSPO/LGR5 signaling has a tumor suppressive activity in colon cancer (8, 13) whereas others showed that LGR5 promotes tumorigenicity (14). Therefore, the role of LGR5 in colon cancer tumorigenesis remains ambiguous, although in a transposon mutagenesis study RSPO2 scored second only to APC as a colon cancer tumor suppressor locus (15). Moreover, it is yet to be determined whether LGR5 contributes to colon cancer progression and metastasis.

TGFβ regulates many aspects of cellular function. Upon ligand binding, TGFβ type II receptor (RII) recruits and activates TGFβ type I receptor (RI), which phosphorylates and activates Smad2/3. Activated Smad2/3 form complexes with Smad4 and translocate to the nucleus, where they regulate gene expression (16). The TGFβ axis has been shown to inhibit cell proliferation, induce apoptosis, and suppress tumorigenicity in many colon cancer cell lines (17) and in intestinal adenomas (18). Abrogation of TGFβ signaling increases metastasis whereas enhanced TGFβ signaling suppresses metastasis in an orthotopic model of colon cancer (19, 20). In genetic mouse models, Smad3 or Smad4 mutation increases malignancy and invasiveness of intestinal tumors of Apcmin/+ or knockout mice (21, 22), indicating a suppressive role of TGFβ signaling in malignant progression of colon tumors. Indeed, TGFβ signaling is defective in 30% to 40% of colon cancer patients due to defects in TGFβ RI or Smads (23, 24), and loss or reduction of TGFβ signaling is tightly associated with metastasis development (25, 26).

To address the potential function of LGR5 in colon cancer metastasis, we examined LGR5 expression in clinical specimens, and found that LGR5 expression decreased in advanced stages of colon cancer as compared to early stages. Knockdown of LGR5 expression in colon cancer cells increased their clonogenicity, survival capacity in vitro, and liver and lung metastasis in an...
orthotopic model in vivo. Investigation of potential cross-talk between RSP01/LGR5 and TGFβ signaling revealed that RSP01/LGR5 activated the canonical TGFβ pathway and elicited its inhibitory effect on clonogenicity and cell survival of colon cancer cells through the activation of TGFβ signaling. Mechanically, LGR5 colocalized and formed complexes with TGFβ receptors upon RSP01 stimulation. Importantly, LGR5 expression positively correlated with Smad2 activation in colon cancer specimens. Therefore, our studies identify LGR5 as a novel component/modulator of the TGFβ signaling pathway and demonstrate that RSP01/LGR5 functions as a suppressor of colon cancer metastasis.

Materials and Methods

Colon cancer cell culture

Human colon carcinoma cell lines (HCT116, RKO, FET, CBS, HCT116b, and TENN) were originally established in Dr. Brattain’s lab in 1981 (27). All cell lines were validated by short-tandem repeat (STR) analyses at the University of Nebraska Medical Center Human DNA Identification Laboratory. STR profiles were cross-checked with the ATCC database. HCT116 and RKO cell lines with ≥80% match with the ATCC online STR database are considered valid (28). STR profiles of FET, CBS, HCT116b, and TENN cell lines have never been reported before. Their STR profiles were confirmed in ATCC database to be of human origin and contain no mammalian interspecies contamination (Supplementary Fig. S1A). There were 5–8 passages between thawing and use in the described experiments for all the cell lines.

Cells were maintained at 37°C in a humidified incubator with 5% CO2 and cultured in McCoy’s 5A serum-free medium (M4892; Sigma) supplemented with 10 ng/mL EGF, 20 µg/mL insulin, and 4 µg/mL transferrin (29). When the cells were subjected to growth factor deprivation stress, they were cultured in the medium in the absence of growth factor or serum supplements.

Antibodies and reagents

Anti-LGR5 (ab75850) and anti-phospho-Smad2 (Ser465/467; ab3849) antibodies were purchased from Abcam. Anti-cleaved PARP (5625), anti-cleaved caspase-9 (9502), anti-Smad4 (9515), anti-TGFβ RI (11888), and anti-actin (4970) antibodies were obtained from Cell Signaling Technology. Anti-Flag (F1804) and anti-HA (MMS-101P) antibodies were purchased from Sigma-Aldrich. The retroviral expression vectors for C-terminal-tagged or non-tagged full-length human LGR5 were gifts from Dr. Keith Johnson (University of Nebraska Medical Center, Omaha, NE).

Dominant-negative TCF expression vector (EdT) was a gift from Roel Nusse (24310; Addgene). Transfection and infection were performed as described previously (30).

RT-PCR assays

Total RNA was isolated from colon cancer cells, liver, or lung of mice using TRIzol reagent (15596026) from Thermo Fisher Scientific. Two micrograms of total RNA was reverse-transcribed to cDNA with M-MLV reverse transcriptase (1701; Promega) using a random primer 2 µL of cDNA product was used to amplify specific genes. Actin was used as a loading control. Sequences of primers for each gene were listed in Supplementary Table S1.

