Wingless homolog Wnt11 suppresses bacterial invasion and inflammation in intestinal epithelial cells

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Liu X, Wu S, Xia Y, Li XE, Xia Y, Zhou ZD, Sun J. Wingless homolog Wnt11 suppresses bacterial invasion and inflammation in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 301: G992–G1003, 2011. First published September 8, 2011; doi:10.1152/ajpgi.00080.2011.—Wnt11 plays an essential role in gastrointestinal epithelial proliferation, and previous investigations have focused on development and immune responses. However, the roles of how enteric bacteria regulate Wnt11 and how Wnt11 modulates the host response to pathogenic bacteria remain unexplored. This study investigated the effects of Salmonella infection on Wnt activation in intestinal epithelial cells. We found that Wnt11 mRNA and protein expression were elevated after Salmonella colonization. Wnt11 protein secretion in epithelial cells was also elevated after bacterial infection. Furthermore, we demonstrated that pathogenic Salmonella regulated Wnt11 expression and localization in vivo. We found a decrease in Salmonella invasion in cells with Wnt11 overexpression compared with cells with normal Wnt11 level. IL-8 mRNA in Wnt11-transfected cells was low; however, it was enhanced in cells with a low level of Wnt11 expression. Functionally, Wnt11 overexpression inhibited Salmonella-induced apoptosis. AvrA is a known bacterial effector protein that stabilizes β-catenin, the downstream regulator of Wnt signaling, and inhibits bacterially induced intestinal inflammation. We observed that Wnt11 expression, secretion, and transcriptional activity were regulated by Salmonella AvrA. Overall, Wnt11 is involved in the protection of the host intestinal cells by blocking the invasion of pathogenic bacteria, suppressing inflammation, and inhibiting apoptosis. Wnt11 is a novel and important contributor to intestinal homeostasis and host defense.

AvrA; intestine; Frizzled; infection; Salmonella

THE WNT GENE FAMILY, which encodes secreted glycoproteins, consists of 19 members in mammals. The Wnt pathway controls cell fate during embryonic development and homeostasis in adult self-renewing tissues (1, 12, 14). The intestinal epithelium represents the best-understood example for the closely linked roles of Wnt signaling in homeostatic self-renewal and in malignant transformation. It is well documented that mutational subversion of the Wnt cascade can lead to colorectal cancer (18, 50). Recently, several studies have implied the involvement of Wnt-Frizzled (Fz) signaling in the activation of proinflammatory mediators in inflammatory disorders (6, 45). Enhanced Wnt5A were observed in lesions of psoriasis and atherosclerotic plaques (4, 15, 41). However, these studies primarily focused on the response of the monocytes to the inflammatory cytokines in the immune system through Wnt5A or Wnt3A (3, 44).

Recent studies have demonstrated that Wnt11 plays an essential role in gastrointestinal epithelial proliferation and cancer cell migration (8, 37). Patients with inflammatory bowel disease (IBD) had significantly higher Wnt11 expression in their intestines (60). Moreover, the expression of the Wnt receptors, Fz3 and Fz4, was increased significantly in IBD patients while the expression of the Fz1 and Fz5 receptors was decreased significantly (60). A recent study indicated a reduced expression of T cell factor 4 (TCF-4), the Wnt-signaling transcription factor, in ileal Crohn’s disease (52). However, the molecular mechanism of intestinal inflammation by the Wnt family remains largely unknown.

Human cells are constitutively exposed to commensal and pathogenic bacteria. Some studies have shown that Wnt/β-catenin is involved in bacterial infection. Mycobacterium tuberculosis-infected macrophages had elevated Fz1/Wnt3A expression (36). Helicobacter pylori increases the function of proximal Wnt signaling components, such as low-density lipoprotein receptor-related protein and Dishevelled, in gastric epithelial cells (9, 11). Bacteroides fragilis activated the β-catenin pathway in intestinal epithelia cells (55–56). DNA microarray studies have found that uropathogenic Escherichia coli can suppress transforming growth factor-β and Wnt5A signaling, which promotes the subsequent differentiation of basal/intermediate cells (34). Chibby, a conserved component of the Wnt-β-catenin pathway, is involved in clearing Pseudomonas aeruginosa from the nasal cavity (51). We have reported that Salmonella activates the Wnt/β-catenin pathway to modulate intestinal inflammation, cellular proliferation, and intestinal stem cell niches (7, 23, 47–48, 58). However, it remains unknown as to whether Wnt11 is directly involved in bacterial infection.

