Lgr4 deficiency increases susceptibility and severity of dextran sodium sulphate-induced inflammatory bowel disease in mice*

Shijie Liu¹⁴, Yu Qian¹⁴, Liang Li¹, Gaigai Wei¹, Yuting Guan¹, Hongjie Pan¹, Xin Guan¹, Long Zhang¹, Xiaoling Lu², Yongxiang Zhao², Mingyao Liu¹,³ and Dali Li¹

¹ Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China
² Biological Targeting Diagnosis and Therapy Research Center, Guangxi Medical University, 22 Shuang Yong Rd., Nanning, Guangxi 530021, China
³ The Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, Texas 77030, USA
⁴ These authors contributed equally to this work

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To whom correspondence should be addressed: Dr. Dali Li and Dr. Mingyao Liu The Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China E-mail: dlli@bio.ecnu.edu.cn or myliu@bio.ecnu.edu.cn

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Background: Lgr4 as a transmembrane receptor binds with R-spondins to modulate Wnt signaling.

Results: Loss of epithelial barrier function in Lgr4 deficiency mice which is highly susceptible to inflammatory bowel disease.

Conclusion: Lgr4 mediated Wnt/β-catenin signaling is required for intestinal homeostasis and regeneration.

Significance: Lgr4 has the potential implications for diagnosis or drug discovery for human colitis.

SUMMARY

Lgr4 is one of the newly identified R-Spondins receptors and potentiates Wnt signaling which regulates intestinal homeostasis. We used a hypomorphic mouse strain to determine the role of Lgr4 in intestinal inflammation and recovery. Intestinal inflammation was induced with dextran sulfate sodium (DSS), followed by a recovery period. Intestinal inflammation symptoms and molecular mechanisms were examined. We found that Lgr4⁻/⁻ mice exhibited dramatically higher susceptibility and mortality to DSS-induced inflammatory bowel disease (IBD) than WT mice. Lgr4 deficiency resulted in greatly reduced numbers of either Paneth cells or stem cells in the intestine. During the intestinal regeneration process, cell proliferation but not apoptosis of intestinal epithelial cells (IECs) was significantly impaired in Lgr4⁻/⁻ mice. When Wnt/β-catenin signaling was re-activated by crossing with APC⁺/- mice or by treating with a GSK-3β inhibitor, the number of Paneth cells was partially restored and the mortality caused by DSS-induced IBD was strikingly reduced in Lgr4 deficient animals. Thus, Lgr4 is critically involved in the maintenance of intestinal homeostasis and protection against inflammatory bowel disease through modulation of the Wnt/β-catenin signaling pathway.

Inflammatory bowel disease (IBD) refers to...
several inflammatory conditions of the colon and small intestine. Clinically, there are two main forms of IBD—Crohn’s disease, which can affect any part of the gastrointestinal tract, and ulcerative colitis which is restricted to the colon and the rectum (1,2). The precise etiology of IBD remains unclear, but several factors have been identified to play major roles in intestinal homeostasis, including microbial defence, innate/adaptive immunity, and autophagy among others (3,4). Disorders of barrier function and epithelial restitution are the major intrinsic pathogenic factors which are regulated by specific signaling pathways in response to harmful microenvironmental factors.

In mammals, intestinal epithelial cells (IECs), establish tight contacts with numerous adjacent epithelial cells to form a single layer that functions as a physical barrier (5). The intestinal epithelium undergoes constant cell turnover through shedding into the intestinal lumen and is the most vigorously renewing adult tissue. Like other somatic stem cells, multipotent intestinal stem cells (ISCs) constitute a long-lived population of cells which have both self-renewal and differentiation abilities (6). Bmi1 and Lgr5 mark two distinct types of ISCs based on their location and cycling properties (7-10). Both of these two populations give rise to all the differentiated cell lineages of the intestinal epithelium, and the interconversion between +4 (quiescent) and crypt base columnar (fast-cycling) stem cells in their niches was reported (7-9). ISCs and their transit-amplifying daughter cells give rise to enterocytes, goblet cells and enteroendocrine cells that occupy the villi and are renewed about every 3 to 5 days in mice (5). Paneth cells are terminally differentiated cells which move opposite to the crypt-villus flow and reside in the bottom of crypts for several weeks as a potential niche for ISCs (11). In addition, Paneth cells secrete numerous antimicrobial peptides, which include lysozymes, defensins, cathelicidins and RegIIIγ, and play important roles in innate immune defence (12,13). Several studies on mice deficient in different Crohn’s disease-associated genes involved in reactive oxygen species (14) generation, autophagy and endoplasmic reticulum (ER) stress, have implicated Paneth cell dysfunction in susceptibility to intestinal inflammation (15,16).

As defective epithelial restitution is an important risk factor for IBD, it is not surprising that dysfunction of genes involved in intestinal development, proliferation and differentiation will increase susceptibility to IBD. The Wnt/β-catenin signaling cascade is the single most dominant pathway in controlling proliferation and differentiation of IECs (17). Genetic variants of TCF4, a Wnt signaling pathway transcription factor which is critical for the maintenance of the crypt progenitor phenotype, has been reported to be associated with Crohn’s disease (18). However, little is known about how Wnt signaling functions during intestinal inflammation.