Colony formation and soft agarose assays

For colony formation assays, cells were seeded in six-well plates at 500 cells per well. After 2 weeks, colonies were stained with MTT and dissolved in DMSO. Relative cell numbers were determined by OD values read at 570 nm. For soft agarose assays, cells were seeded in culture medium containing low melting point agar (BMA50101; Lonza) at a density of 3,000 cells per well in 6-well plates. Colonies were stained with 1% iodonitrotetrazolium violet (17375; Sigma-Aldrich) after 3 weeks and the numbers of colonies were counted.

Apoptosis assays

Cells were plated and allowed to grow to 80% confluence before switching to medium in the absence of growth factor or serum supplements for 2–5 days. Apoptosis was determined by measuring cleaved PARP or cleaved caspase-9 or by performing DNA fragmentation ELISA assays (1158504501; Roche Diagnostics) following the manufacturer’s instruction. Cleaved caspase-9 and PARP are indicators of induction of apoptosis, whereas DNA fragmentation assays can relatively quantify the amount of histone-complexed DNA fragments during apoptosis.

Luciferase assays

SBE4-Luc (16495; Addgene) is a reporter plasmid containing multiple copies of Smad-binding site and encoding the firefly reporter gene. TOPFlash (12456; Addgene) reporter consists of multiple TCF-binding sites and encodes the firefly reporter gene. When they are transfected into the cells, the firefly luciferase activity reflects the activation of the TGFβ or Wnt signaling pathway, respectively. The pRL-CMV plasmid encoding Renilla luciferase (E6621; Promega) was cotransfected as an internal control. Luciferase assays were performed using the Dual Luciferase Assay System (E1910; Promega). The luminescent signal of firefly and Renilla was detected using the BioTek Synergy 2 Multi-Mode Reader according to the manufacturer’s instruction. Values of firefly luciferase were divided by those of Renilla luciferase for the normalization.

RNA sequencing

FET cells in triplicates were treated with RSP01 or TGFβ1 for 4 hours. RNA was isolated using RNAasy Mini Kit (47104; Qiagen). RNA sequencing was performed by the City of Hope Integrative Genomics Core facility. Poly(A) RNA-seq libraries were constructed using TruSeq mRNA Library Preparation Kit (RS-122;
and FDR

Illumina). Purified libraries were validated using Bioanalyzer 2100 system with DNA High Sensitivity Chip (Agilent) and quantified with Qubit Fluorometer (Invitrogen). All libraries were sequenced on the Illumina Hiseq 2500 with single read 51-bp reads following the manufacturer's recommendations. The 51-bp-long single-ended sequence reads were mapped to 51-bp reads following the manufacturer's recommendations. /C21

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method (31) and compared using Bioconductor package "edger" (32). The average coverage for each gene was calculated using the normalized read counts from "edger." Differ 

entially expressed genes were identified if the average coverage 

in at least one sample, log2-based fold change ≥1 or ≤-1 and FDR <0.05. The RNA-seq data have been submitted to GEO repository (accession no. GSE103650).

Immunoprecipitation assays

HCT116 cells expressing Flag-tagged TGFβ RII and HA-tagged LGR5 (HCT116-RII/LGR5) were seeded at 1.0 × 10^6 cells/dish in 100-mm dishes and allowed to attach for 4 hours. Cells were then treated with 200 ng/ml RSPO1 or 4 ng/ml TGFβ1 for 30 minutes. Whole-cell lysates were made in NP-40 lysis buffer (20 mmol/L Tris-HCL, pH 8.0, 137 mmol/L NaCl, 10% glycerol, 1% nonidet P-40, 2 mmol/L EDTA) with protease inhibitor cocktail (P8340, Sigma). Three hundred micrograms of the cell lysate was incubated with a primary antibody for overnight at 4°C on a rotator followed by incubation with secondary antibody at room temperature. Protein complexes were captured on magnetic beads (LSKMAGA10; Millipore, EMD Millipore) for 30 minutes. As a negative control, a normal IgG antibody was used. Immunoprecipitated proteins were resolved by SDS-PAGE followed by Western blot analysis as described previously (30).

Immunofluorescence and confocal microscopy

HCT116-RII/LGR5 cells were plated onto glass coverslips and treated with RSPO1 or TGFβ1 for 30 minutes. The cells were then washed with PBS and fixed with 4% ice-cold paraformaldehyde for 10 minutes. The fixed cells were blocked with 3% BSA-PBS for 1 hour at room temperature, followed by incubation with primary antibodies (anti-Flag, anti-HA, anti-LGR5, anti-TGFβ RII) for overnight at 4°C. On the next day, the cells were washed and incubated with secondary antibodies conjugated with Alexa Fluor 488 or 594 (Invitrogen) for 1 hour at room temperature. Nuclei were counterstained with DAPI. Double stained images were obtained by sequential scanning for each channel to eliminate the cross-reactivity of chromophores. Images were captured and analyzed using confocal laser scanning microscope (LSM800; Carl Zeiss Microscopy GmbH). Quantification of fluorescence signal was performed using an algorithm direct Gaussian fit in the ZEN blue version 2.3 software.