Enteric bacteria play a crucial role in the pathogenesis of many diseases, such as IBD (43) and colon cancer (10, 42). A recent population-based cohort study demonstrates an increased risk of IBD in individuals with Salmonella infection (13). However, it is unknown how bacterial infection directly activates a specific Wnt protein and modulates the inflammatory response of the intestinal epithelial cells. The function and mechanism of Wnt11 in bacterial inflammation has not been explored. In the present study, we hypothesize that Wnt11 is involved in the host protection by preventing bacterial invasion and affecting the inflammatory response to Salmonella typhi-murium infection. Using in vitro and in vivo models, we have identified the importance of Wnt11 in modulating inflamma-
tion of intestinal epithelial cells during host-bacterial interactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study included wild-type Salmonella SL1344 (SB300), AvrA mutant SB1177 derived from SL1344 (17, 29), and nonpathogenic Salmonella mutT strain PhoP⁺ (33), PhoP⁻ AvrA⁻, and PhoP⁻ AvrA⁺/AvrA⁻. Nonaggregated microaerophilic bacterial cultures were prepared by inoculating 10 ml of Luria-Bertani broth with 0.01 ml of a stationary phase culture and were incubated overnight (~18 h) at 37°C, as previously described (28–29).

Cell culture. Human epithelial Caco2-BBE and HT29Cl29A cells were maintained in DMEM supplemented with 10% FBS, penicillin-streptomycin, and l-glutamine. Human colonic epithelial HCT116 cells were cultured in McCoy’s 5A medium supplemented with 10% (vol/vol) FBS, as previously described (47). The rat small intestinal IEC-18 cell line was grown in DMEM (4.5 g/l glucose) containing 5% (vol/vol) FBS, 0.1 U/ml insulin, 50 μg/ml streptomycin, and 50 U/ml penicillin (30, 47).

Streptomycin-pretreated mouse model. Animal experiments were performed using specific-pathogen-free female C57BL/6 mice (Tac- onic, Hudson, NY) that were 6–7 wk old, as previously described (7). The protocol was approved by the University Committee on Animal Resources at the University of Rochester. Water and food were withdrawn for 4 h before oral gavage with 7.5 mg/mouse of streptomycin. After gavage, the animals were supplied with water and food ad libitum. Twenty hours after streptomycin treatment, water and food were withdrawn again for 4 h before the mice were infected with 1 × 10⁶ colony-forming units (CFU) of S. typhimurium [100 μl suspension in Hanks’ balanced salt solution (HBSS)] or treated with sterile HBSS (control) by oral gavage, as previously described (7, 21, 28). At the indicated times after infection, the mice were killed, and tissue samples from the intestinal tracts were removed for analysis. The pCMV-emyc-Wnt1l and pCDNA-Wnt1l plasmids were constructed in the Sun laboratory.

Mouse colonic epithelial cells. Mouse colonic epithelial cells were collected by scraping the tissue from the colon of the mouse, including the proximal and distal regions (7). The cells were sonicated in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium orthovanadate, and protease inhibitor cocktail). The protein concentration was measured using the Bio-Rad Reagent (Bio-Rad, Hercules, CA).

AvrA clone. The avrA gene was isolated from wild-type S. typhimurium strain SL3201. DNA sequencing analysis revealed that the avrA allele used in our study is identical to the allele from S. typhimurium LT2 (GenBank accession no. AE008830).

Transient transfections. Transient transfections were performed with LipofectAMINE2000 (Invitrogen, San Diego, CA) in accordance with the manufacturer’s instructions. Cells were seeded on 60-mm dishes overnight before transfection with DNA and were mixed with liposome reagent at a ratio of 1:1 before addition to cells. After a 24-h transfection period, the proteins were extracted for immunoblot analysis.

Isolation of Wnt11 protein in the cell culture media. IEC-18 cells were seeded in six-well tissue culture plates and grown in regular medium before use. The cells were treated with Salmonella or AvrA protein in DMEM without FBS for 60 min. The conditioned medium (CM) was harvested and concentrated to 22 times by Amicon Ultra filter devices (Millipore, Carrigtwohill, Ireland) (31, 38). CM was lyzed in 2× protein loading buffer (100 mM Tris, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) and followed the steps for immunoblot analysis. β-Actin in the total lysate was used as a control to show the equal protein loading in each group. β-Actin was not detectable in the CM.

Immunoblot analysis. Mouse epithelial cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium orthovanadate, and protease inhibitor cocktail), and the protein concentration was measured. MEF cells were rinsed two times in ice-cold HBSS, lysed in protein loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothyretol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol), and sonicated. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with primary antibodies. The following antibodies were used: anti-Fz5 (Abcam, Cambridge, MA), anti-Fz7 (Millipore, Temecula, CA), anti-c-Myc or HA anti-IκBα, anti-villin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-pho-c-jun, anti-JNK (Cell Signal, Beverly, MA), anti-Wnt11 (Abcam), anti-β-catenin (Transduction Laboratories), and anti-β-actin (Sigma-Aldrich, Milwaukee, WI) antibodies. Membranes that were probed with more than one antibody were stripped before reprobing. For Figs. 1–8, the images were arranged and labeled in Adobe Photoshop and Adobe Illustrator (Adobe Systems, San Jose, CA). Reassembly of noncontiguous gel lanes was demarcated by black lines (see Figs. 1C and 4C). Such arrangements do not alter the information contained therein. The digital images are representative of the original data. Bands were quantified using Kodak MI software (version 4.0.3).