Lgr4, also known as GPR48, belongs to the leucine-rich, G protein–coupled receptor (LGR) family (19). Genetically modified mouse models have demonstrated that Lgr4 plays broad roles in embryonic development as well as postnatal physiological processes in multiple organs (20-22). With a gene trap strategy, we obtained an Lgr4 hypomorphic mouse strain, and 40% of Lgr4 hypomorphic mice are viable and with no significant reduction of lifespan. Using this unique strain, we demonstrated that Lgr4 plays important roles in various organs, including the liver, reproductive tract, bone and eye (23-25). Recently, Lgr4 and its homologue Lgr5 have been identified as receptors of R-spondins, secreted Wnt pathway agonists, and potentiators of Wnt/beta-catenin and Wnt/PCP signaling (14,26). In the intestine, Lgr4 is required for Paneth cell differentiation (27) and maintenance of intestinal stem cells (26). Here, we demonstrate that Lgr4 deficient mice are more
susceptible to DSS-induced colitis. When Wnt/β-catenin signaling is re-activated by crossing with $APC^{min/+}$ mice or by treating with a GSK-3β inhibitor, the decreased number of Paneth cell was partially restored and the mortality caused by DSS-induced IBD was dramatically reduced in $Lgr4$ mutant mice. Taken together, these results underscore the importance of $Lgr4$ mediated Wnt signaling in intestinal homeostasis and inflammation.

**EXPERIMENTAL PROCEDURE**

Mouse lines-$Lgr4^{-/-}$ mice, initially generated on a mixed 129×C57BL/6 background as previously described (25), were backcrossed with C57BL/6 mice for at least 6 generations at the beginning of this study. Heterozygous mice were intercrossed to generate homozygous $Lgr4^{-/-}$ mice and wild-type littermate controls. $Lgr5$-EGFP-IRES-creERT2 mice (Stock NO. 008875) were obtained from The Jackson Lab. $APC^{min}$ mice (Stock NO. J002020) were obtained from the National Resource Center of Mutant Mice (NRCMM). All experiments conformed to the regulations drafted by Association for Assessment and Accreditation of Laboratory Animal Care in Shanghai and were approved by East China Normal University Center for Animal Research.

**Induction of DSS-Induced inflammation**—Acute colitis was induced in mice by the administration of 2.5% (w/v) DSS (molecular weight, 36–50 kilodaltons; MP Biomedicals, Irvine, CA) in the drinking water for 5 days and then changing to regular drinking water.

**Colonic Injury Scoring**—Entire colons were rolled up and fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and stained with hematoxylin/eosin. The degree of colonic injury was assessed by a histology scoring system (28) with minor modifications. Briefly, the score was performed with a combined score of inflammatory cell infiltration (score, 0-3), loss of crypts (score, 0-4), and tissue damage (score, 0-6). The inflammatory cell infiltration score was defined as follows: 0 = inflammatory cells occasionally presented in the lamina propria, 1 = increased number of inflammatory cells in the lamina propria, 2 = confluence of inflammatory cells, extending into the submucosa, 3 = transmural extension of the infiltrate. Loss of crypts: 0 = no involvement, 1 = <25%, 2 = <50%, 3 = <75%, 4 = < 100%. Tissue damage including parameter of mucosal ulceration and depth of injury, for mucosal ulceration: 0 = no injury, 1 = focal injury, 2 = multifocal injury, 3 = diffuse ulceration/infiltration; for the depth of injury: 0 = no injury, 1 = mucosal involvement only, 2 = mucosal and submucosal involvement, 3 = transmural involvement.

**Bone Marrow Transplantation**—WT mice were irradiated with 9Gy and received donor bone marrow ($10^7$ cells) by injection from the vein of tail, and then reconstituted with donor bone marrow for 8 weeks. The success of bone marrow reconstitution was confirmed by PCR.

**β-galactosidase (LacZ) staining**—To perform LacZ staining, intestine was washed with ice-cold LacZ fixation buffer (2% formaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P-40 in PBS), then incubated for 2 hours in fixing buffer at 4°C on a shaking platform. After washing in LacZ washing buffer (2mM MgCl2, 0.01% deoxcholate, 0.02% Nonidet P-40 in PBS), tissues were then fixed and sectioned for histological analysis.