For each sample, approximately 20 different cell images were acquired and quantified. After cell cropping, background correction and intensity thresholds were applied, colocalization and correlation were calculated using Pearson's coefficient.

In vivo orthotopic transplantation

Experiments involving animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC; protocol no. 1509711FC) and IACUC regulations were followed. Orthotopic transplantation was performed as described previously (33). Briefly, exponentially growing GFP-labeled CBS cells (5 × 10^6) were inoculated subcutaneously into athymic nude mice (male, 5–6 weeks, 20–25 g; Harlan Laboratories). Once xenografts were established, they were excised and minced into 1-mm^3 pieces, two of which were then transplanted onto the subserosal layer of the cecum of other mice. Seventy days posttransplantation, mice were euthanized. Organs were explanted, imaged, and immediately frozen or placed in buffered 10% formalin. Tissues were then processed and embedded in paraffin. Four-micron thick sections were cut for IHC or H&E staining.

Human tissue specimens

The patient studies were conducted in accordance with Belmont Report and US common rule ethical guidelines. Informed written consent from the subjects (wherever necessary) were obtained. Following approval by the Institutional Review Board of the University of Nebraska Medical Center (UNMC), a tissue microarray (TMA) was made by the Tissue Science Facility. The TMA contained replicate samples obtained from formalin-fixed paraffin-embedded (FFPE) blocks of normal human colorectal mucosa from specimens removed for reasons other than malignancy (i.e., diverticulosis) and colonic adenocarcinomas at stages I, II, III, or IV retrieved from the files of Department of Pathology and Microbiology. The ages of all patients (including both men and women) were between 55 and 85 years.

IHC staining

FFPE blocks of human/mouse colon tumors were cut into 4-

μm-thick tissue sections. IHC staining was performed to examine LGR5 and pSmad2 expression in TMA and MSI samples as well as primary tumors of CBS control or LGR5 knockdown cells following Novolink Min Polymer Detection System Kit protocol (RC7290-CE; Leica). Briefly, slides were subjected to antigen retrieval using Novoceastra Epiotope Retrieval Solutions, pH 6 (RE7113; Leica), followed by incubation with primary antibodies (anti-LGR5 and anti-pSmad2) overnight at 4°C. One the next day, slides were developed with DAB after incubation with Novolink polymer for 30 minutes. Finally, the sections were counterstained with hematoxylin. The TMA slide was scanned at 40× using Ventana iscan Coreo Au Scanner. Ten fields were randomly selected from each slide for quantification. The staining intensity and density were quantified with Imagescope Software (V12.1.0.5029; Leica).

Statistical analyses

Statistics analysis was performed in GraphPad Prism 5 after figures were generated. Two-sided paired Student t test or one-way ANOVA was used to analyze the differences among groups. Pearson correlation coefficient analysis was used to determine the correlation between two sets of samples. Statistical signifi 

cance of metastatic incidence was determined by Fisher exact test in the orthotopic transplantation experiments. A P value <0.05 was considered statistically significant.

Results

LGR5 expression decreases in advanced stages of colon tumors

To determine the potential function of LGR5 in colon cancer, we examined LGR5 expression in clinical specimens. We first
analyzed the Oncomine database obtained from Skrzypczak colorectal dataset (34), which showed that LGR5 mRNA expression is higher in colon adenomas than in normal colon, but is lower in carcinomas than in adenomas (Supplementary Fig. S1B). Furthermore, we assayed LGR5 protein expression in human colonic adenocarcinomas using IHC staining. We first verified the specificity of the anti-LGR5 antibody used successfully for IHC staining (Supplementary Fig. S1C and S1D; refs. 35, 36). Paraffin blocks from 12 normal colon and 73 colon cancer patients at different stages were stained. LGR5 staining varied significantly in different groups of samples (Fig. 1A). Quantification of the staining showed that, although there were overlaps in LGR5 expression between groups, the average intensity and density of LGR5 staining were higher in stage I/II tumors than in normal crypts and lower in stage III/IV tumors than in stage I/II tumors (Fig. 1B and C). These results indicate that expression of LGR5 decreases in advanced stages of colon cancer as compared with early-stage disease, suggesting that there may be selection for active LGR5 expression early in colon tumorigenesis, or it simply reflects the origin of the tumors as derived by clonal expansion of LGR5-positive stem cells, but suppression thereafter during tumor progression.