Immunofluorescence. Intestinal tissues were freshly isolated and embedded in paraffin wax after fixation with 10% neutral buffered formalin. Immunohistochemistry was performed on paraffin-embedded sections (1 μm) of mouse colons. After preparation of the slides as previously described (59), the slides were incubated in 3% hydrogen peroxide for 20 min at room temperature to block endogenous peroxidase activity and then incubated for 20 min in 5% BSA with 0.1% saponin in PBS to reduce nonspecific background. The cultured cells were rinsed three times in PBS, fixed for 10 min in 3.7% paraformaldehyde, permeabilized for 10 min with 0.2% Triton X-100, and washed three times with PBS containing 10% BSA. The permeabilized cells or tissue samples were incubated with the anti-Wnt11 antibody (Santa Cruz Biotechnology) overnight at 4°C. Samples were incubated with goat anti-rabbit Alexa Fluor 594 (Molecular Probes) and DAPI (Molecular Probes) for 1 h at room temperature. Tissues were mounted with SlowFade (SlowFade AntiFade Kit; Molecular Probes), followed by a cover slip, and the edges were sealed to prevent drying. Specimens were examined with a Leica SP5 Laser Scanning confocal microscope.

Transcriptional activation ELISA. After a 24-h transfection period, the cells were lysed, and luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega) with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Firefly luciferase activity was normalized to Renilla luminescence activity, and the activity was expressed as relative units.

S. typhimurium invasion of human epithelial monolayers. Infection of HCT116 cells was performed as previously described (32). The bacterial solution (~20 bacteria/epithelial cell) was added, and bacterial invasion was assessed after 1 h. Cell-associated bacteria were bacteria that were adhered to and/or internalized into the monolayers and were released by incubation with 100 μl of 1% Triton X-100 (Sigma). Bacteria that were internalized by the epithelial cells with 1% Triton X-100 were determined by the addition of gentamicin (50 μg/ml) for 20 min. Gentamicin, an aminoglycoside antibiotic, does not permeate eukaryotic plasma membranes and is cytolytic only to the extracellular population of bacteria while the intracellular bacterial population remains viable (22). For both the cell-associated and the internalized bacteria, 0.9 ml of LB broth was added, and each sample was mixed vigorously and quantified by plating for CFUs on MacConkey agar medium.

Real-time quantitative PCR analysis. Total RNA was extracted from epithelial cell monolayers using TRIZol reagent (Invitrogen, Carlsbad, CA). The RNA integrity was verified by electrophoresis. RNA reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s protocol. The
RT cDNA reaction products were subjected to quantitative real-time PCR using the MyiQ single-color real-time PCR detection system (Bio-Rad) and the iQ SYBR green supermix (Bio-Rad) according to the manufacturer’s protocol. Interleukin (IL)-8 cDNA was amplified using primers to the human IL-8 gene that were complementary to regions in exon 1 (5’-TGCTAATAAGCATACCTCCAAACCT) and overlapped the splice site between exons 3 and 4 (5’-AATTCT-CAGCGGCTCTTCAAAAA). All expression levels were normalized to the GAPDH levels of the same sample using the forward (5’-CTTTACCACCATGGAGAAGGC) and reverse (5’-GGCATGG-CAGTGGTGTCATG) primers for GAPDH. Percent expression was calculated as the ratio of the normalized value of each sample to that of the corresponding untreated control cells. All real-time PCR reactions were performed in triplicate. All PCR primers were designed using Lasergene software (DNASTar, Madison, WI) (Table 1).

Apoptosis assays. After 24 h of treatment with Salmonella, 1 × 10⁶ adherent cells were trypsinized and incubated with fluorescein isothiocyanate (FITC)-conjugated annexin V (binds to phosphatidylserine on the cytoplasmic surface of the cell membrane) and propidium iodide (PI) for 15 min in the dark according to the manufacturer’s protocol (Annexin V kit; Oncogene Research Products, San Diego, CA). Cells were analyzed by flow cytometry as previously described (49).

Statistical analysis. All data are expressed as means ± SD. All statistical tests were two-sided. P values of less than 0.05 were considered to be statistically significant. Differences between two samples were analyzed by Student’s t-test. Statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC).

RESULTS

Increased mRNA expression of Wnt11 in pathogenic Salmonella-infected cells. To determine whether Salmonella colonization had an effect on Wnt signaling, we used real-time PCR to investigate Wnt mRNA expression in cells before and after wild-type S. typhimurium treatment. We chose the rat small intestinal epithelial cell line because it has wild-type APC and β-catenin (30, 47). As shown in Fig. 1A, mRNA expression of Wnt11 in Salmonella-colonized IEC-18 cells was elevated after bacterial colonization. The Wnt11 level is also higher than the other Wnt proteins, such as Wnt5b.

