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Original Article

Resveratrol enhances MUC2 synthesis via the ANRIL-miR-34a axis to mitigate IBD

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Abstract: Background/aims: Resveratrol (RSV) is a natural polyphenol with strong biological activity that confers a measure of protection against the development of inflammatory bowel disease (IBD), and the long noncoding RNA (lncRNA) antisense non-coding RNA in the INK4 locus (ANRIL) is closely related to inflammation. The present study determined whether resveratrol attenuated IBD by regulating ANRIL and its specific molecular mechanism. Methods: In vivo model of IBD was induced by dextran sulfate sodium (DSS). In total, 60 BALB/c mice were randomly divided into 3 groups (normal control - NC, DSS, RSV), and their weight changes, fecal traits, colon length and tissue hematoxylin-eosin (H&E) were observed. Moreover, human colonic epithelial cells (HCoEpiC) treated with lipopolysaccharide (LPS) were used as cell models of IBD. The tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, IL-10, lncRNA ANRIL, and miR-34a levels were measured by RT-PCR. The expression of mucin 2 (MUC2) and an enzyme associated with MUC2 synthesis, polypeptide N-acetylgalactosaminyltransferase 7 (GALNT7), was measured by RT-PCR and western blot analysis. Results: Resveratrol treatment mitigated colitis by significantly decreasing the expression of pro-inflammatory cytokines (i.e. TNF-α, IL-1β, IL-6) and miR-34a, and increasing the levels of anti-inflammatory cytokine (i.e. IL-10), MUC2, GLNAT7, and lncRNA ANRIL in mice and HCoEpiC (all $P<0.05$). The elevated synthesis of MUC2 could be attributed to the ANRIL-miR-34a axis. Conclusions: Resveratrol attenuates IBD by promoting MUC2 synthesis via the ANRIL-miR-34a axis.

Keywords: Resveratrol, colitis, MUC2

Introduction

Inflammatory bowel disease (IBD) is characterized by chronic relapsing inflammatory disorders of the intestinal tract that affects the colon, ileum, and rectum, with clinical manifestations such as tenesmus, abdominal pain, and even hematochezia, and mainly includes Crohn's disease (CD) and ulcerative colitis (UC). In recent years, the number of patients with IBD is increasing, particularly in western developed countries and newly industrialized countries [1]. IBD has gained widespread attention in the field of gastroenterology because of the difficulty to cure completely and the easy progression of malignant tumors if prolonged. Therefore, effective treatment of IBD is of great clinical significance in alleviating patients' pain and preventing colorectal tumors caused by IBD. Since there is no effective cure for IBD, researchers continue to explore new therapeutic methods to improve the treatment efficacy. Our previous study found that resveratrol inhibits SUMO1 through the Wnt/β-catenin pathway, thus alleviating colitis in mice [2]. In a similar study, Sabzevary-Ghahfarokhi, et al. [3] also concluded that resveratrol appears to have wide potential in IBD treatment.

Resveratrol is a natural polyphenol with strong biological activity and mainly comes from peanuts, grapes (red wine), mulberry, and other plants. Current studies have shown that resveratrol can prevent tumorigenesis, reduce platelet aggregation, and prevent atherosclerosis, and cardiovascular and cerebrovascular diseases [4-6]. In addition, resveratrol exhibits high anti-inflammatory and antioxidant activity.

Tang, et al. [7] reported that resveratrol inhibits colitis by improving glucose metabolism. Hu, et al. [8] found that the regulation of intestinal microbiota by resveratrol supplementation opens a new promising approach in the treatment of IBD. In another study, Kim, et al. [9] applied gamma-irradiated resveratrol to treat UC, with results showing that gamma-ray-treated resveratrol possesses anti-inflammatory activity and dendritic cell differentiation, indicating its promising potential treatment for IBD. Nunes, et al. [10] reported that resveratrol effectively prevents intestinal inflammation by improving intestinal mucosal inflammation disorder. These findings along with the results of our research team showed that resveratrol has a therapeutic effect on IBD [2, 11]. Regardless, the exact mechanism involved in the mitigation effect of resveratrol in IBD remains unclear.

