Novel Insights into Gastric Cancer: Methylation of R-spondins and Regulation of LGR5 by SP1

Franziska Wilhelm, Eva Simon, Christine Böger, Hans-Michael Behrens, Sandra Krüger, and Christoph Röcken

Abstract

Recently, it was shown that leucine-rich repeat-containing receptor 5 (LGR5)–expressing stem cells are the cellular origin of intestinal-type gastric cancer. The aim of our study was to uncover regulatory mechanisms of LGR5 expression in gastric mucosa and their implications for cancer development. Reporter assays identified an LGR5 promoter fragment, which is highly relevant for active LGR5 expression. Chromatin immunoprecipitation verified that SP1 is bound within this region, and reporter activity increased in SP1 transfected cells. Subsequently, the expression of R-spondins (RSPO1 and RSPO2), ligands of LGR5, was explored in neoplastic and nonneoplastic gastric tissue and gastric cancer cell lines. Using IHC, distinct spatial expression patterns of LGR5, RSPO1, and RSPO2 were found in nonneoplastic stomach mucosa and gastric cancer. RSPO expression was lower in gastric cancer compared with nonneoplastic mucosa on both the transcriptional ($P = 0.003$ for RSPO1 and $P = 0.000$ for RSPO2; $n = 50$) and the translational level. Methylation-specific PCR showed higher methylation levels of RSPO1/2 and reexpression of RSPOs in the gastric cancer cell lines MKN45 and MKN74 were induced by demethylating 5-aza-C treatment. Finally, expression patterns of LGR5 and RSPO were similar in gastric cancer.

Implications: This report identifies a regulatory mechanism of LGR5 expression in gastric carcinogenesis, with SP1 as an important component of the transcriptional complex and LGR5 activity, which is modulated by its ligands RSPO1 and RSPO2, whose expression is modulated by methylation.

Visual Overview: http://mcr.aacrjournals.org/content/early/2017/05/01/1541-7786.MCR-16-0472/F1.large.jpg. Mol Cancer Res; 1–10. ©2017 AACR.

Introduction

Despite a declining incidence, gastric cancer still belongs to the leading causes of cancer mortalities worldwide due to its late diagnosis at advanced stages impeding curative treatments (1–3). As reliable biomarkers for early detection and risk grouping are essentially missing, studies on the tumor-promoting cell populations mediating therapeutic resistance and recurrence of gastric cancer might raise new possibilities for clinical interventions.

Cancer stem cells (CSC) are generally accepted as drivers of cancer initiation and progression (20), and their transdifferentiation, tumor tissues still mirror many features of cell type differentiation as well as tissue composition hierarchies, but are equipped with outstanding possibilities for de- and transdifferentiation (5). To regulate this plasticity, microenvironmental cues may preserve stem cell properties and direct lineages of differentiation to allow for tightly controlled homeostasis; in a similar manner, the tumor microenvironment is believed to support CSC populations.

In the hepato-gastrointestinal tract, several stem cell populations have been postulated, which are characterized by the expression of stem cell markers such as CD44, Bmi-1, villin, or CD133 (6, 7). With the identification of leucin-rich G-protein–coupled receptor 5 (LGR5) as a modulator of the Wnt signaling cascade, another promising biomarker was established, linking the transmembrane receptor LGR5 with functional consequences for intracellular stem cell signaling (8, 9). In lineage tracing experiments, LGR5– cells have been shown to regenerate the whole epithelial structure of the stomach (10) as well as the intestine (11), hair follicle (12), or ovary (13). LGR5 acts as a receptor for R-spondins (RSPO), a family of secreted agonists of the Wnt signaling pathway (14, 15). LGR5 and RSPOs potently enhance an active Wnt signal by generating a ternary complex with the ubiquitin ligase Rnf43, leading to Rnf43 inactivation, thereby stabilizing Wnt receptor density on the membrane (16). Together with an orchestra of extra- and intracellular ligands known to define Wnt signaling strength (17), LGR5 is an important component of the fine-tuned interplay of signaling pathways within the stem cell compartment, allowing for a tightly controlled homeostasis by stem cell self-renewal as well as differentiation processes (18, 19).