Wnt11 protein expression is increased in intestinal epithelial cells with Salmonella infection. We focused on the Wnt11 protein, which is known to stimulate proliferation, migration, cytoskeletal rearrangement, and contact-independent growth of intestinal epithelial cells (8, 37). Using the Western blot assay, we found that expression of Wnt11 significantly increased after being infected with Salmonella SL1344 over a time course of 60 min in IEC-18 cells (Fig. 1, B and C), and the increase of Wnt11 expression was time-dependent. Wnt secretion is stimulated by extracellular signaling in colonic epithelial cells (31, 38). We further measured the amount of Wnt11 protein in the cell culture media before and after Salmonella colonization. The secreted Wnt11 protein was increased significantly after bacterial colonization for 60 min in IEC18 cells (Fig. 1D). Taken together, we found that pathogenic Salmonella increased protein expression of Wnt11 in the intestinal epithelial cells in vitro.

Fz (transmembrane Wnt receptors) and Wnt interact with other structural components at the cell surface to initiate complex signal transduction cascades that culminate in the transcriptional regulation of gene expression (27, 45). We further investigated the expression of Fz receptors in human epithelial cells after S. typhimurium colonization. The expression of Wnt receptors, Fz2, -7, and -8, was increased upon bacterial colonization (Fig. 1E). The expression of the Fz family was examined using cultured epithelial cells. Fz7 expression was increased by Salmonella colonization (Fig. 1F). We tested the other Fz proteins; however, we did not detect an alteration in expression of these proteins, or we did not have good enough antibodies to detect these proteins (data not shown).

Bacterial colonization increases Wnt11 expression in mouse intestines in vivo. To assess the physiological relevance of Wnt during Salmonella infection, we investigated signaling in intestinal epithelial cells using a streptomycin-treated Salmonella colitis mouse model (2). We selected this model because it is a well-accepted model for the host-bacterial interactions in colitis (2), 2 we have used this model in our previous studies and reported that the β-catenin pathway, the downstream target of the Wnt signaling, was involved in Salmonella infection (7, 23, 59), and 3 streptomycin pretreatment did not change intestinal Wnt11 expression or distribution in the wild-type mice (Fig. 2, A and B).

In the present study, we focused on the streptomycin-pretreated mice with or without Salmonella infection. At the mRNA level, Wnt11 expression in intestinal mucosa increased significantly 8 h post-Salmonella infection (Fig. 2C). Protein expression of Wnt11 was also increased significantly in Salmonella-infected intestines (Fig. 2D). Furthermore, we investigated the distribution of Wnt11 in the intestines before and after Salmonella infection. We observed positive staining of Wnt11 in intestinal epithelial cells lining the crypt region, as well as the villus and surface epithelium (intestine without bacterial infection; Fig. 2, E and G). In the colon, pathogenic Salmonella infection increased Wnt11 staining (Fig. 2E). High-

Table 1. Primers for real-time PCR

<table>
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<tr>
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Fz, Frizzled; r, rat; m, mouse; and h, human.
resolution images of the *Salmonella*-infected colon further showed the localization of Wnt11 at the cell membrane and in the cytoplasm (Fig. 2F). In addition, we observed strong staining of Wnt11 at the bottom of the crypts in the small epithelial cells (Fig. 2G). Therefore, our data show that *Salmonella* infection not only increased the mRNA and total Wnt11 expression levels but also changed the distribution of Wnt11 in the intestine.

*Overexpressed Wnt11 protects cells from Salmonella invasion.* We examined whether the expression level of Wnt11 contributes to the activity of the host’s defense, such as inhibition of bacterial invasion. We counted the number of *Salmonella* that invaded human intestinal epithelial HCT116 cells with normal or overexpressed Wnt protein levels. We found that Wnt11-overexpressed cells reduced the number of internalized *Salmonella* SL1344 bacterium into the epithelial cells, whereas the control HCT116 cells with regular Wnt11 expression levels had significantly more *Salmonella* invasion (Fig. 3A). We examined the number of cell-associated bacteria, including bacteria that were adhered to and/or internalized into the epithelial monolayers. Green fluorescence-tagged *Salmonella* were used to detect bacterial association in cells with different Wnt11 protein expression levels (Fig. 3B). In vitro data indicated that Wnt11 expression did not change the number of associated *Salmonella* bacteria to the host cells (Fig. 3C). To test the specificity of Wnt11 in inhibiting bacterial invasion, we investigated Wnt2, another Wnt protein family member. However, we did not observe a protective role for Wnt2 in bacterial invasion (data not shown). These results indicate that Wnt11 plays a specific role in inhibiting bacterial invasion. Our data show a significantly lower number of invaded *Salmonella* bacteria in Wnt11-overexpressed epithelial cells compared with control intestinal epithelial cells.