Mucin plays an important role in intestinal mucosa as an important line of the intestinal barrier [12]. There are many kinds of mucins distributed in the mucosal surface of the body, among which MUC2 is the main one in the gastrointestinal tract [13]. MUC2 is the protein characterized by a large amount of O-glycosylation modification under the action of the N-acetylgalactoseaminotransferases (GalNAc-T) family of polypeptides. GALNT7, one of the 20 isoenzymes of the GALNTS family, acts after the initiation of o-glycosylation [14]. Therefore, GALNT7 is likely to play a protective role in the gut.

Long noncoding RNA (lncRNA) is a class of noncoding RNA molecules, which switch gene expression levels in the form of RNA instead of encoding proteins. Studies have shown that lncRNA is involved in the occurrence and progression of intestinal diseases [15, 16]. For instance, Mirza, et al. [17] identified dysregulation of lncRNA (such as KIF9-AS1, DIO3OS, ANRIL, and MMP12) in CD and UC patients. However, none of the lncRNA has been included in the study of resveratrol ameliorating IBD.

LncRNA ANRIL, which serves as a potential marker for estimating disease risk, is downregulated in colonic mucosa tissues of IBD patients and inversely associated with TNF-α and IL-6 levels [18]. ANRIL is also intertwined with miR-323b in UC [19]. Nonetheless, the significance of miRNAs in ANRIL's ameliorating effect on IBD remains to be explored. Interestingly, ANRIL-sponged miR-34a is downregulated by ANRIL in acute myeloid leukemia [20] and up-regulated by ANRIL to promote the apoptosis of human glioma cells [21]. Moreover, miR-34a/c functions as a tumor suppressor by reducing GALNT7 expression [22]. GALNT7, as a glycosyltransferase, is involved in the synthesis of MUC2 and the repair of IBD [23]. Li, et al. [24] found that lncRNA SNHG7 regulates GALNT7 expression through sponging miR-34a. Although ANRIL may be involved in IBD, our knowledge of the specific mechanism and whether it is involved in the protective effect of resveratrol against IBD is still limited.

Therefore, this work aims to determine whether resveratrol attenuates IBD through the ANRIL-miRNA axis-mediated glycosylation of intestinal epithelial cells, which might provide novel insight into treatment for IBD.

Materials and methods

Animal model

In total 60 BALB/c mice (6-8 weeks old, male) were purchased from the Laboratory Animal Research Center of Jiangsu University (Jiangsu, China). All experimental procedures were in accordance with the guidelines of the Animal Care and Nursing Committee of Jiangsu University.

BALB/c mice were divided into three groups (n = 20/group): normal control group (NC), DSS induced colitis group (DSS), and resveratrol treated group (RSV). Resveratrol (Sigma, U.S.A.) for intragastric administration of mice was dissolved in 10% (v/v) ethanol in distilled water with a dose of 100 mg/kg/day. Mice in the NC were given autoclaved-purified water, the DSS and RSV groups were given 3% DSS water prepared by autoclaved-purified water, and the RSV group was given RSV by drinking water/gavage. When obvious hematochezia and weight loss were observed in the positive group, all mice were sacrificed by neck amputation, and their colons and spleens were harvested. The fecal traits and disease activity indicators of mice were documented. The expressions of inflammatory and anti-inflammatory factors and related indexes were measured by QRT-PCR and western blot analysis, and the repair effect of RSV on IBD was comprehensively evaluated. The disease activity index (DAI) score

Table 1. The clinic disease activity index (DAI) score

Score	% Weight loss	Consistency of feces	Visible blood in feces	Appearance
0	None	Normal stool	None	Lively/normal
1	1-5	Soft stool	None	Hunched
2	6-10	Very soft stool	Blood in stool	Starey coat
3	11-20	Fluid stool	Blood in stool	Lethargic
4	>20	Empty, wet colon	Gross bleeding	Lethargic

Table 2. Primers for genes

Genes	Forward (5'-3')	Reverse (3'-5')
IL-6	AAGTCCGGAGAGGAGACTTC	GGATGGTCTTGGTCCTTAG
IL-1β	AGCTTCAGGCAGGCAGTATC	TCATCTCGGAGCCTGTAGTG
TNF-α	AACTCCAGGCGGTGCCTATG	TCCAGCTGCTCCTCCACTTG
IL-10	GTTGTTAAAGGAGTCCTTGCTG	TTCACAGGGAAGAAATCGATGA
ANRIL	CCTTTCTGCTACGAAGACCACACTC	CAAACTGCTCAGGGCTGTCATCC
MiR-34a	AGCCGCTGGCAGTGTCTTA	CAGAGCAGGGTCCGAGGTA
GALNT7	GTTGGGCACATCTACCGTCTTGAG	ATCCCACCAGACTTCCACGACTC
MUC2	CCTTCCTCTGTGCTTATCTGCTGTG	GGTGTCTCCGTATGTGCCGTTG
U6	GCTTCGGCAGCACATATACT	ACGCTTCACGAATTTGCGTG
β-actin	GCACTCTTCCAGCCTTCCTTCC	GCGGATGTCCACGTCACACTTC

was used to evaluate the progression of colitis (**Table 1**).