**Immunohistochemistry (IHC)**—The intestine was dissected and flushed gently with cold 2% PFA and rolled up into a compact circle then fixed in 4% PFA-PBS at 4°C in the dark on a rolling platform, dehydrated, embedded in paraffin, and cut into 4 μm sections. Sections were deparaffinized in xylene and rehydrated in gradient alcohol.
activity was quenched with 3% H2O2 in methanol for 20 min and washed in PBS. Antigen retrieval was performed by boiling slides in 10 mM sodium citrate buffer, pH 6.0 or 10mM Tris-base, 1mM EDTA solution, pH 9.0. Sections were blocked with 1% BSA and incubated with the primary antibodies overnight at 4 °C. After incubation with secondary antibody for 30 minutes, sections were developed with DAB and counterstained with hematoxylin, then dehydrated and mounted in neutral resins. For BrdU staining, a similar procedure was performed with the addition of a 30 minute treatment with 2N HCl after antigen retrieval. Antibodies: lysozyme (1:2000; Dako), β-catenin (1:100; BD Transduction), c-Myc (1:100; SantaCruz), cyclinD1 (1:100; Cell Signaling), p-S6 (1:400; Cell Signaling), cleaved-caspase3 (1:200; Cell Signaling), Ki67 (1:2000; Neomarker), Bmi1 (1:2000; SantaCruz).

Flow cytometry-A standardized 3-cm segment of duodenum was isolated, exposed longitudinally and washed with cold PBS. Then tissue was chopped into approximately 5mm pieces, washed with cold PBS and incubated in 5 mM EDTA in PBS for 5 min at room temperature. The tissue fragments were suspended vigorously, the supernatant, including most villi, was discarded. The sediment was resuspended with 5mM EDTA in PBS and incubated for 30 min on ice. After vigorous resuspension, the supernatant was filtered through a 70μm cell mesh (BD Bioscience) and incubated in 1mg/mL trypsin for 30 min at room temperature. For FACS, dead cells were excluded by scatter characteristics and propidium iodide. Lgr5+ ISCs were identified by their endogenous GFP expression.

Electron Microscopy-Small intestine tissue samples were fixed with 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide in 100 mM phosphate buffer. Tissue was dehydrated, embedded in epoxy resin, and visualized with a JEOL transmission electron microscope at 120 kV (model JEM-1210).

Quantitative RT-PCR-Total RNA was extracted using Trizol reagent (Invitrogen). RNA concentration was determined spectrophotometrically and quality was assessed by agarose electrophoresis. cDNA was synthesized with the Superscript RNase H2 Reverse Transcriptase Kit (Invitrogen) using 2.5 mM random hexamers (Invitrogen). Real-time PCR was performed using SyberGreen probe with theI-cyker IQ5 (Bio-Rad).

Statistical Analysis-Data are expressed as means ± SEM. Means of 2 groups were compared using Student’s t test (unpaired, 2-tailed). Comparisons between multiple groups were made using 1-way ANOVA followed by a post-hoc Bonferroni correction, P<0.05 considered to be statistically significant. Unless indicated in the figure legends, all the experiments were performed at least 3 times with similar results.

RESULTS

Lgr4 in the intestinal epithelium protects mice from susceptibility and mortality to DSS-induced IBD-To explore the roles of Lgr4 in intestinal inflammation, Lgr4 hypomorphic mice were subjected to the DSS-induced experimental IBD model. Notably, after DSS administration a more severe colitis developed in Lgr4 mutant mice than in wild-type (WT) sex matched littermates. Strikingly, following five days of treatment with 2.5% DSS, no mortality was observed in WT mice during the experimental period, however all Lgr4 mutant mice died within 8 days after treatment (Figure 1A). None of the Lgr4 mutant mice could survive even when we decreased the DSS concentration to 1% (Data not shown). Higher body weight loss, a hallmark of intestinal inflammation, was observed from day 4 in Lgr4 mutant mice, concordant with a more severe anemia as reflected by the decreased circulating
Lgr4 protects against inflammatory bowel disease

peripheral blood hematocrit concentration (Figures 1B, C). Moreover, an increased daily activity index (DAI, as described in Table 1) was observed in Lgr4 mutant mice, indicating more severe clinical symptoms (Figure 1D). Although the small intestine is not the major target of DSS-induced tissue damage (29), the relative length reduction after DSS-induced inflammation was significantly increased in the small intestine of Lgr4 mutant mice (Figure 1E) indicating critical functions of Lgr4 in the small intestine. Histological examination showed dramatically increased signs of colitis which is characterized by the loss of crypts and the infiltration of leukocytes into the colons of Lgr4 mutant mice (Figures 2A, B). Accordingly, we found that almost all crypts throughout the small intestine were lost in Lgr4 mutant mice but remained intact in WT littermates (Figure 2C). Infiltration of neutrophils in the colon was quantified by the determination of MPO activities. At day 8, MPO levels were significantly higher in Lgr4 mutant mice than in WT mice (Figure 2D), which is consistent with the increase of leukocytes visualized in H&E stained sections (Figure 2A). Additionally, inflammatory cytokines such as TNFα, IL6 and IL1β were significantly increased in Lgr4 mutant mice after DSS administration (Figure 2E), suggesting a more severe inflammatory response in Lgr4 deficient mice.