*Wnt11 is involved in the regulation of inflammatory pathways in Salmonella-epithelial cell interactions.* To assess the functional role of Wnt11 in *Salmonella*-epithelial cell interactions, we hypothesized that Wnt11 signaling contributes to the inhibition of the inflammatory response. IL-8 level is a well-accepted inflammatory readout for the epithelial-bacterial interaction (35, 54). We found that cells that overexpress Wnt11 downregulate the expression of the inflammatory cytokine IL-8 (Fig. 4A). After *Salmonella* colonization, IL-8 expression was significantly lower in cells that overexpressed Wnt11 compared with cells with normal expression levels of Wnt11 (Fig. 4B).
Fig. 2. *Salmonella* infection alters the expression and distribution of Wnt11 in mouse intestinal cells in vivo. 

**A**: antibiotic treatment did not change the expression and distribution of intestinal Wnt11 in a streptomycin-pretreated colitis mouse model. Villin was a marker for the intestinal epithelial cells; *n* = 3 mice in each group. 

**B**: streptomycin pretreatment did not change the distribution of intestinal Wnt11 in wild-type mice. 

**C**: transcriptional levels of Wnt11 that were altered by *Salmonella* infection in vivo. Total RNA from intestinal epithelial cells was extracted for real-time PCR. *P* < 0.05 significance; *n* = 3. 

**D**: Wnt11 protein expression levels regulated by *Salmonella* in mouse intestine. Wnt protein expression in the small intestine with or without *Salmonella* infection was determined by Western blot analysis. Relative Wnt11 band intensity in normal mice is shown. Data are presented as means ± SD; *n* = 3 mice/group. *P* < 0.05. 

**E**: Wnt11 distribution in mouse colon with or without *Salmonella* infection. 

**F**: high-resolution images of Wnt11 localization in *Salmonella*-infected colon cells. Note the localization of Wnt11 to the cell membrane and cytoplasm. 

**G**: Wnt11 distribution in the small intestine with or without *Salmonella* infection. White arrows show the enhanced Wnt11 staining at the intestinal crypts.
NF-κB activity, was stabilized in cells treated with PDTC. Furthermore, we used the proteasome inhibitor MG-262 to inhibit the activation of NF-κB at the later stages. However, pretreatment with MG-262 had no effect on protein expression of Wnt11 (data not shown). Therefore, these data indicate that the Wnt11-NF-κB interaction may occur at the early stages of NF-κB activation.

Fig. 4. Wnt11 levels and the inflammatory response in intestinal epithelial cells. A: Salmonella-induced interleukin (IL)-8 expression in cells with Wnt11 overexpression. B: the NF-κB inhibitor Pyrrolidine dithiocarbamate (PDTC) abolished the effect of Salmonella in increasing the expression of Wnt11. The image is rearranged because of noncontiguous lanes. Please note the insertion of a line. C: Wnt11 overexpression inhibited protein levels of pho-c-jun in intestinal epithelial cells. D: Wnt11 expression suppressed the transcriptional activity of activator protein (AP)-1. *P < 0.05 and **P < 0.01. Data are means ± SD from 3 separate experiments.

4A). IL-8 is regulated by the activity of the proinflammatory pathways, such as NF-κB and JNK.

To determine the effect of Wnt11 on NF-κB activity, we chose inhibitors to block the activity of NF-κB at different levels and steps. Pyrrolidine dithiocarbamate (PDTC) is an antioxidant that has been used to block the NF-κB signaling pathway at the early stages (39, 53). We pretreated HCT116 cells with PDTC and infected these cells with Salmonella. Our results show that Salmonella-induced Wnt11 expression was inhibited by PDTC (Fig. 4B). In contrast, IκBα, an inhibitor of NF-κB activity, was stabilized in cells treated with PDTC.
Wnt11 has been shown to inhibit JNK activity (40). Therefore, we tested the effect of Wnt11 on the JNK pathway in intestinal epithelial cells. As shown in Fig. 4C, cells with Wnt11 overexpression had lower phosphorylated c-Jun. c-Jun and JNK are involved in activator protein (AP)-1 activity, and transcriptional reporter data have shown that Wnt11 overexpression significantly inhibits AP-1 reporter responses (Fig. 4D).

Salmonella effector AvrA upregulates Wnt11 expression in vitro. AvrA is a known bacterial effector protein that inhibits bacteria-induced intestinal inflammation by stabilizing β-catenin, the downstream target of Wnt signaling (7, 59). Recent studies

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**Fig. 5. Salmonella AvrA enhances Wnt11 in vitro.**

A: AvrA status in bacteria altered the protein level of Wnt11 in HCT116 cells. Cells were infected with wild-type *Salmonella* (SL1344; SB300) or SB1117 mutant AvrA (without AvrA expression) for the indicated times. Total cell lysates were analyzed for protein levels by immunoblot analysis. Control (c), without any treatment. B: AvrA protein expression increased Wnt11 expression in HCT116 cells after bacterial colonization for 1 h. Densitometry analysis showed Wnt11 expression in HCT116 cells after bacterial colonization for 1 h. The blot image is rearranged because of noncontiguous lanes. *P < 0.05 significance; n = 3 separate experiments.

