Master's Thesis of Hosun Yu

Neuron-Specific Gene 2 (NSG2) knockout relieved colitis and colitis-associated cancer in mice

NSG-2 유전자에 의한 DSS 유도 장염 및 대장암에 대한 효과 연구

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Abstract

Inflammatory bowel disease (IBD), characterized by chronic intestinal inflammation, remains challenging to treat. It is the result of abnormal immune responses to host gut microbes.[1] Recently abnormalities of the nervous system, communicating with the immune system and gut microbiota, have been associated with IBD.[2,3] Neuron-specific gene 2 (Nsg-2) encodes alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor regulating excitatory neuron, which might be associated with colitis.[4] However, the role of Nsg-2 in colitis has yet to be well known.

Using a murine colitis model, we studied its role and underlying mechanism in intestinal inflammation. Abscence of Nsg-2 attenuated Dextran sulfate sodium (DSS)-induced colitis and colonic CD4+ T lymphocyte infiltration. Nevertheless, amelioration of colitis in Nsg-2 deficient mice did not disappear after cohousing with wild-type mice. The role of Nsg-2 in colitis-associated colon cancer induced by Azoxymethane (AOM) and DSS was also evaluated. Suppresion of Nsg-2 decreased the number and size of tumors, phosphatidylinositol-3 kinase-related kinases (pIKK) and β-catenin signaling compared to wild-type. However, there is no

difference in immune cells in colon tissues between Nsg-2 KO mice and wild-type mice. Nsg-2 gene expression was also increased more in the colon tissue of UC patients than in healthy control subjects.

Thus Nsg-2 can be a mediator in intestinal acute and chronic inflammation, bridging between the immune systems and nervous systems. It is a potential novel therapeutic target for IBD.

Keywords: Inflammatory bowel disease, neuron-specific gene2, cohousing, dextran sulfate sodium(DSS) induced colitis, colitis-associated colon cancer

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Introduction

Inflammatory bowel disease(IBD), comprised of Ulcerative colitis and Crohn's disease, is characterized by chronic inflammation of the digestive tract. The disease has recently been increasing in young adulthood ,especially in Asia, including Korea, causing a lot of social costs.[5] Despite the significant improvement in the prognosis of the disease due to the recent introduction of various biological agents and small molecules, there are still patients who do not respond to treatment or lose their response.[6] Accordingly, there is a need to develop new drugs based on understanding disease's pathophysiology.

Emerging evidences suggest the association between IBD and other neurological diseases. Patients with IBD have a higher risk of developing Parkinson's disease and dementia than non-IBD individuals.[7,8] In other words, the gut-brain axis, in which the brain and intestines interact with each other, can be the new concept that can explain the pathophysiology of IBD.[9] Intestinal immune cells and gut microorganisms would interact with the central nervous system (e.g., Brain) and enteric nervous system in the gut-brain axis.[10] The vagus nerve from the brain forms a synapse with the enteric nervous system and regulates immune

cells in lamina propria via neurotransmitters.[11] Immune cells transmit signals from the enteric nervous system to the brain through cytokines production responding to gut microorganisms.[10] Thus, Understanding the interaction of several components of the gut-brain axis may be essential to understand IBD.

A recent study has shown that Neuron-Specific Gene 2 (Nsg-2) encodes an alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor binding protein in the developing postsynaptic neuron which plays an important role in regulating the excitatory neuronal signal, in the brain. When deleting Nsg-2, the excitatory signal decreased due to suppressing AMPA receptors. When Nsg-2 becomes overexpressed, an increase in AMPA receptors and the excitatory signal are also confirmed.[4] AMPA receptor was increased in the hippocampus of the TNBS colitis model in the previous study.[12] However, the exact role of Nsg-2 in colitis has yet to be studied.

Using the Nsg-2 knocked-out(KO) mouse model, we studied the role of Nsg-2 in intestinal inflammation in interaction with intestinal microorganisms and the immune system.

Materials and Methods

1. Mice

Nsg-2 KO mice with C57BL/6 background aged 7 to 8 weeks were used. In addition, Age- and gender-matched wildtype mice (C57/6NCrljBgi), purchased from Orient (Seongnam, Korea), were used as control. The mice were maintained under specific pathogen-free housing with standard conditions of humidity, temperature, and a light/dark cycle in the Laboratory of Experimental Animal Research of Boramae Medical Center. According to the protocol, the mice were euthanized with isoflurane inhalation on the day or early when mice exhibited severe body weight loss (25% of their pre-experimental body weight).

2. Induction and evaluation of DSS-induced colitis

DSS (MP Biochemical, Irvine, CA, USA), a water-soluble, negatively charged sulfated polysaccharide, can cause intestinal inflammation. 3% DSS dissolved in drinking water was administrated for six or seven days.[13] Five mice were randomly assigned to each group. It has already been proven that the control group was treated with normal water.

The mice were assessed every day for behavior, water/chow consumption, body weight, stool consistency, and evidence of gross hematochezia. Disease activity index(DAI) for assessment of colitis severity was determined as the sum of parameters consisting of the changes in body weight loss, stool consistency, and presence of rectal bleeding.[14] Weight loss was calculated as the percent difference between the original weight (day 0) and the weight on any particular day. On day 7, colon tissue samples were obtained under anesthesia, fixed in 10% buffered formalin, and embedded in paraffin. Sections were stained with H&E. The severity of colitis was scored by two examiners not involving experimental procedures as described previously[15]. In brief, three independent parameters were measured: inflammation severity (0, none; 1, mild; 2, moderate; 3, severe), inflammation extent (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural), the extent of crypt damage (0, none; 1, damage to the basal one-third portion; 2, damage to the basal two-thirds portion; 3, damage to the entire crypt with surface epithelium intact; 4, erosion). The sum of these scores was quantified as to the percentage of tissue involvement (0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%).