LGR5 inhibits cell survival and clonogenicity of colon cancer cells in vitro

These observations promoted us to explore whether LGR5 might play a suppressive role in tissue culture models of colon cancer progression and metastasis. We first examined LGR5 expression in a panel of human colon cancer cell lines with differing metastatic capability. Highly metastatic cells (TENN, RKO, and HCT116) express lower amounts of LGR5 than moderately, weakly, or nonmetastatic CBS, HCT116b, and FET cells, respectively, at both mRNA and protein levels (Fig. 2A), indicating an inverse correlation between LGR5 expression and metastatic potential. Accordingly, HA-tagged LGR5 (LGR5/HA) was introduced into CBS cells expressing intermediate level of LGR5 (Fig. 2B, top). Because aberrant survival capacity of tumor cells is an important determinant of metastatic potential (37, 38), we first evaluated the ability of colon cancer cells to withstand stress-induced apoptosis in vitro. Under growth factor and nutrient deprivation stress (GFDS), LGR5-expressing cells became more sensitive to GFDS-induced apoptosis than control cells, reflected by increased caspase-9 cleavage (Fig. 2B, bottom) and enhanced apoptosis in DNA fragmentation assays (Fig. 2C). In addition, increased expression of LGR5 reduced clonogenicity in colony

Figure 1.
Evaluation of LGR5 expression in colon cancer patient specimens. A, IHC staining with an anti-LGR5 antibody was performed in normal colon and colon cancer specimens at different stages. Representative images are shown. Scale bar, 100 μm. B and C, Quantification of percentage of LGR5-positive cells (B) and LGR5 staining intensity (C) was performed. The values of individual samples are shown. Error bars, SEM of the values in each group. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
in vitro results indicate that RSPO1/LGR5 inhibits cell survival and clonogenicity in soft agarose assays (Fig. 2H). These results indicate that RSPO1/LGR5 inhibits cell survival and clonogenicity in vitro.

Complementarily, LGR5 expression was significantly reduced with each of two independent shRNAs (#5 and #6) in FET and CBS cells (Fig. 3A, top). LGR5 knockdown cells showed increased resistance to GFDS-induced apoptosis, reflected by decreased PARP cleavage (Fig. 3A, bottom) and reduced apoptosis in DNA fragmentation assays (Fig. 3B). In addition, clonogenicity assays indicated that knockdown of LGR5 expression led to almost two-fold of increase in colony forming capacity when plated at sparse densities (Fig. 3C) and 2.5- to 4.5-fold of increase in anchorage-independent clonogenicity assays (Fig. 3D). These results demonstrate that reduced LGR5 expression increases cell survival and clonogenicity in vitro.

LGR5 suppresses metastasis of colon cancer cells in vivo

We next examined the role of LGR5 in colon cancer metastasis using an orthotopic mouse model that substantially recapitulates human colon cancer metastasis to the liver and lungs (39, 40). CBS cells, moderately metastatic in vivo (20), were used. CBS control and LGR5 knockdown cells were stably transfected with GFP. Mice implanted with either control or LGR5 knockdown cells (LGR5 KD: combined results of two shRNAs) showed 100% primary tumor growth. Knockdown of LGR5 expression resulted in a modest 23% increase in primary tumor weight (Supplementary Fig. S2A). However, it significantly increased the incidence of liver or lung metastasis from 29% to 77%, assessed by histologic analyses (Fig. 4A and B). Fluorescence imaging of explanted liver or lungs showed increased tumor burden of metastases in mice implanted with LGR5 KD cells (Fig. 4C). To confirm it, RNA was extracted from lungs and liver of each mouse and semiquantitative RT-PCR was performed using human-specific GAPDH primers. The level of human GAPDH mRNA expression represents the amount of human RNA, which is reflective of tumor burden in the lungs and liver of mice. RT-PCR results showed that human-specific GAPDH mRNA level was much higher in LGR5 KD group than in the control group (Supplementary Fig. S2B and S2C). These results indicate LGR5 inhibits incidence and tumor burden of colon cancer metastasis in vivo.

LGR5 activates TGFβ signaling in colon cancer cells

To explore the mechanism(s) of LGR5 suppression of metastasis, we first investigated the effect of LGR5 on procarcinogenic Wnt signaling, given that RSPO1/LGR5 is a well-established activator of this pathway in many cell types including HEK293T (4). We were able to recapitate the finding that RSPO1 increased Wnt activity in HEK293T cells as reflected by increased phosphorylation of LRP6, a co-receptor of Wnt, and β-catenin expression (Supplementary Fig. S3A ref. 4). However, no similar effect was observed in FET cells (Supplementary Fig. S3B), which are responsive to the RSPO1/LGR5 biological effects shown earlier (Fig. 2G and H). The luciferase activity of TOPFlash reporter consisting of multiple TCF-binding sites confirmed these observations (Supplementary Fig. S3C). These results were unexpected and remain under investigation, but in the meantime encouraged the exploration of other potentially relevant pathways, particularly TGFβ signaling, a well-established tumor suppressor pathway in colon cancer, but not previously interconnected with LGR5.