B: AvrA protein expression increased Wnt11 expression in CaCo2-BBE cells after bacterial colonization for 1 h. D: AvrA status in *Salmonella* is associated with Wnt11 secretion in the cell medium. IEC-18 cells were treated with *Salmonella* SL1344 or its derived AvrA mutation (AvrA–) for 60 min. The CM was harvested and concentrated for immunoblot analysis. β-Actin in the total lysate was used as a control to show the equal protein loading in each group. *P < 0.05 significance; n = 3 separate experiments. E: purified AvrA protein modulates Wnt11 secretion in a dose-dependent manner. IEC-18 cells were treated with AvrA (2.5 μg/ml) for indicated time. F: AvrA protein significantly enhances Wnt11 secretion in the LiCl-treated IEC-18 cells. Data are presented as means ± SD; n = 3 mice/group. *P < 0.05.

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**Fig. 6. AvrA enhances intestinal Wnt11 in vivo.**

A: transcriptional levels of Wnt11 were altered by *Salmonella* with or without AvrA in vivo. The total RNA from intestinal epithelial cells was extracted for real-time PCR. Data are presented as means ± SD; n = 3 mice/group. *P < 0.05. B: Wnt11 protein expression is regulated by AvrA in a mouse model. Wnt protein expression in the small intestine with or without *Salmonella* infection was determined using Western blot analysis. Villin is a marker for epithelial cells. C: the expression of Wnt11 and Fz7 in mice infected with PhoPC, AvrA–/AvrA+ or AvrA+ (without AvrA expression). Total cell lysates were analyzed for protein levels by immunoblot analysis. Relative Wnt11 band intensity is shown as Wnt11 vs. β-actin in the LiCl-treated IEC-18 cells. Data are presented as means ± SD; n = 3 mice/group. *P < 0.05.
have shown that *Salmonella* strains with AvrA expression block the activation of the JNK pathway (5, 20, 29). Moreover, we hypothesized that AvrA is involved in Wnt11 signaling during bacterial colonization. We examined the effects of AvrA on the induction of Wnt11 expression using a human epithelial cell model. SL1344 is a strain with AvrA expression, whereas SB1117AvrA\(^-\) is a mutant-derived strain from SL1344 (17, 29). We tested the Wnt11 levels in cells colonized with bacteria with or without AvrA over 1 h (Fig. 5A). We found that the SL1344 strain with AvrA expression significantly increased expression of Wnt11 after 1 h of exposure, whereas AvrA\(^-\) did not increase expression of Wnt11 in the human intestinal epithelial cells (Fig. 5B). In the human intestinal Caco2-BBE cell line, Wnt11 protein expression was upregulated by AvrA expression in SL1344, whereas Wnt11 expression was significantly lower in mutated AvrA\(^-\) bacteria-infected cells (Fig. 5C). Moreover, the secreted Wnt11 in media was increased significantly in epithelial cells colonized with AvrA-expressed *Salmonella* for 1 h. In contrast, AvrA\(^-\) bacteria induced less Wnt11 secretion in IEC-18 cells (Fig. 5D).

Bacterial effectors could function independent of the type three secretion system (19). Therefore, we purified the AvrA wild-type protein and tested its effects on Wnt11. We observed that purified AvrA (2.5 \(\mu\)g/ml) did increase Wnt11 expression in a time-dependent manner (Fig. 5E). We also used AvrA at different concentrations. Treatment of AvrA protein significantly enhanced Wnt11 secretion in cell culture media in a dose-dependent manner (Fig. 5F).

AvrA upregulates Wnt11 expression in vivo. In *Salmonella*-infected mouse intestinal cells, we found that SL1344 significantly increased expression of Wnt11, whereas SB1117AvrA\(^-\) had significantly less Wnt11 expression at the transcriptional level (Fig. 6A) and at the protein level (Fig. 6B). In a previous study, we used the *Salmonella* mutant strains Pho\(^+/+\), Pho\(^-/-\), and Pho\(^-\) AvrA\(^-/-\)AvrA\(^-\) to determine the effect of AvrA on the host response (17, 40–41). Our Western blot data showed that AvrA expression in *Salmonella* increased Wnt11 protein expression in the intestines compared with the control without infection or with test groups infected with low AvrA-expressing *Salmonella* strains (Fig. 6C). However, we found that Fz7 expression increased in all infection groups, and this increase was not dependent on AvrA. These data indicated that AvrA may not be the only bacterial protein that contributes to the increased Wnt/Fz signaling. Moreover, we tested the expression levels of Wnt3 and Wnt9A in Pho\(^+/+\), Pho\(^-\) AvrA\(^-\)-, and Pho\(^-\) AvrA\(^-/-\)/AvrA\(^-\)-infected mouse intestines. However, AvrA expression did not alter expression of Wnt3 and Wnt9A (data not shown).