Within the intestinal epithelium, aberrant Wnt signaling has been correlated with cancer initiation and progression (20), and its participation in gastric cancer pathogenesis has been postulated for a considerable proportion of gastric cancer cases as well (21). We and others have already shown LGR5 to be a putative molecular marker of cancer-initiating cells in gastric cancer and have shown its association with invasion and survival (22–24).
For LGR5, no driver mutations are known in gastric cancer (25–27); therefore, it is tempting to speculate that changes in the LGR5 expression profile in gastric cancer are dependent on transcriptional regulation. With this report, we describe a specificity protein 1 (SP1)-mediated elevation of LGR5 expression and dysregulation of RSPO1 and RSPO2 expression partly mediated by hypermethylation in the neoplastic gastric mucosa as well as functional consequences in disease-representative cell lines.

Materials and Methods

Study cohort selection

Corresponding neoplastic and nonneoplastic tissues from 51 gastric cancer patients were retrieved from the archive of the Institute of Pathology, UKSH (Kiel, Germany); these tissues comprised formalin-fixed paraffin-embedded (FFPE) material as well as unfixed, fresh-frozen samples. All human tissues were obtained as part of a therapeutic surgery. Gastric cancer classification was evaluated according to the WHO classification (28) and the seventh edition of the Union International Contre le Cancer (29). The study was approved by the local ethics committee of the University Hospital in Kiel, Germany (ref. number D452/11 and D472/15). Survival information was obtained from the Epidemiological Cancer Registry of the state Schleswig-Holstein, Germany. Before analysis, all patient data were pseudonymized.

IHC and evaluation of immunostainings

After deparaffinization, whole-mount FFPE tissue sections were boiled in citrate buffer for antigen retrieval and subsequently washed with Tris-buffered saline. To avoid unspecific reactions, slides were treated with hydrogen peroxide block and Ultra V Block Staining (both Thermo Scientific). For chromogenic staining, slides were incubated with the primary antibody prepared in antibody diluent (ZYTOMED Systems) for 30 minutes at room temperature and overnight at 4°C in a moist chamber [LGR5, 1:1,000 from (24); RSPO1 HPA046154, 1:50; RSPO2 HPA024764, 1:100; both Sigma-Aldrich]. Immunoreactions were visualized by incubation with ImmunPRESS universal-HRP-polymer for 30 minutes and application of DAB or NovaRED polymer for 30 minutes and application of DAB or NovaRED chromatin immunoprecipitation (ChIP) validated SP1 antibody (Merck Millipore). Immunoprecipitation was carried out with a chromatin immunoprecipitation (ChIP)-validated SP1 antibody (Merck Millipore), the anti-H3K4 control antibody or an IgG negative control, and magnetic protein A/G beads. Resulting DNA fragments were quantified by qRT-PCR (primers are given in Supplemental Materials S1).

Reverse transcriptase reaction and qRT-PCR

Total cellular RNA was isolated with the RNA Pure Kit (Roche) from cell culture samples. RNA from gastric tissues was prepared with the miRvana Isolation Kit, followed by DNase treatment with Turbo DNA-free Kit (both Life Technologies). Nucleic acid concentration and purity were assessed on a NanoDrop 2000 (Thermo Fisher Scientific) and reverse transcribed with Maxima First Strand cDNA Synthesis Kit (Life Technologies) according to the manufacturer's instructions. qRT-PCR was done with the QuantiTect SYBR Green PCR Kit (Qiagen) on a 1.5 LightCycler System (Roche) for cell culture experiments, and results were normalized to succinate dehydrogenase subunit A (SDHA). Expression analysis of gastric cancer tissues was performed with the LightCycler 480 Probes Master and the following probes and primer sets (RSPO1: fw- cgttagagggtagtcgcttcccaaattactcaag and rev- tcccaaattactgaagtccaggaattgtttgg and ttcccaaattactcaatctacaaa for unmethylated [Hs_LGR5_02_PM covering three potential CpG sites within the first intron was utilized and analyzed on the PyroMark Q24 (all from Qiagen). For analysis of RSPO1 and 2, methylation-specific PCR (MSP) on converted DNA from matching malignant and nonmalignant fresh-frozen tissues was established using the HotStarTag Kit (Qiagen). The following primers were designed with MethPrimer (31) for RSPO1: tcggttagagtagggtattcgttc and tcccaataactaatgga for the methylated sequence; tcggttaggtaggttttcg and tcccaataactaatcaca for unmethylated RSPO1. For RSPO2, primers were adopted (also see Supplemental Material S1; ref. 32). Commercial control DNA minimally/maximally methylated (Qiagen) served as control.