3. LPMC isolation

Lamina propria mononuclear cells (LPMCs) were isolated as previously described with minor modifications.[16] Briefly, colons were cut longitudinally and washed in Hank's balanced salt solution (HBSS) supplemented with 1% fetal bovine serum (FBS). Tissues were cut into 0.5−cm2 pieces and incubated at 37° C for 20 min in HBSS containing 3 mM ethylenediaminetetracetic acid and 1 mM dithiothreitol to remove the epithelial cell. After the incubation, epithelial cells in the supernatant were discarded by passing through a 100−μm cell strainer. Then, the remaining pieces were minced and incubated with 1× minimum essential medium-a containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS, and supplemented with 1 mg/ml collagenase IV (Biosharp, China), 1 mg/ml dispase II (Roche, Basel, Swiss), and 0.5 mg/ml deoxyribonuclease I (Roche, Basel, Swiss) for 60 min in a shaking incubator at 37° C. The resulting cell suspension was filtered through a 70−mm cell strainer (BD, USA), and LPMCs were collected after centrifugation at 300g for 10 min.

4. FACS analysis

For cell surface staining, cells were stained with antibodies

against CD45 (Percp/cy5.5, clone#30-F11), CD3 (Alexa Fluor 700, clone#17A2), and CD4 (Pacific Blue, clone#GK1.5) at 4° C for 30 min and then washed with phosphate-buffered saline (PBS) twice. All the fluorescence-activated cell-sorting antibodies were from Abcam (UK). Flow cytometry was performed on a BD FACS Aria(USA). The data were analyzed with FlowJo software (Tree Star).

5. Induction of colitis-associated tumorigenesis in mice

Modeling and analysis of colitis-associated tumorigenesis were performed as described previously.[17] Briefly, 12.5 mg/kg azoxymethane (Sigma-Aldrich, St Louis, MO) was intraperitoneally injected into mice at day0. Dextran sulfate sodium (DSS) was diluted to a concentration of 2% in their drinking water and then supplied to mice from day 7 to day 12. Mice were administered untreated, free water for the next 16 days. A total of three cycles of DSS administration were conducted. Body weight was checked weekly in a blinded manner. Mice were killed on day 64, after the third cycle of DSS administration, using isoflurane inhalation.

6. Gross and histological assessment

After euthanasia, mouse colons were extracted and cut

longitudinally. Digital photos were obtained. The number of tumors was counted and size was measured. Tumor dimensions were also assessed using ImageJ(National Institutes of Health, Bethesda, MD). The colon tissues were fixed in 10% formalin and embedded in paraffin. Hematoxylin and eosin staining were performed. The severity of intestinal inflammation was assessed by independent researchers blinded to the study protocol.[18] Briefly, the distal colon was examined because DSS induced severe damage to the distal colon compared with the proximal colon. The severity was assessed in terms of crypt damage (0–4), the extent of inflammation (0–3), the severity of inflammation (0–3), and the total percent of involvement (0–4). The sum of all features was calculated.

7. Immunohistochemical analysis for mouse and human colon samples

Immunohistochemistry was performed as described previously.[18] Antibody to a phosphorylated inhibitor of IκB kinase (pIKK)-α/β (Sigma- Aldrich), β-catenin (Sigma-Aldrich), and Nsg-2(Abcam) were used. Quantitative measurements were performed as described earlier.[15] Briefly, immunoreactivity was assessed by determining the percentage of positive cells using a visual

scoring system and was classified as 0 (no staining), 1+ (<10%), 2+ (10-30%), 3+ (31-60%) and 4+ (61-100%). Immunohistochemical intensities were calculated in each of the three nonadjacent tumor fields (magnification: ×400), which were randomly selected.[15] To evaluate β-catenin translocation into the nucleus, immunopositive nuclei of β-catenin were calculated in three high-power fields on each slide

8. Statistical analysis

Data are expressed as the mean and standard error of the mean (SEM). Non-parametric Mann-Whitney test was performed to compare values between the groups using SPSS 25 statistical software (SPSS, Chicago, IL, USA). The P-values less than 0.05 were considered statistically significant

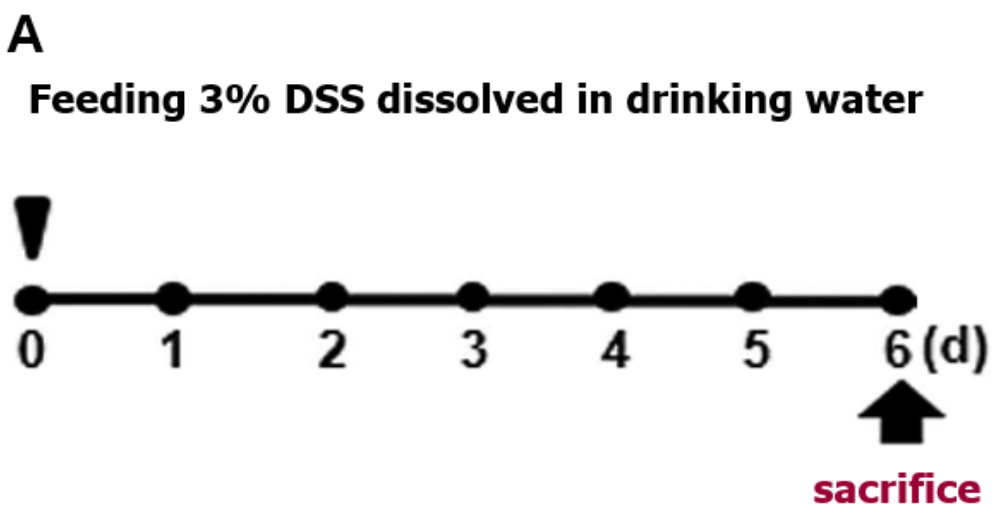
9. Ethics approval

All procedures were approved by the Animal Care Committee at SMG-SNU Boramae Medical Center (IACUC No.2016-0017). All animal experiments were conducted by the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health.