Expression of AvrA significantly increases Wnt/TCF activity. Bacterial AvrA is known to activate the \(\beta\)-catenin pathway (23, 48, 59). Upon Wnt activation, \(\beta\)-catenin is stabilized and translocated to the nucleus where it binds with TCF. TCF transcriptional activity is a key feature of Wnt activity. We investigated Wnt/\(\beta\)-catenin transcriptional activity in human intestinal CaCo2-BBE cells and found that AvrA significantly increased Wnt/\(\beta\)-catenin transcriptional activity (Fig. 7A). LiCl, a positive activator of the Wnt/\(\beta\)-catenin pathway, significantly increased Wnt11 activity, whereas \(\beta\)-catenin accumulated in the cells (Fig. 7, B and C).

Wnt11 plays an essential role in gastrointestinal epithelial proliferation. The activation of Wnt11 could be a strategy for the host to promote cell proliferation and replace tissues damaged due to infection. BrdU staining data showed an increase in proliferative cells in SL1344-infected intestines in vivo (Fig. 7D).

![Figure 7](http://ajpgi.physiology.org/)

**Fig. 7.** Wnt11 and its downstream effector proteins were altered by *Salmonella* infection. *A:* overexpression of AvrA increases Wnt/TCF-responsive reporter (Re) in epithelial cells. Intestinal epithelial Caco2-BBE cells were transfected with pCMV-myc-AvrA for 24 h. Ne, negative control. Luciferase activity was normalized to the internal control (Rluc activity). LiCl treatment, which activates the Wnt/\(\beta\)-catenin pathway, was used as a positive control. *B:* Wnt11 expression increased in epithelial cells of the small intestine that were treated with LiCl. C: relative Wnt11 band intensity is shown as Wnt11 vs. \(\beta\)-actin in IEC-18 cells. Data are presented as means ± SD. *D:* representative BrdU labeling of small intestine epithelial cells after *Salmonella* infection; \(n = 3\) in each experimental group.
Wnt11 overexpression inhibits apoptosis in intestinal cells. We further hypothesized that Wnt11 signaling promotes viability in intestinal epithelial cells during bacterial infection. We determined the percentage of apoptotic cells during Wnt11 overexpression. In Fig. 8, A–F, Salmonella induced 14.2% apoptosis in HCT116 and 15.6% in cells that were transfected with an empty pCMV-myc plasmid. In contrast, Wnt11-overexpressed cells had only 10.8% apoptotic cells (Fig. 8F). At the later stage of Salmonella-induced apoptosis, HCT116 cells with pcDNA had ~14% apoptotic cells, whereas HCT116 cells with pcDNA-Wnt11 were significantly reduced to only 9% apoptosis (Fig. 8G). In conclusion, these data indicate that Wnt11 overexpression protects cells from apoptosis.

DISCUSSION

We have demonstrated that Salmonella infection induces an increase in Wnt11 mRNA and protein expression in intestinal epithelial cells both in vitro and in vivo. Overexpression of Wnt11 lowers IL-8 expression induced by Salmonella infection, and Wnt11 overexpression inhibits Salmonella invasion. Therefore, Wnt11 is involved in the regulation of intestinal inflammation and the inhibition of bacterial invasion induced by 10.220.33.1 on June 26, 2017 http://ajpgi.physiology.org/ Downloaded from

Fig. 8. Protective role of Wnt11 in apoptosis. A–F: apoptotic cells in cells with or without Wnt overexpression after Salmonella infection. Apoptosis in Salmonella-colonized cells with or without Wnt11 overexpression. After 24 h of treatment with Salmonella, 1 × 10⁶ adherent cells were trypsinized and incubated with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) for 15 min in the dark. Cells were analyzed using flow cytometry. The percentage of cells in each group within the gated areas is indicated. In the gated areas, the top right panel is for the population of later apoptotic cells, and the bottom right panel is for the early apoptotic cells. Dot plots of early apoptotic cells have increased Annexin V-FITC fluorescence only, whereas the necrotic and late apoptotic cells have increased annexin V and PI fluorescence. Axes are labeled with arbitrary fluorescence units. Data are from a representative experiment from 3 independent experiments that gave similar results. G: statistical analysis of the early apoptotic percentages after Salmonella colonization. Data are from 3 separate experiments. H: the working hypothesis of the role of Wnt11 in bacterially induced intestinal inflammation. Wnt11 expression protects the host at different levels, including blocking pathogenic bacterial invasion, suppressing inflammation, and inhibiting apoptosis. Salmonella AvrA protein enhances the expression and function of Wnt11.
by \textit{Salmonella}. Our in vivo studies further demonstrate that pathogenic bacteria regulate Wnt11 expression and localization. We determined the effects of the bacterial protein AvrA on the induction of Wnt11 expression in human epithelial cells. Wnt11 expression, distribution, transcriptional activity, and target genes were regulated by \textit{Salmonella}. Our study indicates that Wnt signaling is involved in the complex networks for mucosal defense.