Methylation analysis

For methylation analysis, cell cultures were treated with 5-aza-2-deoxycytidine (5-aza-C, 1 μmol/L, BioCat) that was daily renewed over a period of 72 hours. DNA was extracted according to the protocol delivered with the QIAamp DNA Extraction Kit, and the resulting DNA was converted with the EpiTect Bisulfite Kit (both Qiagen). Samples from gastric cancer patients were prepared according to the manufacturer’s specifications. For analysis of the LGR5 gene from FFPE material, the PyroMark CpG Assay Hs_LGR5_02_PM covering three potential CpG sites within the first intron was utilized and analyzed on the Pyromark Q24 (all from Qiagen). For analysis of RSPO1 and 2, methylation-specific PCR (MSP) on converted DNA from matching malignant and nonmalignant fresh-frozen tissues was established using the HotStarTag Kit (Qiagen). The following primers were designed with MethPrimer (31) for RSPO1: tcggttagagggtagtcgcttcccaaattactcaag and rev- tcccaaattactgaagtccaggaattgtttgg and ttcccaaattactcaatctacaaa for unmethylated RSPO1. For RSPO2, primers were adopted (also see Supplemental Material S1; ref. 32). Commercial control DNA minimally/maximally methylated (Qiagen) served as control.

Chromatin immunoprecipitation analysis

To investigate promoter regions bound by proteins, cells were cultured to subconfluence in a 10-cm dish for 3 days before crosslinking with freshly prepared paraformaldehyde solution (final concentration, 1%). The cell number was determined from a replicate dish. Chromatin was fragmented by sonication in 20 cycles (20 seconds on/40 seconds off) applied by the Sonopuls GM70 homogenizer (Bandelin), leading to fragments between 150 and 1,000 bp (Fig. 2B), and purified according to the protocol of the EZ-Magna ChiP HiSens Kit (Merck Millipore). Immunoprecipitation was carried out with a chromatin immunoprecipitation (ChIP)-validated SP1 antibody (Merck Millipore), the anti-H3K4 control antibody or an IgG negative control, and magnetic protein A/G beads. Resulting DNA fragments were quantified by qRT-PCR (primers are given in Supplemental Materials S1).

Cell culture, transfection, and clonogenic assay

MKN45 were obtained from the German Collection of Microorganisms and Cell cultures (DSMZ) and cultured in RPMI1640 Medium (PAA) supplemented with 20% FCS (FCS GOLD, Invitrogen). For transfection experiments, FCS concentration was lowered to 10%. MKN74 cells were purchased from the Japanese Health Science Research Resource Bank and grown in RPMI containing 10% FCS GOLD. HEK293eRNA cells (Invitrogen) were maintained in DMEM with 10% FCS GOLD, all in a

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Humidified atmosphere. After receipt, the gastric cell lines were reauthenticated using the AmpFISTR Identifiler PCR Amplification Kit (Thermo Fisher Scientific) and a 3500 Genetic Analyzer (Applied Biosystems). Cells were split upon subconfluency by short treatment with trypsin once or twice a week and were not cultured for longer than 20 passages. To stimulate Wnt signaling, recombinant Wnt3a (BioCat), RSPO1 (Life Technologies), or RSPO2 (antibodies-online) were added at a final concentration of 10 nmol/L each. To inhibit SP1-mediated gene expression, mithramycin A (MMA, 10 nmol/L, Biomol) was applied.