Lgr4 in nonhematopoietic cells is more important for protection against DSS-induced IBD-To determine the cell populations that are critical for Lgr4-dependent protection against DSS-induced bowel inflammation. Bone marrow transplantation was employed to determine the contribution of immune and/or non-immune cells to the IBD phenotype observed in Lgr4−/− mice. After 8 weeks of bone marrow reconstitution, bowel inflammation was induced by DSS administration. Interestingly, WT mice reconstituted with Lgr4−/− bone marrow did not exhibit an exacerbated inflammation response compared with WT mice transplanted with WT bone marrow (Figure 2F). Upon DSS-induced colitis, the body weight was decreased more dramatically in Lgr4−/− recipients than that of WT recipients despite the genotype of transplanted bone marrows. Furthermore, all these Lgr4−/− recipients died in 6 days but most of the WT recipients were survived (Figure 2F). These results indicate that Lgr4 expression in nonhematopoietic cells is more important for protection against DSS-induced IBD.

Impaired proliferation but not apoptosis of intestinal crypts in Lgr4 mutant mice during tissue regeneration-Lgr4 mutant mice exhibited much severer disease symptoms and disrupted intestinal integrity in DSS-induced intestinal inflammation suggesting a potential role of Lgr4 in regulation of epithelial cell behavior. In the DSS-induced IBD model, after 5 days of DSS treatment the intestinal tissue transits to regeneration process. By IHC against Ki67-positive proliferating cells, we observed significantly decreased cell proliferation in Lgr4 mutant intestines during tissue regeneration (Figures 3A, B and C). However, we observed no significant alteration of apoptosis either in normal condition (Figures 3D, F) or in the recovery period between Lgr4 mutant and WT mice (Figures 3E, F). These results indicated that Lgr4 is responsible for epithelial cell proliferation but not apoptosis during DSS-induced tissue regeneration.

Lgr4 is required for intestinal homeostasis under normal conditions-Taking the advantage of our gene trapped mice with the LacZ reporter integrated in the first intron of the Lgr4 locus (25), we examined the expression pattern of Lgr4 in the intestine by X-gal staining in heterozygous mice. In the small intestine, dense staining signals were observed in Paneth cells which are easily distinguished by large secretory granules in the cytoplasm. In the colon, Lgr4 was mainly expressed in the zone of transit amplifying cells (Figure 4A). By using an
Lgr4 protects against inflammatory bowel disease

unambiguous Paneth cell marker, we observed that the number of Paneth cells was 80–85% lower in Lgr4 mutant mice compared to WT mice which is consistent with a previous report (27) (Figures 4B, C). Furthermore, the mRNA levels of Paneth specific antimicrobial genes, such as Lysozyme, Mmp7, Defa5, Crs1c and Crs4c, were also dramatically downregulated (Figure 4D). Transmission electron microscopy also confirmed that the ultrastructure of the few remaining Paneth cells in mutant mice was obviously altered, suggesting that the secretory function of the remaining Paneth cells in Lgr4 mutant mice was largely impaired (Figure 4E).

With different strategies we also confirmed that the number of Lgr5+ ISC was dramatically reduced (Figure 5A-C). Furthermore, we found that Bmi1+ cells were decreased slightly but significantly in Lgr4 mutant mice (Figure 5D, E). However, the other intestine cell types including Goblet cells and enteroendocrine cells were not significantly altered between WT and Lgr4 mutant mice (data not shown). These data suggest that Lgr4 modulates both Lgr5+ and Bmi1+ ISC either directly or indirectly to maintain intestinal homeostasis, and the deficiencies of ISC in Lgr4 mutant mice may be a reason for the impaired cell proliferation and intestine integrity during DSS-induced inflammation.

Lgr4 deficiency impairs Wnt/β-catenin signaling in intestinal epithelial cells-Lgr4 is one of the receptors of R-spondins which amplify the Wnt/β-catenin signaling pathway (26,30). Given that Wnt/β-catenin signaling is required for intestinal homeostasis and ISC self-renewal (31), we examined how Lgr4 regulates the activity of Wnt signaling in Lgr4 mutant mice. Using immunohistochemical staining, we found markedly less cells with nuclear-localized β-catenin (active form) at the bottom of crypts in Lgr4 mutant mice while higher Wnt/β-catenin activity was observed in the intestinal epithelium in WT mice (Figure 6A). The protein levels of the direct Wnt target genes, such as c-Myc and CyclinD1, were both downregulated in Lgr4-/- mice (Figures 6B, C). Moreover, the activity of mTor signaling, which is demonstrated to be downstream in the β-catenin pathway, was also inhibited as shown by the decreased phospho-S6 level in Lgr4 mutants (Figure 6D). To determine whether Lgr4 affects the mRNA levels of the target genes, we evaluated the expression of β-catenin target genes, including EphB2, Axin2, CyclinD1, Ascl2 and Sox9 using quantitative real-time PCR analysis. Our data indicate the mRNA levels of all these target genes decreased in Lgr4 mutant mice (Figure 6E), suggesting that Lgr4 is required for the maintenance of Wnt/β-catenin signaling in the intestinal epithelium.