Hematoxylin-eosin staining

All mice were fasted for 24 hours before being sacrificed. Colon tissue was washed with 4°C pre-cooled PBS to remove fecal matter and then cut along the longitudinal axis with scissors for protein and RNA extraction. A small portion of colon and spleen tissues were fixed in 4% (wt/vol) paraformaldehyde, decalcified, dehydrated, permeabilized, embedded in wax, and 4-μm-thick sections were mounted on Silanized Slides. After being deparaffinized with xylene, it was rehydrated through decreasing concentrations of ethanol, stained with hematoxylin and eosin, and sealed with neutral resin.

Cell culture

Human colonic epithelial cells (Nanjing Saihongrui Biotechnology Co., Ltd, 2022031700-34P) were inoculated in minimum Eagle's medium (MEM) containing 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA) and 1% penicillin-streptomycin, under the conditions of 37°C and 5% CO_2.

Cell treatment with LPS and RSV

Resveratrol for the cells was dissolved in dimethyl sulfoxide (DMSO), and the concentration was 20 μmol/L. HCoEpiC were treated in three ways: 1) LPS group: 1 μg/ml LPS (Sigma, USA) was added to the cell culture medium, 2) NC group: PBS was added to the cell culture medium, and 3) RSV group: 20 μmol/l resveratrol was pretreated for 2 h before adding 1 μg/ml LPS. Protein and RNA from HCoEpiC were extracted and measured after 24-h of cell culture.

Real-time (RT) PCR

After the RNA extraction from colon and HCoEpiC using the TRIzol method (Vazyme, China), the quality of RNAs was measured by ultraviolet spectrometer (Thermo, U.S.A.). Then cDNAs of ANRIL, MUC2, and GALNT7 were transformed from RNAs according to the manual of the HiScript 1st Strand cDNA Synthesis Kit (Vazyme, China), and cDNAs of miR-34a were synthesized using the miRNA PrimeScript cDNA synthesis kit (Gene pharma, China). The cDNAs were amplified according to the SYBR Premix Ex Taq II kit (Vazyme, China) instructions; the primers are shown in **Table 2**. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Colon tissue and HCoEpiC were lyzed in radio-Immunoprecipitation Assay (RIPA) buffer (Solarbio, China) containing protease inhibitors at 4°C. An equal amount of protein was separated on 10% SDS-PAGE gels and transferred to polyvinyl-difluoride (PVDF) membranes. After blocking with 5% skimmed milk, the membranes were incubated with anti-GALNT7 polyclonal (1:1000, proteintech) and anti-β-catenin (1: 1000, Cell Signaling Technology) antibodies overnight. This was followed by incubating with secondary antibodies for 45 min at 37°C, visualizing the band using an enhanced chemiluminescence reagent (Mitro, China), and taking