Promoter studies by luciferase reporter assay

Fragments of the human LGR5 promoter were PCR amplified from MKN45 or MKN74 genomic DNA and cloned into the pGL3prom vector using the restriction sites of KpnI and HindIII (Fig. 1B, primer sequences in Supplementary Material S1). The SV40 promoter fragment in the recipient vector was thereby removed. The religated vector without insert was named pGL3basic and served as control. Constructs were verified by sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Potential transcription factor–binding sites were determined using TFsearch (http://diyhpl.us/~bryan/irc/protocol-online/protocol-cache/TFSEARCH.html). The plasmid pN3-SP1 was gratefully received from Prof. Suske (Philips University Marburg, Marburg, Germany). HEK293ebna cells were seeded at 10,000 cells/well density in white sterile 96-well plates and transfected at day 2 with Lipofectamine LTX including PLUS reagent (Invitrogen) with molar ratios of 100:20:10 (ng, promoter construct:pN3-SP1:pRenilla). Promoter activity was analyzed 24 hours later by detection of firefly and Renilla luciferase activity with the DualGlo-Assay (Promega) according to the manufacturer’s protocol using the Synergy Mx luminometer (BioTek).

Statistical analyses

Statistical analyses were done with the PASW 20.0 statistical software (IBM SPSS Statistics) and Prism4 software (GraphPad Software Inc.). Cell culture data were analyzed by the paired t test (Fig. 1, 5) or the Mann–Whitney U test (Fig. 6), whereas histochemical data were evaluated using the two-sided Fisher exact test for nominal variables or Kendall tau rank-order correlation for trends within a group. Effects of multiple testing were accounted for by applying the explorative Simes (Benjamini–Hochberg) procedure (33). The median overall survival was determined by the Kaplan–Meier method, and significance was assessed by the log-rank test. The results were considered significant with P < 0.05.

Results

Role for SP1 in LGR5 expression

Dysregulation of promoter methylation is a common feature in carcinogenesis. We therefore investigated a possible epigenetic
regulation of LGR5 expression by analyzing the first intronic region of the LGR5 gene from 48 gastric cancer patients (Table 1; three missing values) with bisulfite sequencing. Comparing tumor regions against the matching nonmalignant controls, we were unable to detect significant changes in the methylation pattern of the LGR5 gene (Fig. 1A). Similarly, treatment of gastric cancer cell lines with 5-aza-C for demethylation did not lead to differential expression of LGR5 (see Fig. 6C), arguing against direct or indirect effects of methylation in regulating LGR5 expression under these conditions.

Therefore, we aimed to identify promoter regions responsible for active LGR5 expression. A 1.5-kb fragment of the human LGR5 promoter was cloned into the regulatory space of the pGL3 vector coding for firefly luciferase, and serial deletions were made to detect critical binding regions. Interestingly, omission of the T-cell factor/lymphoid enhancer factor (TCF/LEF)–containing fragment appeared to be scarcely relevant for transcriptional activity under these conditions, whereas the region +638 to +349 was shown to be crucial for transcription of LGR5 (Fig. 1B). Within this region, one SP1-binding site was predicted. By cotransfection, SP1 proved relevant for LGR5 expression, although inhibition of SP1-mediated gene expression by MMA did not significantly decrease the luciferase signal (Fig. 1C). This result was further substantiated by cell culture experiments, in which SP1 overexpression in HEK293 cells (Fig. 1D) led to increased LGR5 mRNA levels (Fig. 1D).

To rule out any indirect effect, binding of SP1 to the LGR5 promoter was verified by ChIP assays. With an antibody directed against SP1, DNA promoter fragments from ultrasound fragmented chromatin preparations (Fig. 2B) were precipitated and analyzed by qRT-PCR. As positive control, the SP1-binding domains of the ADAM17 promoter (Szalad 2009/2012) were used (Fig. 2D). In both MKN45 and HEK293 cells, the SP1-binding fragment of the LGR5 promoter was specifically enriched with the SP1-directed antibody. K-ras does not contain any SP1-binding site and was only unspecifically precipitated. According to the expression levels (A), isolation of the LGR5 fragment was superior from HEK293 cells (n = 4) compared with MKN45 (n = 2).