The defect of Lgr4-/- mice is partially rescued by the reactivation of the Wnt/β-catenin signaling cascade-To determine whether the functions of Lgr4 in intestinal homeostasis and inflammation are mediated by the Wnt/β-catenin signaling pathway, we generated Lgr4-/-;APCmin/+ mice and examined the effects of APC mutation in restoring the defects caused by Lgr4 deletion. APCmin/+ mice contain a germ-line mutation in the APC gene, which spontaneously activates β-catenin. As shown in Figure 7A, mutation of APC partially restored the activation of β-catenin signaling in Lgr4-/-;APCmin/+ mice. The number of cells with nuclear-localized β-catenin increased significantly in Lgr4-/-;APCmin/+ mice. Furthermore, the APC mutation increased the expression of c-Myc in the Lgr4-/- crypts (Figure 7B), suggesting re-activation of the Wnt/β-catenin target genes. Moreover, the number of Paneth cells was restored in Lgr4-/-;APCmin/+ mice compared with Lgr4 mutant mice (Figures 7C, D). Interestingly, the deficiency of Lgr4 in Apcm+/+ background did not delay or decrease adenoma formation (Figure 7A-C right panel). To further confirm that Lgr4 functions through Wnt/β-catenin
signaling in intestines, we reactivated β-catenin by administration of a GSK3β inhibitor SB216763 in Lgr4 mutant mice. As shown in Figure 7E, addition of SB216763 partially restored the nuclear localization and activation of β-catenin in Lgr4 mutant mice. Moreover, treatment of SB216763 largely restored the number of Lysozyme positive Paneth cells (Figure 7F). These data suggest that reactivation of Wnt/β-catenin signaling in Lgr4 mutant mice partially rescued the intestinal homeostasis defects, and we also hypothesize that Wnt signaling reactivation helps protect the mice from intestinal inflammation.

To address this issue, we pretreated Lgr4 mutant mice with SB216763 for two days then subjected them to DSS treatment to induce IBD (Figure 8A). The survival rate was significantly increased compared with Lgr4 mutant mice injected with vehicle (83% versus 0) (Figure 8B). The body weight of SB216763 treated Lgr4-/- mice increased slightly from day 10-11, indicating the improvement from colitis (Figure 8C), which was also confirmed by histology analysis (Figure 8D, E). Accordingly, we observed re-activated Wnt/β-catenin signaling in SB216763 treated Lgr4-/- mice (Figure 8F), and the infiltration of neutrophils also reduced in SB216763 treated Lgr4-/- mice compared with controls through histological analysis (Figure 8D inserts) and MPO studies (Figure 8G). These observations strongly suggest that the defects in Lgr4-/- mice are mainly due to hypoactivation of Wnt/β-catenin signaling, and support the hypothesis that Lgr4 is a key receptor in intestinal homeostasis and inflammation.

**DISCUSSION**

Our studies demonstrate that Lgr4 plays a crucial role in recovery from inflammatory bowel disease. Using an Lgr4 hypomorph gene-trap mouse strain (25), we found a striking susceptibility to DSS-induced IBD in Lgr4-/- mice. We confirmed that Lgr4 deficiency causes a dramatic loss of Paneth cells as well as Lgr5+ ISCs in mice. Furthermore, when Wnt/β-catenin signaling is re-activated by using APCmin/-Lgr4-/- mice or by treating with a GSK-3β inhibitor, the number of Paneth cells was partially restored and the mortality caused by DSS-induced IBD was strikingly reduced. Moreover, adenoma initiation is not altered between APCmin/+ and APCmin/-;Lgr4-/- double mutant mice suggesting that not only is Lgr4 regulation of the Wnt/β-catenin signaling pathway important for intestinal homeostasis and wound repair, but also that Lgr4 functions upstream of the APC/GSK-3β complex.

Lgr4 is one of the receptors for R-spondin proteins which synergize with low levels of Wnt to enhance Wnt signaling activity by inhibition of ZNRF3 mediated turnover of frizzled and LRP6 (32). In Tcf4-/- neonatal mice, the crypt progenitor compartment is entirely absent (33), but in adult Lgr4-/- mice only Paneth cells and Lgr5+ ISCs are affected. Since Lgr4 binds to R-spondins and is responsible for the hyperactivation of Wnt signaling, it suggests that Paneth cells and Lgr5+ ISCs are dependent on higher Wnt activities than other compartment of the crypt. Upon DSS-induced intestinal inflammation, all Lgr4 mutant mice died within 8 days even at the low DSS concentration of 1% which usually does not induce significant IBD symptoms in wild-type mice. These observations again suggested that higher Wnt signaling activity is critical for intestinal regeneration and protection against IBD.