Results

1. Nsg-2 KO is protective against 3% DSS-induced colitis

To evaluate the role of Nsg-2 in acute colonic inflammation, we studied a dextran sulfate sodium (DSS)-induced murine acute colitis model. We evaluated the phenotypic characteristics of the DSS-induced mice, such as body weight and disease activity index (DAI), every six days. Nsg-2 KO mice showed significantly lower weight reduction and DAI than wild-type mice (Fig1 B, C). In addition, there were blood stool contents in the gross appearance of extracted colons of wild-type mice, not marked in Nsg2 KO mice (Fig1 D). Histological analysis showed that Nsg-2 KO mice developed less severe colitis than wild-type mice(Fig1 F-G).



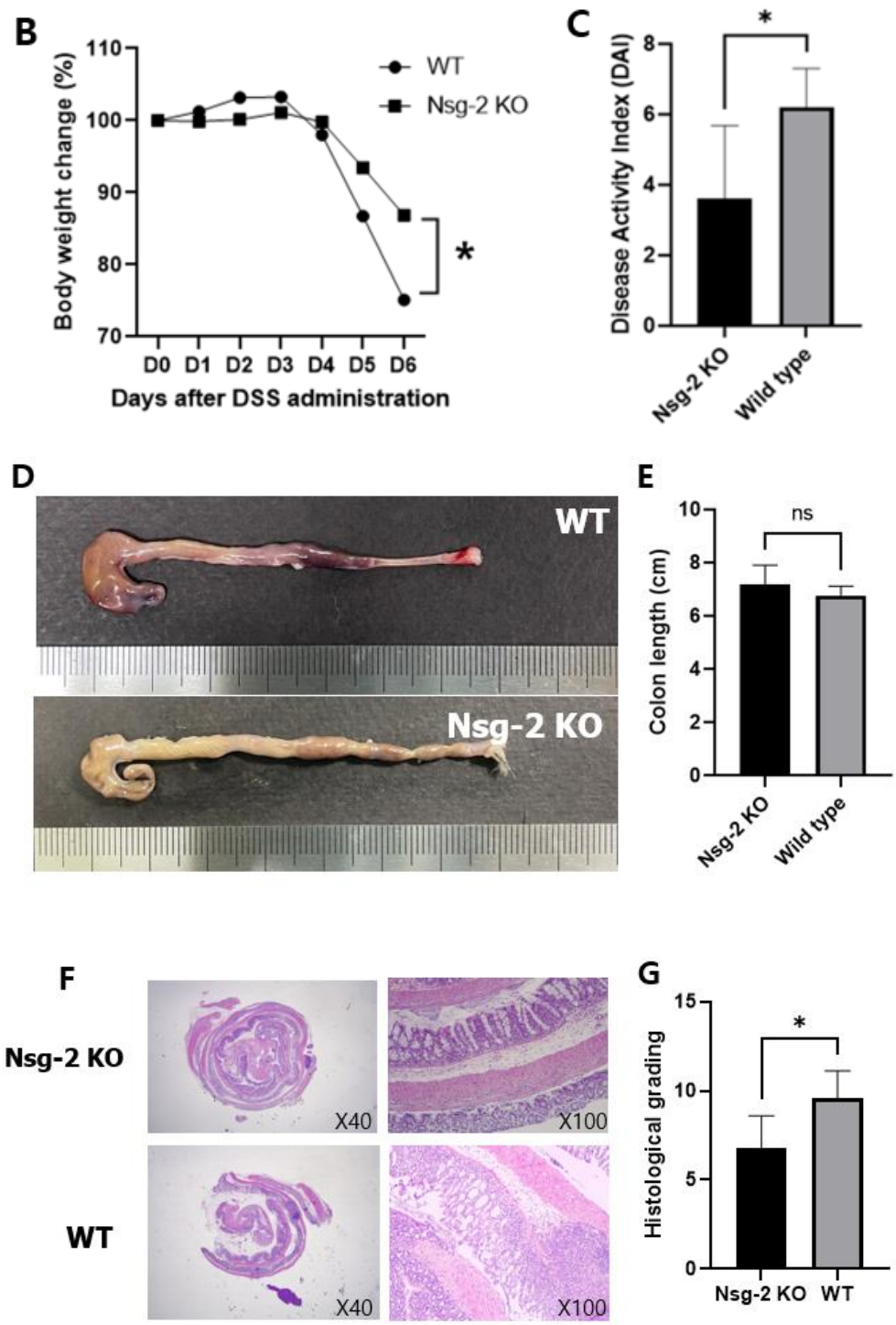

B
Body weight change (%)
110
100
90
80
70
WT
Nsg-2 KO
*
D0 D1 D2 D3 D4 D5 D6
Days after DSS administration
C
Disease Activity Index (DAI)
8
6
4
2
0
*
Nsg-2 KO
Wild type
D
WT
Nsg-2 KO
E
Colon length (cm)
10
8
6
4
2
0
ns
Nsg-2 KO
Wild type
F
Nsg-2 KO
WT
X40
X100
X40
X100
G
Histological grading
15
10
5
0
*
Nsg-2 KO
WT