Accordingly, we deployed a luciferase plasmid driven by multiple Smad binding elements (SBE4-Luc) as a surrogate marker of TGFβ-dependent promoter activity. FET and CBS cells were treated with RSPO1. As they both also express endogenous TGFβRII, TGFβ1 was used as a reference ligand. RSPO1 exposure increased Smad reporter activity in both cell types in a dose-dependent manner (Fig. 5A and Supplementary Fig. S4A, result in Fig. 4D will be discussed below). A time course study showed that RSPO1 and TGFβ1 activated Smad2 with similar dynamics (Fig. 5B). Besides FET and CBS cells, RSPO1 also activated Smad reporter and increased Smad2 phosphorylation in HT29 cells, which express endogenous LGR5 (Supplementary Fig. S4B). These studies indicate that RSPO1/LGR5 activates TGFβ signaling in colon cancer cells.

To determine whether RSPO1 and TGFβ1 activate similar transcription, RNA-seq analysis was performed to compare gene profiles after treatment by RSPO1 or TGFβ1. FET cells were treated with RSPO1 or TGFβ1 in triplicates for 4 hours and RNAs extracted from these cells were used for RNA-seq analysis. Genes with log2-based fold change ≥1 and FDR <0.05 were considered upregulated genes and those with log2-based fold change ≤−1 and FDR <0.05 were considered downregulated genes. There were 269 genes upregulated and 126 genes downregulated by RSPO1 and 120 genes upregulated and 42 genes downregulated by TGFβ1 (Supplementary Table S2 and S3). The log2, data of genes regulated by TGFβ1 were also regulated by RSPO1. A Venn diagram depicting the numbers of genes commonly or exclusively regulated by RSPO1 or TGFβ1 is shown in Fig. 5D. The RNA-seq data have been validated by semiquantitative RT-PCR of p21 and c-myc, both regulated by RSPO1 or TGFβ1 to similar degrees (Fig. 5E). Taken together, these results indicate that RSPO1 activates TGFβ1-mediated gene transcription.

In a complementary approach, we examined the converse effect of reducing LGR5 expression on Smad activation. LGR5 knockdown in FET and CBS cells (Fig. 3A) decreased the Smad reporter activity by approximately 60% to 80% (Fig. 5F, left). Consistently, induction of Smad2 phosphorylation by TGFβ3 was attenuated in LGR5 knockdown cells as compared to control cells (Fig. 5F, right). Furthermore, IHC staining showed that nuclear Smad2 phosphorylation was reduced in primary tumors derived from CBS LGR5 KD cells by 79% as compared to those derived from control cells (Fig. 4D), suggesting that knockdown of LGR5 decreased Smad activation in vivo.

Next, we determined which recognized components of TGFβ signaling might play a role in pathway activation by RSPO1/LGR5. Expression of RI, RI, Smad2, Smad3, and Smad4, was reduced individually by shRNAs in FET cells (Fig. 5G and H, top). Expression of LGR5 was also specifically knocked down (Fig. 5G, top). Reduced expression of any of these individual genes attenuated or abrogated RSPO1-induced Smad reporter activation (Fig. 5G and H, bottom), indicating that each plays a role in RSPO1-mediated TGFβ activation.

Because TGFβ RI kinase is essential for activating Smads, a potent and selective inhibitor of TGFβ RI kinase, SB525334, was
used. This compound almost completely blocked Smad reporter activation induced by RSPO1 in FET and CBS cells under normal condition (top). Cleaved PARP was determined under GFDS for 48 hours (bottom). B, Apoptosis was measured by DNA fragmentation assays under GFDS for 48 hours. C, Colony formation assays were performed in control and LGR5 knockdown cells with representative images (left) and quantification (right) shown. D, Soft agarose assays were performed in control and LGR5 knockdown cells, with representative images (left) and quantification (right) shown. The data are presented as the mean ± SEM of three replications. *** P < 0.001.

Figure 2. RSPO1/LGR5 inhibits cell survival and clonogenicity of colon cancer cells in vitro. A, LGR5 expression was determined by RT-PCR (top) and Western blot (bottom) analyses in colon cancer cell lines. B, Western blot analyses show that HA-tagged LGR5 was stably expressed in CBS cells under normal condition (top). B and C, Vector- and LGR5/HA-expressing CBS cells were subjected to GFDS for 72 hours. Apoptosis was determined by cleaved caspase-9 (B, bottom) and DNA fragmentation assays (C). D, Colony formation assays were performed in vector- and LGR5/HA-expressing CBS cells, with representative images (left) and quantification (right) shown. E, Soft agarose assays were performed in vector- and LGR5/HA-expressing CBS cells, with representative images (left) and quantification (right) shown. F, TGFβRI expression was knocked down in FET cells. G, FET control and RI knockdown cells were subjected to GFDS while treated with RSPO1 or TGFβ1 for 16 hours. Apoptosis was determined by DNA fragmentation assays. H, FET control and RI knockdown cells were treated with RSPO1 or TGFβ1 in soft agarose assays. Stained plates (top) and quantification (bottom) are shown. I, FET cells were treated with RSPO1 or TGFβ1 and the RI kinase inhibitor under GFDS. Apoptosis was determined by DNA fragmentation assays. J, Soft agarose assays were performed in FET cells treated with RSPO1 or TGFβ1 and the RI kinase inhibitor. Stained plates (left) and quantification (right) are shown. The data are presented as the mean ± SEM of three replications. *** P < 0.001; ***, P < 0.001.