Lgr4 is required for Paneth cell differentiation and the maintenance of Lgr5+ ISCs in normal conditions (26, 27). Recently, several processes have been highlighted to be important to Paneth cell biology and susceptibility to IBD in both humans and mice, such as the regulation of necroptosis, granule exocytosis and the ER stress response (15,16,34). However, ablation of Paneth cells in mice did not produce detectable effects on the proliferation or terminal
Lgr4 protects against inflammatory bowel disease

differentiation programs of the other three lineages or on host-microbial interactions (35). We also found that loss of Paneth cells did not cause spontaneous inflammation. It is seemed that loss of Paneth cell in IBD patients probably is not the cause of IBD. We speculate that under stressed conditions such as DSS treatment, the severity of IBD in Lgr4 mutant mice is mainly due to the dysfunction of the epithelial barrier, since during the recovery process Lgr4 mutant mice exhibited dramatically decreased epithelial proliferation compared with WT mice (Figure 3). Additionally, although we have not examined the function of Lgr5+ ISCs during intestinal inflammation and regeneration, the decreased number of Lgr5+ ISCs in Lgr4 mutant mice might decrease epithelial cell turnover which further leads to the loss of epithelial integrity since this stem cell is the source of epithelial cells in the intestine.

Interestingly, we observed a slightly decreased number of Bmi1+ ISCs, which is different from the report that the number of Bmi1+ cells was elevated when Lgr5 positive stem cells were depleted (36,37). Because the Lgr4 mutant mouse model is different from the Paneth cell depletion models, we speculate that Lgr4 could regulate Bmi1+ stem cell numbers, which was supported by Mustata et al., who demonstrated that the Bmi1 mRNA level was decreased in Lgr4 mutant crypts (27). Moreover, Yan and colleagues reported that Lgr5+ ISCs were dependent on R-spondin1, which was also essential for in vitro culture of Bmi1+ ISCs (36).

The Wnt/β-catenin signaling pathway has an essential role not only in intestinal development but also in homeostasis and regeneration after injury(31). A notable feature of intestinal inflammation is a persistently increased expression of mucosal cytokines which modulate intestinal homeostasis in a biphasic manner (38,39). For example, at the onset of inflammation, INF-γ activates β-catenin through the PI3K-AKT pathway and promotes epithelial proliferation, but activation of β-catenin in turn facilitates the induction of the secreted Wnt antagonist Dkk1 to inhibit cell proliferation and promote apoptosis (38). Deletion of Dkk1 induces a strong proliferative response that promotes wound repair after colitis (40). Moreover, PI3K signaling mediates β-catenin activation in intestinal epithelial stem and progenitor cells in colitis (41). These reports reveal that Wnt/β-catenin signaling is an important regulator of inflammatory cytokine responses and homeostasis during inflammation(17). In the subsequent regeneration process, Wnt/β-catenin signaling also plays an important role by activating its downstream targets such as c-Myc and mTOR(42).

In summary, we have demonstrated that Lgr4 is essential for the maintenance of intestinal homeostasis and protection from DSS-induced IBD in mice. Using different strategies, we also demonstrated that Lgr4 directly modulates Wnt/β-catenin signaling in animal models. Since Paneth cell dysfunction or abnormal expression of Paneth cell associated genes was reported to be correlated with both intestinal inflammation and cancer (34,43), the identification of the essential roles of Lgr4 in IBD represents an advance that has implications for diagnosis or potential drug targets for human colitis.
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FOOTNOTES
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1 Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China

2 Biological Targeting Diagnosis and Therapy Research Center, Guangxi Medical University, 22 Shuang Yong Rd., Nanning, Guangxi 530021, China

3 The Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, Texas 77030, USA

4 These authors contributed equally to this work

Table 1  Scoring of the Disease Activity Index

| Score | Weight loss | Stool consistency | Bleeding |
|-------|-------------|-------------------|----------|
| 0     | None        | Normal            | Normal   |
| 1     | 1–5%        | –                 | –        |
| 2     | 5–10%       | Loose             | Hemoccult + |
| 3     | 10–20%      | –                 | –        |
| 4     | >20%        | Diarrhea          | Gross bleeding |

The DAI is combined with weight loss, stool consistency and rectal bleeding, leading to a maximum DAI of 12.
FIGURE LEGENDS

FIGURE 1. Deletion of Lgr4 increases susceptibility and mortality to DSS-induced inflammation. Mice were subjected to DSS-induced colitis for 5 days, followed by regular drinking water. Survival rate (A), body weight (B), hematocrit values of peripheral blood (C) and mean Disease Activity Index (DAI) (D) were determined. (WT, n=10; Lgr4+/−, n=6; Lgr4−/−, n=12. Data are mean±SEM. *P<0.01; **P<0.001). Mice were euthanized, the entire intestines were obtained in both WT and Lgr4 mutant mice. The lengths of small intestines and colons were measured. The alteration of the length of both small intestines and colons were analyzed after DSS treatment compared with regular drinking water control mice (E). CTR: control, DSS: 2.5% DSS in drinking water for 5 days followed by 3 days of regular drinking water. n≥3 for each group. Data are mean±SEM.