Figure 1. Nsg-2 KO is protective against 3% DSS-induced colitis. These figures are the comparison of clinical indices, gross appearance, and histologic grading in neuron-specific gene 2-deficient (Nsg-2 KO) (n=5) and wild-type mice (n=5). **(A)** To induce acute colitis, 3% DSS dissolved in drinking water was administrated for six days **(B)** Nsg-2 KO mice showed significantly lower weight reduction than wild-type mice. **(C)** Wild-type mice showed significantly higher scores in disease activity index than Nsg-2 KO mice. **(D)** There were blood stool contents in the gross appearance of extracted colons of wild-type mice, not marked in Nsg2 KO mice. **(E)** No significant difference in colon length was observed between Nsg-2 KO and wild-type mice. **(F-G)** Histological analysis showed that Nsg-2 KO mice developed less severe colitis than wild-type mice. Asterisk(*) indicate significant differences (P-values<0.05)

2. CD4+ T cells present at higher frequencies in inflamed colon tissue from Nsg-2 KO mouse

Inflamed colon tissue from Nsg-2 KO mice contains a lower abundance of CD4+ T cells than wild-type mice. (Fig2)

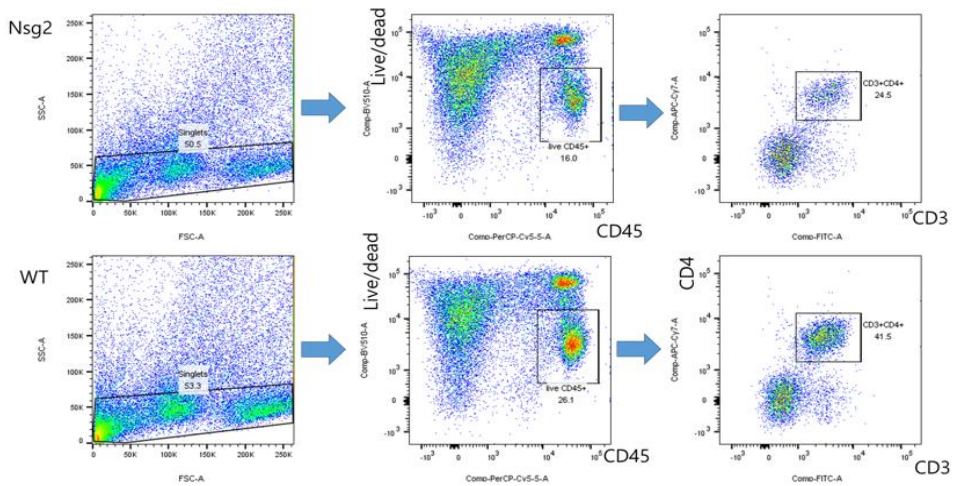
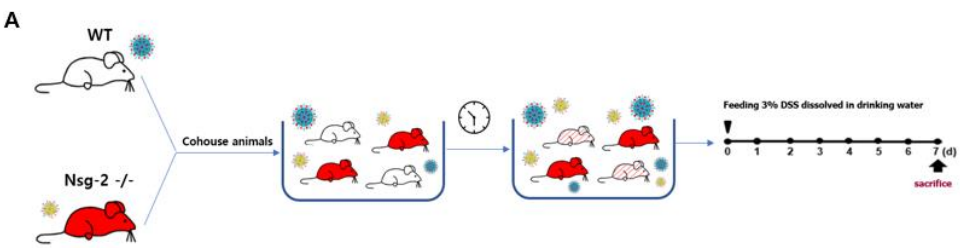
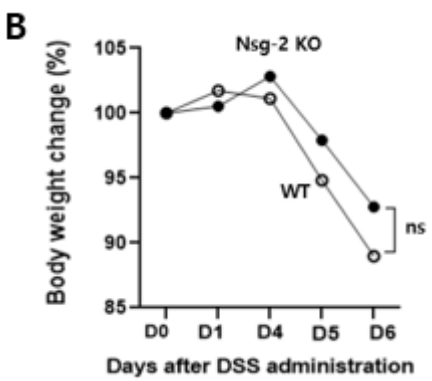


Figure 2. CD4+ T cells present at higher frequencies in inflamed colon tissue from Nsg-2 KO mice compared with inflamed tissue from wild-type. These figures are the flow cytometry picture of lamina propria mononuclear cells (LPMCs). CD4+ T cells were gated after gating lymphocytes from LPMCs. Inflamed colon tissue from Nsg-2 KO mice contains a lower abundance of CD4+ T cells than wild-type mice.

3. Less severe DSS-induced colitis was sustained in Nsg-2 KO mice after cohousing with wild-type mice

For the cohousing experiment, Nsg-2 KO mice (n=5) and wild-type mice (n=5) were housed together after weaning. After 6 weeks of cohousing, the mice were treated with 3% DSS. (Fig3 A) Gross appearance of extracted colons showed rectal bleeding and bowel edema in wild-type mice, but there was no difference in weight loss between the two groups. (Fig3 B-C) Clinical analysis showed that Nsg-2 KO mice developed less severe colitis than wild-type mice. (Fig3 E)