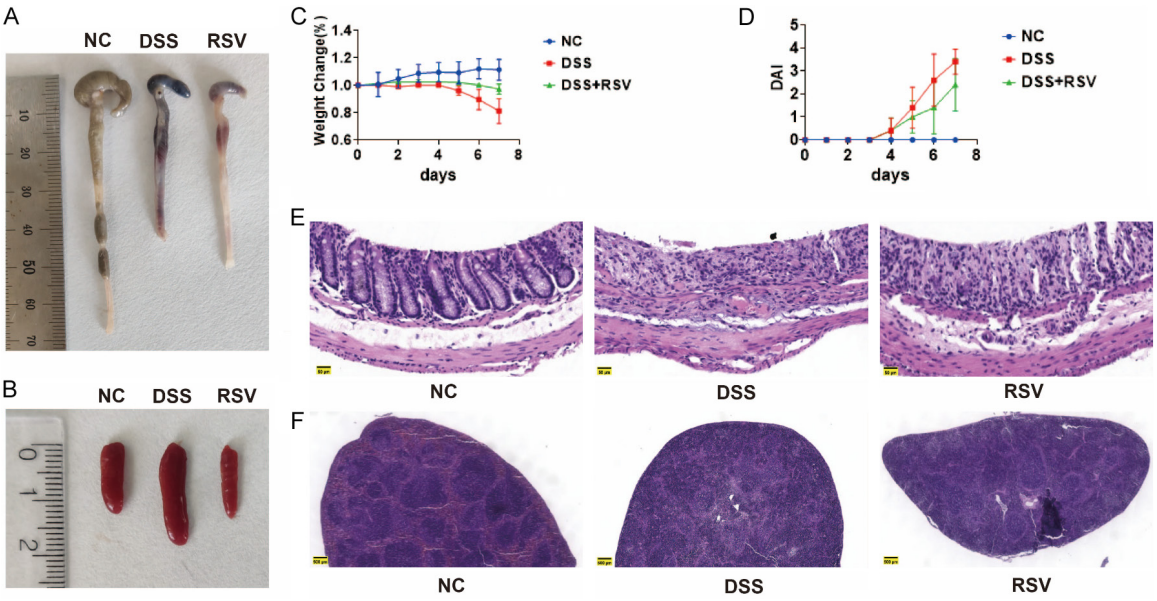

Figure 1. Resveratrol alleviates DSS-induced colitis in mice. A. Colonic length among three groups. B. Spleen appearance among three groups. C. The percentage of body weight of mice among all groups. D. The clinic disease activity index (DAI) of mice. E. H&E staining of colon tissue in mice (400 ×). F. H&E staining of spleen tissue in mice (40 ×). DSS: dextran sulfate sodium.

images using imaging software (ImageQuant, USA).

Statistical analysis

Data are presented as mean ± SD. The differences among groups were valuated using one way ANOVA. Data were analyzed using the GraphPad Prime5 software and $P<0.05$ is considered statistically significant.

Results and discussion

Resveratrol alleviates the macroscopic and microscopic features of DSS-induced colitis

Mice in the NC group had longer colon tissue and intact spleen compared with the other groups. On the contrary, the weight of the DSS group was reduced significantly with slightly bloody stool on the fifth day (**Figure 1A**). Moreover, the DSS group had enlarged and hyperemic spleen (**Figure 1B**). RSV treatment significantly restored colon length, body weight, and splenic blood flow compared to the DSS (**Figure 1C**). While the DAI score of the DSS group increased sharply from day 4, that of the RSV group was significantly lower than that of the DSS group (**Figure 1D**). DSS treatment also caused severe damage to the intestinal structure but was markedly relieved by RSV (**Figure 1E**). H&E staining of the spleen showed that compared with the DSS group, the integrity of the splenic tissue structure of mice in the RSV group was better than that in the DSS group, and inflammatory cell infiltration was reduced (**Figure 1F**).

Resveratrol modulates pro- and anti-inflammatory cytokines to relieve colitis

The colorectum of mice was isolated and mRNA and protein were extracted for further analysis. QRT-PCR results showed that the levels of IL-1β, IL-6, and TNF-α were increased in colon mucosa of the DSS group, while significantly decreased in the RSV group (**Figure 2A-C**). On the contrary, the level of IL-10, an anti-inflammatory factor, decreased in the DSS group but increased in the RSV group (**Figure 2D**). These data suggest that RSV effectively alleviates DSS-induced inflammatory bowel disease in mice.

Resveratrol relieves LPS-induced intestinal epithelial inflammation

HCoEpiC were cultured and LPS used to simulate an inflammatory environment and treated with 20 μmol/LRSV. Results showed that the