FIGURE 2. Severer intestine inflammatory in DSS-induced Lgr4−/− mice. (A, C) H&E staining revealed severer disease activity in both colon (A) and jejunum (C) of Lgr4−/− mice on day 8. Note more infiltration of leukocytes in Lgr4−/− mice. Scale bars: 50μm. The histology score was measured in (B). (D) Colon MPO activity as measure of neutrophil infiltration. (E) Real-time PCR analysis revealed the relative expression of cytokines in colonic mucosa of WT and Lgr4−/− mice at day 5 of DSS administration. Data are mean±SEM. *P<0.01; **P<0.001. n≥5. (F) Mice were reconstituted with donor bone marrow for 8 weeks, then bowel inflammation was induced by 2.5% DSS (w/v) in drinking water for 5 days followed by normal drinking water; weight loss and survival rate were monitored every day. WT receipt mice (n=6), Lgr4−/− receipt mice (n=5). Data are mean±SEM.

FIGURE 3. Impaired proliferation but not apoptosis of intestinal crypts in Lgr4 mutant mice during recovery. Colon (A) and jejunum (B) sections from WT and Lgr4−/− mice on day 8 of DSS-induced colitis, proliferation were identified by Ki67 staining (arrowhead). The numbers of Ki67 positive cells were analyzed in (C), SI: small intestine (40-60 crypts per mouse; n=3 per group. Data are mean±SEM. *P<0.01; **P<0.001). (D, E) Cleaved Caspase3 positive cells (arrowhead) were detected through IHC with specific antibodies in mice in normal conditions (D) or after treatment with DSS (E). Scale bars: 50μm. (F) Statistic indicated the numbers of apoptosis cells in WT and Lgr4−/− mice. CTR: control, DSS: 5 days treatment with DSS followed by 3 days recovery. Data are mean±SEM. ns: no significant.

FIGURE 4. Lgr4 is required for differentiation of Paneth cells. (A) Whole-mount X-gal staining was performed on the ileum (left) and colon (right) of Lgr4−/− mice; note the granular particles observed in Paneth cells in the enlargement. (B) Paneth cells were assessed by IHC staining with anti-Lysozyme antibody. (C) The number of Paneth cells in mice was analyzed by IHC with anti-Lysozyme antibody (40-60 crypts per mouse; n=5 per group. Data are mean±SEM. **P<0.001). (D) Quantitative real-time PCR analysis of the expression level of Paneth cell marker genes. Data are mean±SEM. (E) Ultrastructure of Paneth cells was explored with electron microscopy. CBC cells were easily recognized (white asterisks) and Paneth cell was indicated by white dashed circle.

FIGURE 5. Lgr4 is required for maintenance of intestinal stem cells in adult mice. Endogenous GFP (green) driven by Lgr5 promoter indicate CBC stem cells in Duodenum (A) or Colon (B) of...
Lgr4 protects against inflammatory bowel disease

Lgr5\textsuperscript{GFP/+};Lgr4\textsuperscript{-/-} (left) and Lgr5\textsuperscript{GFP/+};Lgr4\textsuperscript{-/-} (right). (C) FACS analysis demonstrated the ratio of GFP-positive ISCs was significantly decreased in Lgr5\textsuperscript{GFP/+};Lgr4\textsuperscript{-/-} mice. Lgr5\textsuperscript{-/-} (13.2\% ± 1.62\%) compared with Lgr5\textsuperscript{+/-};Lgr4\textsuperscript{-/-} (0.61 ± 0.11\%). (D) Duodenum sections from WT or Lgr4\textsuperscript{-/-} mice were prepared. Bmi1\textsuperscript{+} ISCs were detected by IHCs with Bmi1 specific antibodies (arrowhead). (E) Statistic data indicating the number of Bmi1\textsuperscript{+} ISCs in WT and Lgr4\textsuperscript{-/-} mice. 300 crypts per mouse from 4 different pairs of WT and Lgr4\textsuperscript{-/-} mice were examined. Data are mean ± SEM. *P < 0.01. Scale bars: 50μm

**FIGURE 6.** Downregulation of Wnt target genes in Lgr4\textsuperscript{-/-} mice. (A) Nuclear β-catenin was observed at the bottom of crypts in WT mice (left) but not in that of Lgr4\textsuperscript{-/-} mice (right). (B, C) The Wnt/β-catenin targets, c-Myc and CylinD1, were dramatically down-regulated in Lgr4\textsuperscript{-/-} mice as detected by IHC. (D) mTOR downstream target, phospho-S6, was decreased in Lgr4\textsuperscript{-/-} mutant mice. Scale bars: 50μm. (E) Quantitative real-time PCR analysis of mRNA levels of Wnt target genes in WT and Lgr4\textsuperscript{-/-} littermates. Data are mean ± SEM.