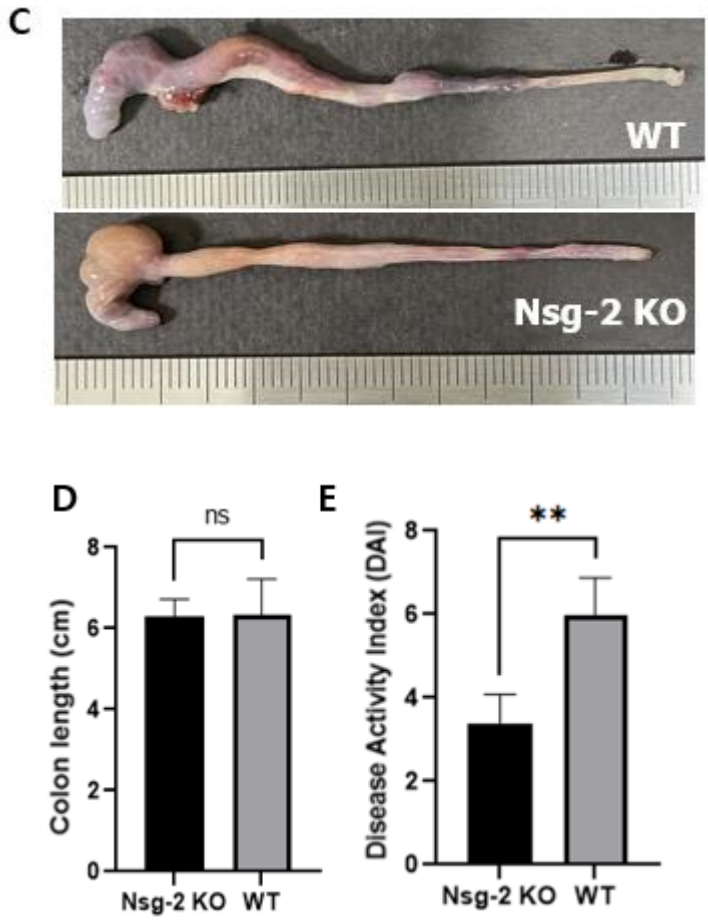
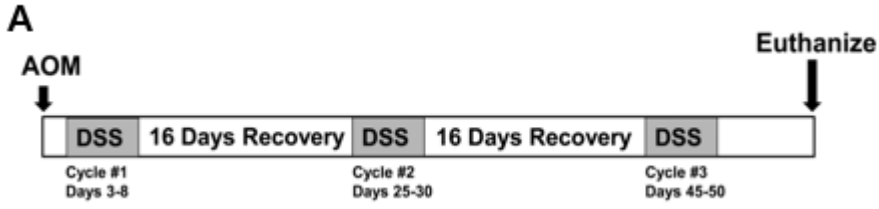


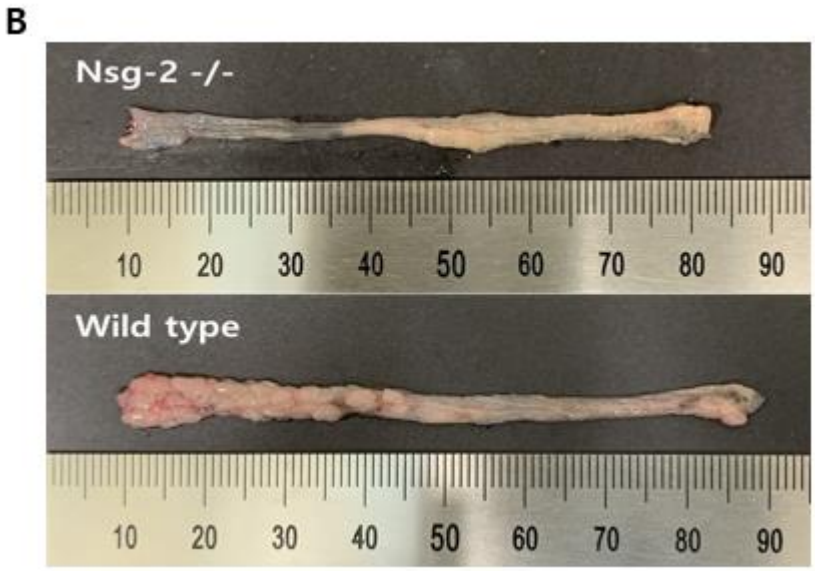
Figure 3. Less severe DSS-induced colitis was sustained in Nsg-2 KO mice after cohousing with wild-type mice (A) For the cohousing experiment, Nsg-2 KO mice (n=5) and wild-type mice (n=5) were housed together after weaning. After 6 weeks of cohousing, the mice were treated with 3% DSS. (B) There was no difference in weight loss between the two groups. (C) Gross appearance of extracted colons showed rectal bleeding and bowel edema in wild-type mice. (D) There was no significant difference in colon length between Nsg-2 KO and wild-type mice. (E) Clinical analysis

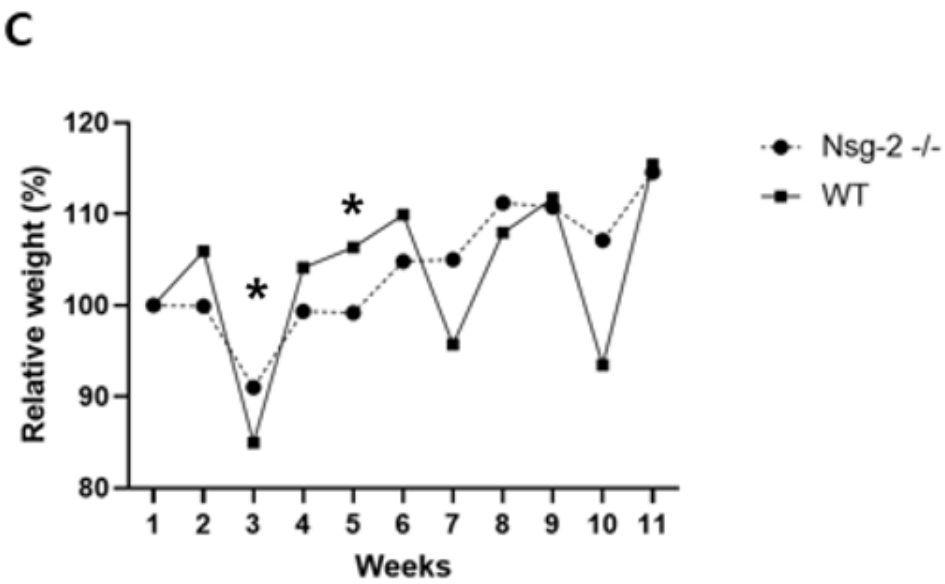
showed that Nsg-2 KO mice developed less severe colitis than wild-type mice. Two asterisks(**) indicate significant differences (*P*-values<0.001)