Figure 3. Knockdown of LGR5 expression increases cell survival and clonogenicity in vitro. A, LGR5 expression was significantly reduced by two LGR5 shRNAs in FET and CBS cells under normal condition (top). Cleaved PARP was determined under GFDS for 48 hours (bottom). B, Apoptosis was measured by DNA fragmentation assays under GFDS for 48 hours. C, Colony formation assays were performed in control and LGR5 knockdown cells with representative images (left) and quantification (right) shown. D, Soft agarose assays were performed in control and LGR5 knockdown cells, with representative images (left) and quantification (right) shown. The data are presented as the mean ± SEM of three replications. *** P < 0.001.
TGFβ1-mediated promoter activation (Fig. 5G). These findings suggest that TGFβ1/RII and RSPO1/LGR5 are intimately interconnected in activating TGFβ signaling. One potential mechanism might comprise signaling through a common physical complex. We therefore investigated whether LGR5 forms complexes with TGFβ R1 and/or RII upon RSPO1 stimulation. To facilitate co-IP assays and IF staining to detect complex formation and colocalization respectively, we engineered HCT116 cells to

![Table](image1.png)

**Figure 4.** LGR5 suppresses colon cancer metastasis in vivo. **A,** Control and LGR5 knockdown CBS cells were orthotopically implanted into athymic nude mice. The incidence of liver and/or lung metastasis is shown. *, *P* < 0.05; **,** *P* < 0.01 with power >80%. **B,** Representative images of H&E staining in primary tumors, liver, and lung metastases are shown. Scale bars, 200 μm. **C,** Representative GFP images of liver and lung metastases are shown. Scale bars, 100 μm. The data are presented as the mean ± SEM. *, *P* < 0.05.

**Figure 4.**

LGR5 suppresses colon cancer metastasis in vivo. A, Control and LGR5 knockdown CBS cells were orthotopically implanted into athymic nude mice. The incidence of liver and/or lung metastasis is shown. *, *P* < 0.05; **,** *P* < 0.01 with power >80%. B, Representative images of H&E staining in primary tumors, liver, and lung metastases are shown. Scale bars, 200 μm. C, Representative GFP images of liver and lung metastases are shown. D, IHC staining of phosphorylated Smad2 was performed in primary tumors. Representative images are shown (left). The intensity of pSmad2 staining was quantified (right). Scale bars, 100 μm. The data are presented as the mean ± SEM. *, *P* < 0.05.
express both Flag-tagged TGFβRI and HA-tagged LGR5 (HCT116-RII/LGR5; Fig. 6A). HCT116 cells express abundant endogenous TGFβRI (Fig. 6A). Treatment of HCT116-RII/LGR5 cells with either RSPO1 or TGFβ1 increased Smad2 phosphorylation (Fig. 6B–I), indicating the activation of the TGFβ pathway. Protein lysates from nontreated control (C), RSPO1 (R)- or TGFβ1 (T)-treated cells were immunoprecipitated with an anti-LGR5, anti-Flag (RII), or anti-RI antibody followed by Western blot analysis of expression of LGR5, RII, and RI in the immunoprecipitated complexes. Co-IP assays showed that, when immunoprecipitated with the anti-LGR5 antibody, RSPO1 or TGFβ1 increased the amount of RII and RI associated with LGR5 as compared to control samples (Fig. 6B–II). Similarly, when immunoprecipitated with the anti-Flag antibody, which pulled down RII, the amount of LGR5 and RI associated with RII was increased by RSPO1 or TGFβ1 (Fig. 6B–III). When immunoprecipitated with the anti-RI antibody, RSPO1 or TGFβ1 increased the amount of LGR5 and RII associated with RI (Fig. 6B–IV). Similar results were observed in FET cells expressing endogenous LGR5 and RII. When immunoprecipitated with the anti-RI antibody, RSPO1 or TGFβ1 increased the amount of LGR5 and RII associated with RI (Fig. 6C). Of note, increased complex formation between TGFβRII and RII induced by TGFβ1 was expected from previous studies (41, 42). We have shown earlier that knockdown of TGFβRI, RII, or LGR5 reduced RSPO1- or TGFβ1-induced Smad reporter activation (Fig. 5G). Taken together, these results suggest that the complex formation of RI/RII/LGR5 may be important for RSPO1- or TGFβ1-mediated TGFβ signaling.