Expression of RSPOs in the gastric mucosa

LGR5 function is regulated by RSPOs, which allow the formation of a complex with the ubiquitin-ligase Rnf43 or its close relative Znrf3. Internalization of the ternary complex LGR5–RSPO–Rnf43 abrogates the inhibitory effect on Wnt receptors, thereby stabilizing a robust Wnt signal (16).
investigate the availability of RSPOs in the gastric epithelium, we performed IHC stainings on whole-mount nonneoplastic stomach sections from sleeve gastrectomy specimens. Expression of both RSPO1 and 2 was readily detected in epithelial cells of the gastric neck and gland (Fig. 3) with contrasting intensities. RSPO1 showed a cytoplasmic staining with a gradual increase of staining intensity from negative luminal areas to the immunopositive gland base and scattered strongly expressing cells mostly residing in the lower gland section (Fig. 3A and 3A2). In contrast, RSPO2 staining identified distinct immunopositive cells within the isthmus and neck areas to the immunopositive gland base and scattered strongly expressing cells mostly residing in the lower gland section (Fig. 3A and 3A2). This arrangement of RSPO1 and 2 expression, together covering the proposed stem cell regions (Fig. 3C), led us to speculate about a possible role for RSPOs in defining the stem cell compartment and influencing differentiation directions within the corpus epithelium.

Role of RSPOs in gastric cancer

In gastric cancer specimens, RSPO expression was found more homogenously distributed among tumor cells with often lower staining intensities compared with nonmalignant tissues (Fig. 4). Although the Histoscore analysis detected no difference between gastric cancer and normal tissue (Fig. 5A and B; Table 1), RSPO expression levels evaluated by qRT-PCR were clearly decreased in gastric cancer and normal tissue (Fig. 5A and B; Table 1), RSPO expression in the gastric cell lines MKN45 and MKN74 was hardly detectable. Accordingly, MSP analysis showed no methylation at either locus in HEK293ebna cells. In contrast, in MKN45 and MKN74, the RSPO2 locus was highly methylated; for RSPO1, both PCR products were detected (Fig. 6B).

Table 1. Correlation of RSPO1-2 expression with clinicopathologic characteristics in gastric cancer patients analyzed with the two-sided Fisher exact test, for trends with the Kendall tau test and significance after multiple testing as indicated.

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Methylation controls the expression of RSPOs

The overall decrease in RSPO staining intensities in gastric cancer prompted us to check the methylation state of RSPO1 and 2. We therefore established MSP (Fig. 6A) and compared the results of MSP (Fig. 6B) to qRT-PCR expression values for three different cell lines. Whereas HEK293ebna cells showed distinct expression of RSPO1 and RSPO2 (Fig. 6C), RSPO expression in the gastric cell lines MKN45 and MKN74 was hardly detectable. Accordingly, MSP analysis showed no methylation at either locus in HEK293ebna cells. In contrast, in MKN45 and MKN74, the RSPO2 locus was highly methylated; for RSPO1, both PCR products were detected (Fig. 6B).
We then subjected the gastric cancer cell lines to treatment with 5-aza-C, having determined that 1 μmol/L 5-aza-C was the optimal dose to inhibit methylation without toxic effects in a dose–response analysis (data not shown). After 3 days, a shift to the unmethylated PCR product was detected by MSP (Fig. 6B), correlating with reliable induction of RSPO1 expression in both cell lines. The effect for RSPO2 was even greater in MKN45, whereas in MKN74, expression was only partially restored (Fig. 6C). These results suggest methylation within the promoter regions responsible for the decreased RSPO1/2 expression. Indeed, we observed distinct methylation patterns within the gastric mucosa of nonneoplastic and tumor tissues of gastric cancer patients. In a small random selection from our gastric cancer cohort, methylation of RSPO1 and RSPO2 was detected in all five samples (Fig. 6D). For 3 patients, methylation was elevated in tumors, compared with their normal tissue. The other 2 patients showed higher methylation levels already in the normal tissue, possibly arguing for an early onset of epigenetic changes during gastric tumorigenesis. Therefore, methylation seems to be an important mechanism to regulate gastric RSPO expression.