4. Nsg-2 KO is protective against AOM/DSS-induced colitis-associated colorectal cancer.

To evaluate the role of Nsg-2 in colitis-associated colon cancer, we studied an Azoxymethane(AOM)/dextran sulfate sodium (DSS)-induced murine acute colitis model.(Fig4 A) More tumors were formed in wild-type mice, not in Nsg-2 KO mice.(Fig4 B) The number and size of tumors were significantly decreased in the Nsg-2 KO group compared to the wild-type group. (Fig4 D-E) Crypt distortion and inflammatory cell infiltration were significant in wild-type mice than Nsg-2 KO mice.(Fig5 A) Immunohistochemical analysis of the activity of pIKK and beta-catenin in tumor tissues of mice. Tumor tissues of wild-type mice showed more positivity of both pIKK and beta-catenin. (Fig 5 B-C, Brown means positive staining). FACS analysis shows no difference of immune cells in colon tissues between Nsg-2 KO mice and wild-type mice.(Fig5 D)


A
AOM
Euthanize
DSS
16 Days Recovery
DSS
16 Days Recovery
DSS
Cycle #1
Days 3-8
Cycle #2
Days 25-30
Cycle #3
Days 45-50


B
Nsg-2 -/-
10 20 30 40 50 60 70 80 90
Wild type
10 20 30 40 50 60 70 80 90


C
Relative weight (%)
120
110
100
90
80
1 2 3 4 5 6 7 8 9 10 11
Weeks
*
*
Nsg-2 -/-
WT

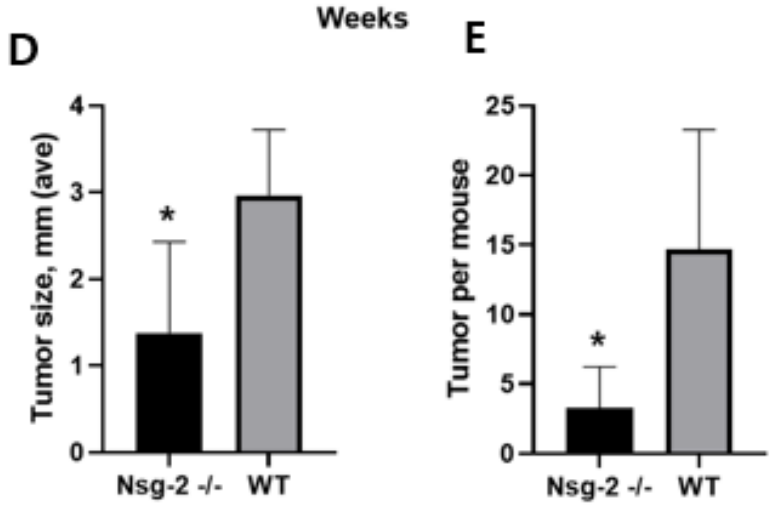
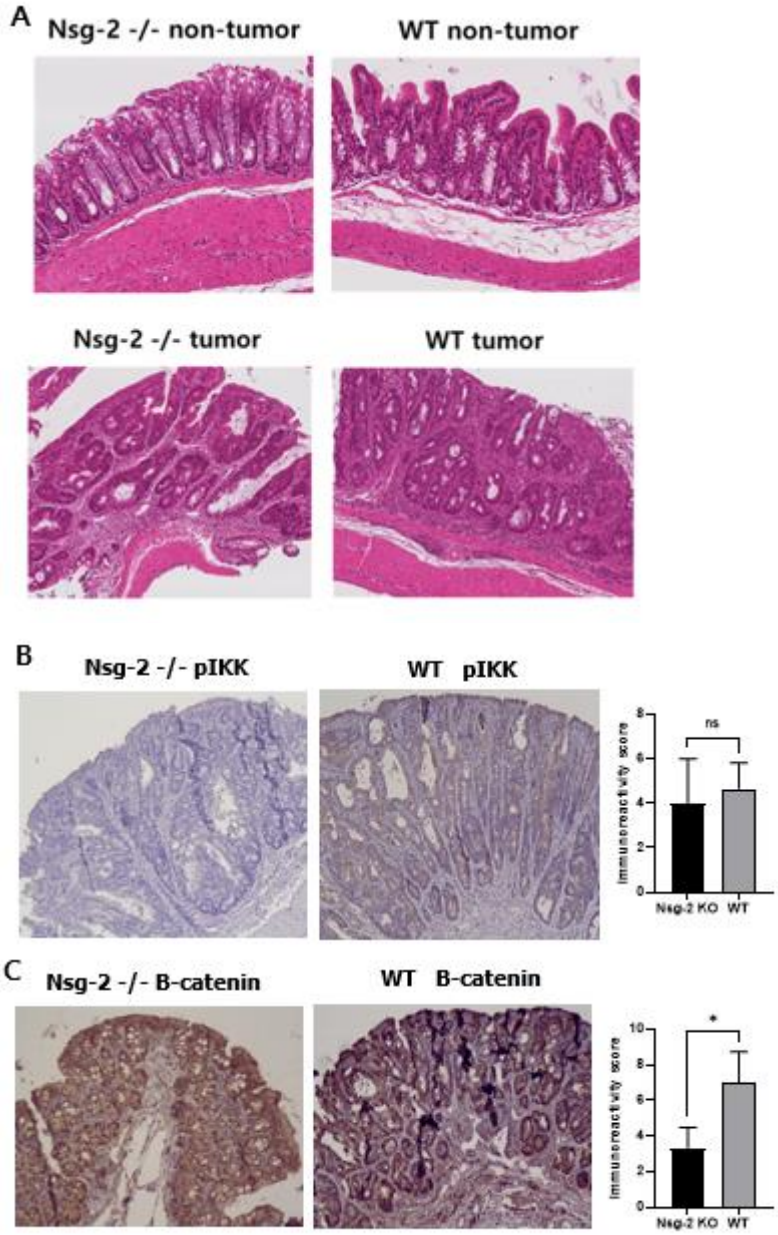