To further explore the hypothesis that LGR5 complexes with TGFβ receptors, immunofluorescence microscopy was used to determine whether LGR5 colocalized with TGFβ RI and RII. HCT116-RII/LGR5 cells treated with RSPO1 or TGFβ1 were labeled with an anti-HA (for LGR5), anti-RI, or anti-Flag (for RII) antibody followed by incubation with a secondary antibody conjugated with either Alexa Fluor 488 (green) or Alexa Fluor 594 (red). Confocal microscopy analysis showed that in control...
Figure 6. LGR5 forms complexes with TGFβ receptors. A, HCT116 cells were stably transfected with Flag-tagged TGFβ RII and HA-tagged LGR5 (HCT116-RII/LGR5). Expression of LGR5, RII, and RI was detected. B, HCT116-RII/LGR5 cells were treated with 200 ng/mL RSPO1 (R) or 4 ng/mL TGFβ1 (T) for 30 minutes. Input (top) and pSmad2 expression (bottom) are shown (B, I). Complex formation between LGR5, RII, and RI was detected by immunoprecipitation, followed by Western blot analyses (B, II–IV). C, FET cells were treated with 200 ng/mL RSPO1 (R) or 4 ng/mL TGFβ1 (T) for 30 minutes. Input (left top) and pSmad2 expression (left bottom) are shown. Complex formation between LGR5, RII, and RI was detected by immunoprecipitation of RI followed by Western blot analyses (right). D, HCT116-RII/LGR5 cells were treated with RSPO1 (R) or TGFβ1 (T) for 30 minutes. Single-channel (green or red) and merged (yellow) microscopic images of distribution of LGR5 (green) and RI (red; top), RII (green) and LGR5 (red; middle), and RII (green) and RI (red; bottom) are shown. Scale bars, 50 μm. E, Statistical analyses of colocalization of LGR5/RI (top), RII/LGR5 (middle), and RII/RI (bottom) using Pearson correlation coefficient were performed. ***, P < 0.001.
cells, LGR5, RII, and RII were each distributed mainly on the cell membrane (Fig. 6D). Treatment with either RSPO1 or TGFβ1 induced internalization of these receptors and increased colocalization of LGR5/RI (top), RII/LGR5 (middle), and RII/RII (bottom) as shown by yellow color in merged images (Fig. 6D). Quantification of fluorescent signals indicated that RSPO1 or TGFβ1 increased aggregation of LGR5 and TGFβRI (Supplementary Fig. S6A). In addition, Pearson’s correlation coefficient used for describing the correlation of intensity distributions between channels was calculated, which showed that the extent of colocalization between green and red channels of LGR5/RI (top) and RII/RII (bottom) was significantly enhanced by RSPO1 or TGFβ1 and extent of colocalization of RII/LGR5 (middle) was markedly enhanced by TGFβ1 (Fig. 6E).

**LGR5 expression positively correlates with TGFβ signaling in human patient specimens**

To explore whether there is any potential clinical relevance of the activation of the TGFβ pathway by RSPO1/LGR5, we determined the relationship between LGR5 expression and Smad2 phosphorylation (pSmad2) in colon cancer patient samples using IHC staining. We found that tumors with high pSmad2 also expressed high LGR5 (Fig. 7A). The study revealed a strong correlation between LGR5 and pSmad2 positivity (expressed as percentage of positive cells; Fig. 7B, left) and a moderate correlation between LGR5 and pSmad2 intensity (expressed as intensity of staining; Fig. 7B, right). Therefore, our data demonstrate the clinical relevance and biological importance of RSPO1/LGR5-mediated activation of TGFβ signaling in colon cancer.

Taken together, our studies suggest a novel model of cross-talk between RSPO/LGR5 and TGFβRII signaling (Fig. 7C). In this model, RSPO or TGFβ increases association of LGR5, RII and RII, enhancing TGFβ downstream signaling. Reduction of LGR5 or RII expression will decrease TGFβ activation, enabling cancer cells to escape TGFβ tumor suppressor function, leading to cancer progression, poor prognosis, and/or poor survival of colon cancer patients.

**Discussion**

In this study, we have shown that RSPO1/LGR5 directly activates TGFβ signaling, inhibits cell survival and clonogenicity of colon cancer cells in vitro, and suppresses colon cancer metastasis in vivo. Mechanistically, upon stimulation by RSPO1, LGR5 colocalizes and forms complexes with TGFβ RI and RII, suggesting that LGR5 may be a novel component of the TGFβ signaling complex (Fig. 7C). Importantly, in support of the results in colon cancer cell lines, studies of clinical specimens show that LGR5 expression positively correlates with the activation of TGFβ signaling in colon cancer and that LGR5 expression decreases in advanced stage of colon cancer. In summary, this study discovers a tumor suppressor function of LGR5 in colon cancer and establishes a novel cross-talk between an intestinal stem cell marker and a well-established tumor/metastasis suppressor pathway, which provides a new avenue for understanding how intestinal stem cells contribute to tumor development and progression.