To clarify the surprising results regarding higher number of expressing cells in gastric cancer versus repression by methylation, we performed cell culture experiments with recombinant RSPO proteins. Incubation of MKN45 with Wnt3a and RSPOs, which increased Wnt target gene expression as already shown (34), also induced morphologic changes in the semiadherent culture favoring the flattened cell type (Fig. 6E). By qRT-PCR analysis, an increase in vimentin expression was detected, whereas no significant change in E-cadherin expression occurred (Fig. 6F), suggesting a stimulating effect of RSPOs for epithelial–mesenchymal transition in this culture system.

In summary, we provide evidence for a possible role of SP1 in elevating LGR5 expression and the dysregulation of RSPO expression by methylation in the diseased stomach mucosa. Positivity for RSPO1+2 correlated with LGR5 positivity, and these changes were associated with morphologic changes in gastric cancer cell culture.
Discussion

The importance of LGR5+ cells in regenerating gastrointestinal epithelia has been supported in many recent studies (35). In the intestine, LGR5+ cells are localized at the base of the glands in close relation to Paneth cells, which provide Wnt and EGFR ligands (36). In the murine stomach, LGR5+ cells mainly reside in the pyloric glands (10). As lineage tracing studies are not available in humans, LGR5 expression has been confirmed by in situ hybridization, RNAscope, or antibodies with controversial results regarding the localization of LGR5+ cells (37–39). With our recently established highly specific LGR5 antibody, we were able to confirm the localization of immunopositive cells in not only the distal but also the neck region of human gastric glands (Fig. 3; 24). This particular localization raises the question of the underlying regulatory and restricting mechanisms maintaining stem cell functions and tissue homeostasis.

As target gene of the Wnt signal pathway, the LGR5 promoter contains two typical TCF/LEF-binding domains. Furthermore, the involvement of GATA-6 was proven for LGR5 expression in the colon (40), and a role for A专项 was identified in the murine intestine (41). Here, we provide evidence for a relevant impact of SP1 in the regulation of LGR5 expression. By rational promoter analysis, we identified a promoter region from +638 to +349 (similar to the region identified by Tsuji and colleagues; ref. 40) containing one binding site for SP1 and providing first evidence for its biological function. SP1 belongs to the family of SP/KLF transcription factors and interacts by its zinc finger motif directly with GC-rich promoter elements. Thereby, SP1 is involved in important biological processes like cell growth and differentiation, immune responses, or chromatin remodeling (42). Aberrant expression of SP1 was positively correlated with tumorogenesis of the stomach (43). Using ChIP experiments, we found a direct interaction of SP1 with the LGR5 promoter. SP1-mediated expression has also been established for other stem cell markers, such as ABCG2, a transporter characterizing the so-called side population and accounting for elevated multi drug resistance (44). As SP1 is hypoxia induced (45), a stimulatory influence of ischemic conditions within the tumor milieu is conceivable, and increased SP1-mediated expression of LGR5 and other proteins accounting for stem cell properties could exert synergistic effects and additionally fuel the cancer stem cell phenotype.