Figure 4. Nsg-2 KO is protective against AOM/DSS-induced colitis-associated colorectal cancer. These figures are comparison of clinical indices and gross appearance in Nsg-2 KO mice (n=5) and wild-type mice (n=5). **(A)** To induce colitis-associated colorectal cancer, Injecting AOM is followed by 3 cycles of 3% DSS drinking with recovery days. **(B)** The macroscopic view of the colon lumen. More tumors were formed in wild-type mice, not in Nsg-2 KO mice. **(C)** There was no significant difference in weight change between the two groups. **(D-E)** The number and size of tumors were significantly decreased in Nsg-2 KO group than wild-type group. Asterisk(*) indicate significant differences (P-values<0.05)


A
Nsg-2 -/- non-tumor
WT non-tumor
Nsg-2 -/- tumor
WT tumor
B
Nsg-2 -/- pIKK
WT pIKK
ns
Immunoreactivity score
Nsg-2 KO
WT
C
Nsg-2 -/- B-catenin
WT B-catenin
*
Immunoreactivity score
Nsg-2 KO
WT

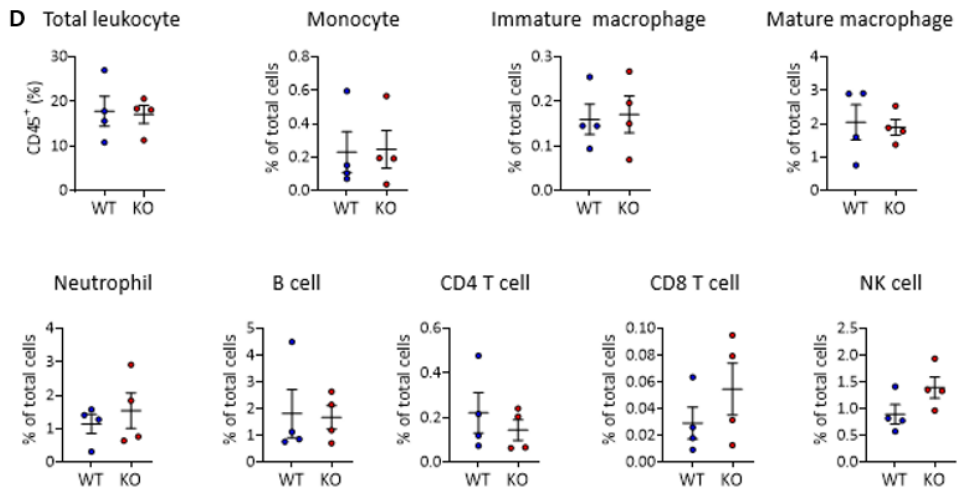


Figure 5. Histopathological and fluorescence-activated cell sorting analysis (FACS) of the colon tissues. **(A)** Crypt distortion and inflammatory cell infiltration were significant in wild-type mice than Nsg-2 KO mice.**(B-C)** These figures are immunohistochemical analysis of the activity of the activities of pIKK **(B)** and beta-catenin **(C)** in tumor tissues of mice. (Brown means positive staining.) Tumor tissues of wild-type mice showed more positivity of both pIKK and beta-catenin. **(D)** FACS analysis shows no difference of immune cells in colon tissues between Nsg-2 KO mice and wild-type mice.

5. Nsg-2 gene expression was increased more in the colon tissue of UC patients than in healthy control subjects.

We studied Immunohistochemical analysis of Nsg-2 expression in the inflamed colon tissues in UC patients and the normal colon tissues in healthy control. Nsg-2 gene expression was increased more in the inflamed colon tissue of UC patients than in healthy control subjects.(Fig 6)

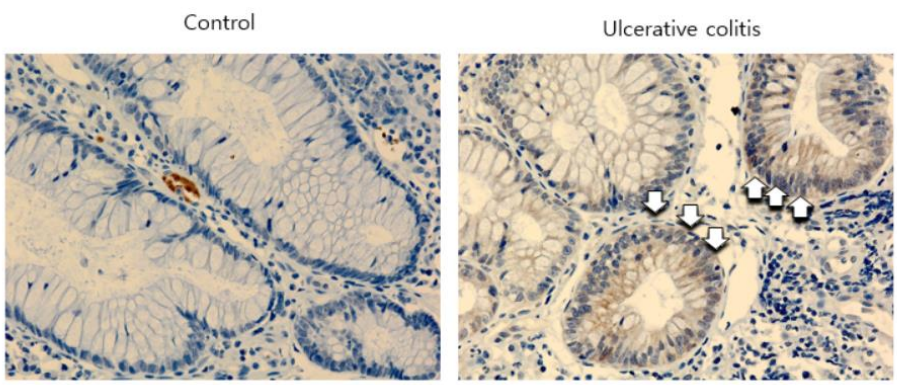


Figure 6 Nsg-2 gene expression was increased more in the inflamed colon tissue of UC patients than in the normal colon tissue of healthy control subjects.