Our data on bacterial invasion, inflammation, and apoptosis indicate that Wnt11 may contribute to the protection of the host in the intestines by affecting various signaling pathways, including NF-κB and JNK, to modulate the inflammatory response. Wnt11 is known to promote proliferation and migration of intestinal epithelial cells (8, 37). Our previous studies demonstrated that β-catenin is a negative regulator of intestinal NF-κB activity in bacterially induced epithelial inflammation (7, 47). Recently, we have shown that Axin, the negative regulator of Wnt/β-catenin (16), is exploited by \textit{Salmonella} (unpublished observations). Therefore, it is clear that Wnt11, its regulators, and β-catenin are all involved in the bacterial inflammation response in the intestines.

Our study provides evidence for the localization and increase in expression of Wnt11 after \textit{Salmonella} infection in the small intestines and colon. The localization of Wnt11 may be associated with the alteration of stem cell niches during infection. Gut homeostasis is maintained through a balance of cell damage that results from the collateral effects of bacterial killing and epithelial repair by stem cell division. The increased epithelial renewal and proliferation upon \textit{Salmonella} infection may be a consequence of a major defense mechanism in the gut (25–26).

Here, we report that the bacterial effector protein AvrA increases Wnt11 expression. Previous studies have demonstrated that AvrA inhibits multiple inflammatory pathways (21, 23, 28–29, 46, 48, 57, 58). Both early and late phases of the host response display specificity for AvrA-expressed strains and provide new insights into the molecular cascade that is mobilized to combat bacteria-associated intestinal inflammation (25–26). We noted that SB1117AvrA	extsuperscript{-} had less Wnt11 expression compared with the parental SL1344 strain. However, Wnt11 expression was still higher in AvrA-infected intestines than that in the control group without bacterial infection (Fig. 5D). These data suggest that AvrA may not be the only bacterial protein that contributes to increased Wnt11 expression. Our recent study on intestinal Wnt2 indicated that its expression and location were regulated by commensal bacteria (24).

The Wnt signaling is modulated by both pathogenic and commensal gut bacteria. Wnt levels in intestine might determine how the gut flora interacts with the immune system. Wnt11 is involved in multiple signaling pathways, including β-catenin, NF-κB, and JNK. It remains unknown which pathway plays the dominant role in regulating inflammatory responses through Wnt11. Dysfunction of Wnts is reported in patients with IBD and may lead to chronic inflammation and cancer (59). Wnt11 is known to promote proliferation, transformation, and migration of IEC (8, 37). In the colitis-associated cancer, Wnt11 may predispose to cancer development through limiting inflammatory responses. It is interesting to investigate how Wnt11 synergistically coordinates these pathways in inflammation and tumorigenesis.

There are complicated interactions among the 19 Wnt members in the signaling pathway. Some of the effects of Wnt11 were not found with other Wnt family members. Inhibition of bacterial invasion and early apoptosis is associated with Wnt11 overexpression but not with Wnt2 status. However, Wnt2B, -3A, -5B, -6, -7A, and -9A all displayed increased expression in IBD patients compared with non-IBD patients (59). These data indicate that other Wnt proteins may synergistically play critical roles in intestinal inflammation. We tried Wnt11 small interfering RNA in vitro; however, the reduction of Wnt11 was not significantly high enough for us to investigate the physiological function of Wnt11 in the cultured cell system. We also tried Wnt inhibitors; however, the inhibition is not specific for Wnt11 (data not shown). This may be because of the complementary mechanisms of the other Wnt proteins. Hence, in the current study, we chose a Wnt11-overexpressed system to confirm the role of Wnt11.

Another limitation of our study is that we had to narrow down our focus on Wnt11. We did not have Wnt11 knockout mice to further investigate \textit{Salmonella} infection in vivo. Aside from \textit{Salmonella}, other pathogenic bacteria and commensal bacteria may regulate Wnt11 and its downstream signaling pathways as well.

As summarized in Fig. 8H, we uncovered several novel aspects of Wnt11: 1) Wnt11 is elevated by bacterial infection at the mRNA and protein level; 2) pathogenic \textit{Salmonella} regulate Wnt11 expression, secretion, and localization; 3) Wnt11 protects cells from bacterial invasion, apoptosis, and inflammation; and 4) the bacterial effector protein AvrA induces Wnt11 activation by increasing transcriptional activity and Wnt11 expression. Wnt family members are important contributors to intestinal homeostasis and protection of the host from bacterial invasion and infection. Our study on the role of Wnt11 in the \textit{Salmonella}-infected model provides molecular insights into host-bacterial interactions in infectious diseases and inflammation.

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GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

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