A recent study discovered that expression of Wnt5a, the noncanonical Wnt ligand, is upregulated during crypt regeneration, which inhibits proliferation of intestinal epithelial stem cells by activation of TGFβ signaling mediated by Wnt5a receptor Ror2 and TGFβ RI kinase activity (43). However, Wnt5a does not mimic RSPO1 effect in activating TGFβ signaling in colon cancer cells (Supplementary Fig. S6B). Therefore, in different contexts, there may be cross-talks between Wnt and TGFβ signaling through different ligand/receptor-mediated mechanisms.

Both LGR4 and LGR5 are co-expressed in the stem cells of intestine, colon, stomach, and hair follicle (44–46). LGR6 marks multipotent stem cells in the epidermis (47). We found that LGR5 is differentially expressed in colon cancer cell lines (Fig. 2A), whereas LGR4 is ubiquitously expressed and LGR6 is almost undetectable in those cells (Supplementary Fig. S6C). When LGR5 expression was knocked down in CBS and FET cells, expression of LGR4 was not affected (Supplementary Fig. S6D), suggesting that LGR4 does not compensate for LGR5 loss. The function of LGR4 in colon cancer remains to be determined.

RNA-seq analysis of gene expression mediated by RSPO1 or TGFβ1 indicates that majority of genes regulated by TGFβ1 are shared by RSPO1. However, approximately 60% of RSPO1-mediated genes are regulated exclusively by RSPO1 (Fig. 5D). These results indicate that, despite of an overlap between RSPO1- and TGFβ1-mediated transcriptional program, RSPO1 has its own specific set of target genes that are not mediated by TGFβ1. It suggests that, in addition to the activation of TGFβ signaling, RSPO1 activates other signaling pathways. RSPO1/LGR5 has been shown to modulate Wnt signaling (5, 8). Analysis of a ChiP-Seq dataset, which identified genes whose genomic upstream regulatory regions exhibit significant β-catenin and TCF binding in intestinal crypt epithelial cells (48), shows that only 19 of 242 RSPO1-exclusive genes (less than 8%) overlap with β-catenin/TCF target genes. These results indicate that the majority of RSPO1-exclusive genes are not subject to Wnt-mediated transcriptional regulation. Further studies show that knockdown of β-catenin or expression of a dnTCF had little effect on RSPO1-mediated upregulation of RASA4 (RAS p21 protein activator 4) and TSPAN2 (tetraspanin 2), two genes exclusively regulated by RSPO1 (Supplementary Fig. S6E). Taken together, these results indicate that RSPO1 regulates downstream gene expression largely independent of Wnt signaling in colon cancer cells.

We also showed that either TGFβ1 or RSPO1 induces the complex formation of RI/RII/LGR5 (Fig. 6E) and that knockdown of either RI or LGR5 reduced TGFβ activation induced by either TGFβ1 or RSPO1 (Fig. 5G). Taken together, we propose a model of TGFβ signaling as shown in Figure 7C. Upon binding their respective ligands, TGFβ RI and LGR5 form a complex with TGFβ RI and activate it, leading to phosphorylation and activation of Smads downstream. Therefore, TGFβ/RII and RSPO/LGR5 can transduce TGFβ signaling collaboratively through the activation of the downstream TGFβ RI–Smad pathway (Fig. 7C). Reduction of RI or LGR5 would decrease TGFβ signaling, enabling colon cancer cells to escape TGFβ tumor suppressor function. In addition to colon cancer, studies in squamous cell carcinoma (SCC) show that inactivation of TGFβ receptors coupled with hyperactivation of RAS/RAF/ MAPK signaling or Tp53 mutation in LGR5-positive stem cells drives rapid development of cutaneous SCC (49), suggests that TGFβ signaling inactivation could be a tumor-initiating event. These studies provide compelling support for the tumor suppressive role of TGFβ signaling in carcinogenesis.
In summary, our studies have significant implications. Studies of colon cancer specimens show that LGR5 expression increases in stage I/II tumors and decreases in stage III/IV tumors. These results suggest that upregulated LGR5 expression, driven by Wnt activation at early stages of colon cancer, enhances TGFβ signaling as a fail-safe mechanism to prevent further tumor development. When tumors progress to late stages, LGR5 expression is suppressed by promoter methylation (50) or other unknown mechanisms, which leads to reduced TGFβ signaling and facilitates colon cancer progression and metastasis. Therefore, LGR5 is another important tumor suppressor of colon cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: X. Zhou, L. Geng, J. Wang
Development of methodology: X. Zhou, L. Geng, J. Wang
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Zhou, L. Geng, D. Wang, J. Wang

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