Discussion

In this study, we assessed the role of Nsg-2 in acute and chronic colitis. Our current study demonstrates that deletion of the Nsg-2 gene ameliorates acute colitis. CD 4+ T cells were less abundant in NSg-2 KO mice than in wild-type mice. However, cohousing-mediated microbiota transfer did not narrow the difference between Nsg-2 KO and wild-type mice. Deletion of the Nsg-2 gene reduced the tumorigenesis of the AOM/DSS model. Knockout of the Nsg-2 gene reduced pIKK immunoreactivity and β-catenin translocation into the nucleus, but made no difference of immune cells in colon tissues. The expression of Nsg-2 was increased in the colon tissue of UC than healthy control. To our knowledge, this is the first study to demonstrate the role of Nsg-2 in colonic inflammation.

Activating GABAa receptors, the main inotropic receptors like AMPA receptors, in colonic Epithelium aggravated DSS-induced colitis.[19] Also, Suppressing the N-methyl-D-aspartate (NMDA) receptor, also known as one of the inotropic receptors, using antagonist therapy suppresses inflammation in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis.[20] In the present study, like other inotropic receptors, the AMPA receptor

regulated acute colitis. Knockout of Nsg-2 gene encoding AMPA receptor has less abundant CD4+ T cell compared to wild-type. Taken together, Nsg-2 may aggravate intestinal inflammation by promoting T cell trafficking.

The role of Nsg-2 protein on T cell modulation remained uncertain. In recent studies, GDNF-family ligands (GFLs) released by glial cells promote innate lymphoid cells to protect the host from bacteria via releasing IL-22.[21] Acetylcholine produced by cholinergic neurons binds to M3R in T cells producing IFNg, and IL-13, which are important to defend against various species.[22,23] Also, Glutamate trigger integrin-mediated migration of human T cell via binding to a subtype of AMPA receptor, GluR3.[24] This study observed fewer CD4+ T cells in the lamina propria of Nsg-2 KO mice. Taken together, CD4 + T cells may have a receptor binding to neurotransmitters from excitatory neurons, promoting the trafficking of T cells to lamina propria.

There is much evidence of communication between the microbiome and neurons. Gut bacteria could produce neurotransmitters, such as gamma-aminobutyric acid (GABA), dopamine, serotonin, and norepinephrine.[25] the Previous study showed that short-chain fatty acid from gut microbiota reduced the

expression of cFos in gut sympathetic ganglia, influencing the brainstem sensory nuclei.[26] Since our experiment has a limitation in that we did not use littermates control and conduct other experiments to see the effect of microbiota (e.g., antibiotics experiments), further research is needed to see the relationship between gut microbiota and Nsg-2 protein.

Previously N-myc-downstream-regulated-gene 4 (NDRG4), controlling vesicular transport in the ENS, could be a valuable biomarker for detecting early-stage colorectal cancer.[27] In a recent study, The B-cell lymphoma9 (BCL9) oncogene, which functions as a co-activator of the β-catenin pathway, accelerated tumor progression by neurotransmitter-dependent interaction among colon cancer cells.[28] fewer colitis-associate cancer was observed in Nsg-2 KO mice with decreased cancer-related signaling, but there is no difference of immune cells in colon tissues between Nsg-2 KO mice and wild-type mice. Overall, neurotransmitters from excitatory neurons related to Nsg-2 aggravate tumorigenesis via increasing cell signaling.

There are some limitations in our study. We demonstrated the association of immune cells in the pro-inflammatory role of Nsg-2 but have yet to show the exact mechanism. Especially other models (e.g., antibiotics treatment models and germ-free mice models) are

needed to show the role of microbiota. More experiments are also needed to find the exact mechanism of how the deletion of Nsg-2 reduced tumorigenesis. More samples from UC patients, healthy control, and other patients (e.g., Crohn's disease), particularly the normal tissue from UC patients, will help to elucidate the function of Nsg-2 in humans.

In conclusion, we have demonstrated that suppression of Nsg-2 attenuated acute colitis in the murine model. This effect is mediated by promoting T-cell colonic infiltration but not by microbiota. Nsg-2 deficient also decreased colitis-associated colon cancer and related signaling. Increased expression of Nsg-2 was also founded in UC patients. Overall, Nsg-2 is a potential novel therapeutic target for inflammatory bowel disease. Nevertheless, further study to evaluate the detailed mechanism and validate it in the human setting will be needed.

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국문 초록

만성 장관내 염증을 특징으로 하는 염증성 장질환은 새로운 약제의 개발에도 불구하고 여전히 치료에 어려움이 있다. 염증성 장질환은 장내 미생물에 대한 이상 면역반응의 결과로 생각되는데, 최근 이들과 상호작용하는 신경계의 이상이 염증성 장질환과 관련 있다고 알려져 있다. Nsg-2는 장염과 관련성이 알려진 AMPA 수용체와 결합하는 단백질을 암호화하는 유전자로 알려져 있다. 하지만 Nsg-2의 장염에서 역할은 여전히 잘 알려져 있지 않다. 이에 본연구에서는 다양한 장염 생쥐 모델을 이용하여 Nsg-2의 장염에서 역할을 살펴보았다

먼저 Nsg-2 유전자 발현을 억제하였을 때 DSS에 의한 장염은 호전되었고 CD4+ T 세포의 장관 내 침윤도 감소하였다. Cohousing에 의해 이러한 효과는 사라지지 않았다. 만성 장관내 염증에 의한 대장암의 발생 또한 Nsg-2 유전자 발현 억제시 감소하였고 pIKK 및 beta-catenin과 같은 암 신호전달체계 또한 감소하였다. 다만, 장관내 면역세포는 양 군에서 큰 차이가 없었다. 또한 건강인의 정상 조직과 비교하여 궤양성 대장염 환자의 염증 조직에서 Nsg-2의 발현이 증가하는 것을 확인하였다. 이를 통해 Nsg-2 가 장관내 염증에서 중요한 역할을 함을 알 수 있고, 염증성 장질환의 새로운 치료 표적이 될 수 있을 것으로 생각한다.