Recently, RSPOs have been introduced as potentiators of LGR5-stimulated Wnt signaling. In vivo, overexpression of RSPO1 increases crypt cell proliferation in the small intestine and colon (46). Furthermore, RSPO1 supply is essential for the maturation of miniguts in organoid cultures (10), implying a fundamental role for RSPOs in the maintenance of LGR5 stem cell function as driver for tissue regeneration. Nevertheless, in colorectal cancer, RSPO2 has been shown to suppress tumor growth, and RSPO2 promoter methylation was reasoned to allow unrestricted tumor growth (32). Our investigations of RSPO1 and RSPO2 expression in gastric cancer cell culture after 5-aza-C treatment as well as promoter methylation in gastric cancer patients. To determine whether elevated methylation is based on an active methylation process or due to the expansion of a certain cell population, carrying the elevated methylation is based on an active methylation process or due to the expansion of a certain cell population, carrying the methylated sequences will necessitate further investigations.

These data fit well with the observation of a decreased RSPO staining intensity in gastric cancer. Aberrant DNA methylation is a common feature of carcinogenesis arising in early stages, accounting for fundamental gain- or loss of function of the affected genes and presumably driving tumor progression. One prominent example is E-cadherin, a tumor suppressor that is silenced in various cancers, including gastric cancer. In contrast, neither in...
gastric cancer cell cultures nor in patient samples did we find evidence for an epigenetic control of LGR5 expression, supporting the hypothesis that mainly the ligands and regulators of the Wnt signaling cascade are controlled by methylation within diverse human cancer entities (47).

We already reported the relevance of specific LGR5⁺ cell localization for the prognostic prediction of gastric cancer patients (24). Although in the nonneoplastic mucosa, only few single LGR5⁺ cells could be detected, in gastric cancer patients, patches of LGR5 tumor cells were detected, including also luminal areas and showing strong staining intensities at the invasive front. These observations might indicate a change in the stem cell compartment, which allows for unorganized growth and aberrant differentiation behavior. With the observation of differential RSPO expression within the stomach epithelium, such mechanism could be identified. It is intriguing to speculate about RSPOs as critical factors determining and restricting the Wnt-dependent stem cell niche and affecting migration direction or differentiation. Which R-spondin is relevant for the preservation of stem cell properties, or whether a combination of both is needed, still needs further investigations. In gastric cancer cell culture, both RSPOs enhanced the mesenchymal phenotype and induced Wnt target gene expression as already shown in HEK293 (8, 9). In contrast to the suggested action of RSPO2 as tumor suppressor in the colon, our results of a concomitant expansion of LGR5⁺ cells in relation to RSPO expression imply a positive stimulation of the Wnt active stem cells by RSPOs in the stomach epithelium (visual overview).

Collectively, our data and recent deep sequencing data provide evidence of a complex and diverse mechanism, by which the WNT signaling pathway and gastric stem cells can be deregulated, for example, mutations of the APC, CDH1, and RNF43 genes or methylation of CTNNB, RSPO1, and RSPO2 (visual overview).

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Figure 5.
Correlation of RSPO expression to LGR5 expansion in gastric cancer. A and B, The Histoscore result for RSPO1 and 2 staining is not changed in tumor tissue (TU) compared with nonmalignant tissue (NT). Individual changes in the score result are shown below (n = 52). C and D, Total RSPO1 and 2 mRNA levels are significantly decreased in gastric cancer patients compared with their matched control tissue. Bar plot depicts the expression value difference for each case (n = 50). E–G, IHC staining of LGR5 (E), RSPO1 (F), and RSPO2 (G) on serial sections of an intestinal-type gastric cancer (×250 magnification). Brownish staining reveals concomitant expression of LGR5 and its ligands in close proximity. H and I, Splitting up the Histoscore result, the overall number of RSPO1/2⁺ cells significantly increases in gastric cancer patients (H). Nevertheless, intensity scores are negatively affected compared with nonmalignant tissue (I). K, Changes in the percentage of immunostained RSPO1 or 2 and LGR5 cells are correlated.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Denisa Hajzeri and Marten Röckendorf for excellent technical support.

Grant Support
This work was supported by a grant of the German Research Foundation (DFG) to C. Röcken (Ro-1173/12-1).

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Received December 19, 2016; revised January 17, 2017; accepted February 4, 2017; published OnlineFirst February 20, 2017.

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Molecular Cancer Research

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Mol Cancer Res  Published OnlineFirst February 20, 2017.

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