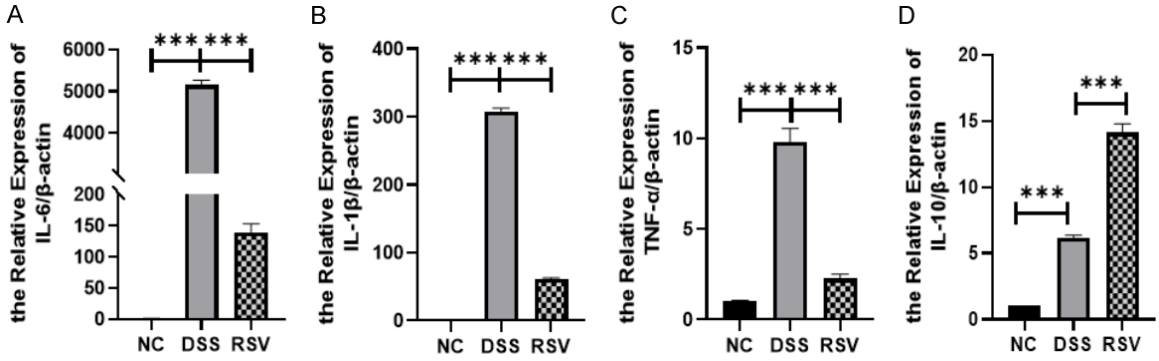

Figure 2. Gene expression levels of inflammation factor in the colon tissues of mice. A. Gene expression levels of inflammatory factor IL-6 in the colon tissues. B. Gene expression levels of inflammatory factor IL-1β in the colon tissues. C. Gene expression levels of inflammatory factor TNF-α in the colon tissues. D. Gene expression levels of inflammatory factor IL-10 in the colon tissues. Bar represent the means ± SD. ***P<0.001.

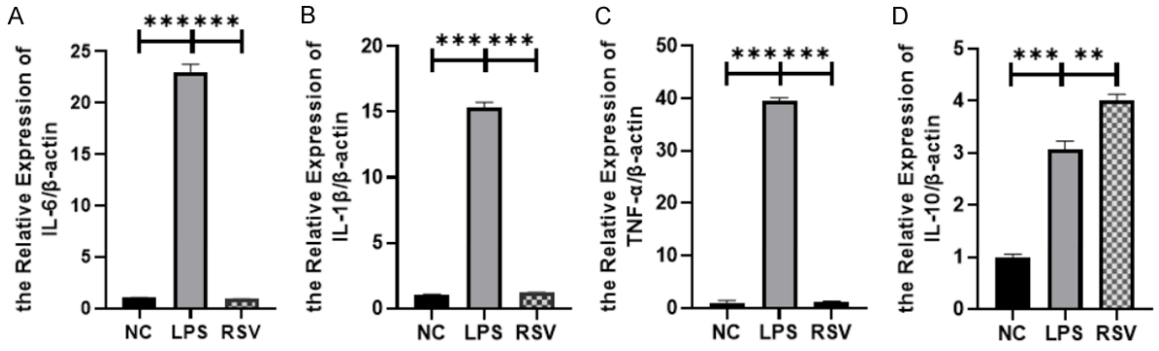

Figure 3. Gene expression levels of inflammation factor in the HCoEpiC. A. Gene expression levels of inflammatory factor IL-6 in the HCoEpiC. B. Gene expression levels of inflammatory factor IL-1β in the HCoEpiC. C. Gene expression levels of inflammatory factor TNF-α in the HCoEpiC. D. Gene expression levels of inflammatory factor IL-10 in the HCoEpiC. Bar represent the means ± SD. **P<0.01; ***P<0.001.

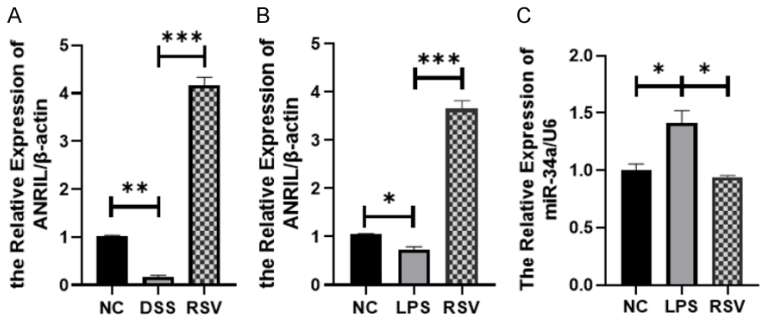

Figure 4. Gene expression levels of lncRNA ANRIL and miR-34a in vivo and vitro. A. The gene expression level of lncRNA ANRIL in colon tissue was detected by qRT-PCR. B. The gene expression level of lncRNA ANRIL in HCoEpiC was detected by qRT-PCR. C. The gene expression level of miR-34a in HCoEpiC was detected by qRT-PCR. Bar represent the means ± SD. *P<0.05; **P<0.01; ***P<0.001.

levels of IL-1β, IL-6, and TNF-α were increased in the LPS group but significantly decreased in the RSV group (**Figure 3A-C**). In contrast, IL-10 levels were significantly higher in the RSV group than those in the LPS group (**Figure 3D**). These data suggest that RSV is effective in alleviating LPS-induced intestinal epithelial inflammation.