**FIGURE 7.** The phenotypes are partially restored in Wnt/β-catenin reactivated Lgr4\textsuperscript{-/-} mutant mice. In Lgr4\textsuperscript{-/-};APC\textsuperscript{min/+} double mutant mice Wnt target genes were reactivated. The number of cells with active (nuclear localized) β-catenin was increased in Lgr4\textsuperscript{-/-};APC\textsuperscript{min/+} compared with Lgr4\textsuperscript{-/-} mice (A). The expression of c-Myc was increased in crypts in Lgr4\textsuperscript{-/-};APC\textsuperscript{min/+} mice (B). IHC with antibody against Lysozyme indicated the recovery of Paneth cells in the epithelium of Lgr4\textsuperscript{-/-};APC\textsuperscript{min/+} mice (C). The right panel presents the microadenoma in small intestines. A: Mean adenoma. (D) The number of Paneth cells in mice was analyzed by IHC with Lysozyme specific antibody (40-60 crypts per mouse; n=3 per group. Data are mean ± SEM. *P<0.01). (E, F) Lgr4\textsuperscript{-/-} mice received 2mg/kg SB216763 intraperitoneally every day for 5 days and euthanasia in day 8. Sections of the small intestine from mice of indicate genotypes were stained with β-catenin antibody (E) or Lysozyme (F). Scale bars: 50μm.

**FIGURE 8.** Inhibition of GSK3β activity ameliorates experimental colitis in Lgr4\textsuperscript{-/-} mutant mice. (A) Scheme of treatment with SB216763 in Lgr4\textsuperscript{-/-} mice during DSS-induced colitis. (B, C) Lgr4\textsuperscript{-/-} mice were treated with SB216763 and then subjected to 1% DSS-induced colitis for 5 days. Survival rates (B) and body weight changes (C) were monitored, SB: abbreviation of SB216763. (n=6 per group. Data are mean ± SEM. *P<0.01). (D) On day 8 of DSS-induced colitis, intestinal sections from mice of WT, Lgr4\textsuperscript{-/-} and Lgr4\textsuperscript{-/-} treated with SB216763 were stained with H&E (Insets indicating the infiltration of leukocytes), and the histological score was measured (E). (n=3 per group. Data are mean ± SEM. *P<0.01). (F) IHC with antibodies of β-catenin and c-Myc indicates reactivated in SB216763 treated Lgr4\textsuperscript{-/-} mice. Scale bars: 100μm. (G) Colon MPO activity was measured for neutrophil infiltration. (n=3 per group. Data are mean ± SEM. *P<0.01; **P<0.001).
Lgr4 protects against inflammatory bowel disease

Figure 1

A. Survival rates (%)

B. Body weight (%)

C. Hematocrit (%)

D. DAI

E. SI length (cm), Colon length (cm), change of SI length (%), change of colon length (%)
Lgr4 protects against inflammatory bowel disease

Figure 2

A

WT

Lgr4−/−

B

Histology score

WT

Lgr4−/−

C

D

MPO (relative units)

E

Relative mRNAs

TNFα

IL6

IL1β

F

Body weight (%)

Survival rates (%)

Days of DSS treatment
Lgr4 protects against inflammatory bowel disease

Figure 3

**A**

WT

Lgr4−/−

**B**

WT

Lgr4−/−

**C**

WT

Lgr4−/−

K67+ cells/crypt

**D**

WT

Lgr4−/−

**E**

WT

Lgr4−/−

Caspase3-cleaved

**F**

WT

Lgr4−/−

Apoptosis cells/villi

CTR

DSS

ns

ns

ns

ns
Figure 4

**Lgr4 protects against inflammatory bowel disease**

A  
X-Gal

B  
WT  
Lgr4-/-  
Lysozyme

C  
Paneth cells/crypt

D  
Relative mRNA level

- WT
- Lgr4-/-

- Lysozyme
- Mmp7
- Defa5
- Cst1C
- Cst4C

**”**
Figure 5

**Lgr4 protects against inflammatory bowel disease**

**A**

**B**

**C**

**D**

**E**

*WT*  *Lgr5^{GFP/+}*  *Lgr4^{-/-}; Lgr5^{GFP/+}*
Figure 6

Lgr4 protects against inflammatory bowel disease

A. \(\beta\)-catenin

B. C-Myc

C. CyclinD1

D. p-S6

E. Relative mRNA

Graph comparing WT and Lgr4-/-.
Figure 7

Lgr4 protects against inflammatory bowel disease
**Figure 8**

Lgr4 protects against inflammatory bowel disease

**A**

Days

| 2 | 0 | 5 | 10 | Day 14 |
|---|---|---|----|--------|
| 1% DSS | | | | |
| 2mg/kg SB216763 | | | | |

**B**

Survival rates (%)

- WT
- Lgr4-/+-SB
- Lgr4/-

**C**

Body weight (%)

- WT
- Lgr4-/+-SB
- Lgr4/-

**D**

WT  Lgr4/-  Lgr4/-+SB216763

**E**

Histology score

- WT
- Lgr4/-
- Lgr4/-+SB

**F**

β-catenin  C-Myc

**G**

MPO (relative units)

- WT
- Lgr4/-
- Lgr4/-+SB
Lgr4 deficiency increases susceptibility and severity of dextran sodium sulphate-induced inflammatory bowel disease in mice
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