Resveratrol regulates lncRNA ANRIL/Mir-34a to repair IBD

The mechanism involved in the repair effect of RSV was further investigated. QRT-PCR results showed that ANRIL level in intestinal tissues of DSS-induced IBD mice was significantly decreased, but elevated in the RSV group (**Figure**

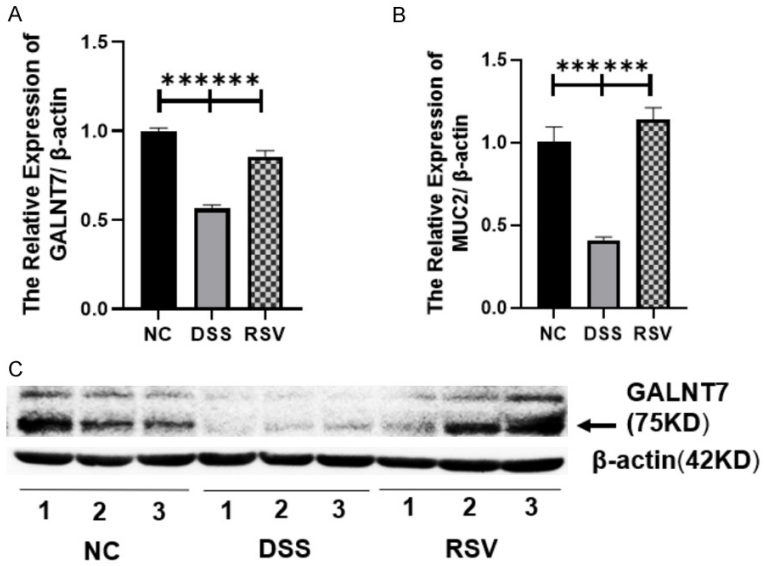

Figure 5. Resveratrol promotes MUC2 to repair IBD in mice. A. The mRNA expression level of GALNT7 in colon tissue was measured by qRT-PCR. B. The mRNA expression level of MUC2 in colon tissue was measured by qRT-PCR. C. The western blot result of GALNT7 in the colon tissue. Bar represent the means ± SD. ***P<0.001. MUC2: Mucin 2; IBD: inflammatory bowel disease.

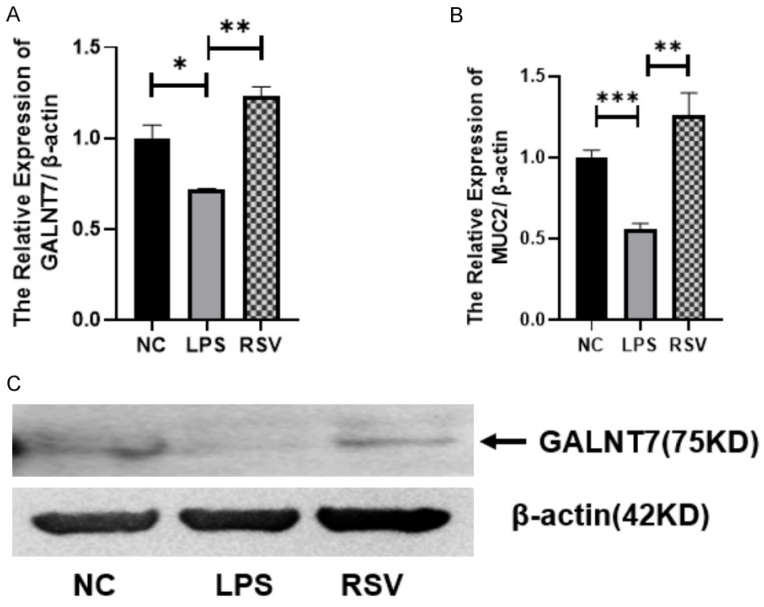

Figure 6. Resveratrol promotes MUC2 to repair IBD in HCoEpiC. A. The mRNA expression level of GALNT7 in colon tissue was measured by qRT-PCR. B. The mRNA expression level of MUC2 in colon tissue was measured by qRT-PCR. C. The western blot result of GALNT7 in HCoEpiC. Bar represent the means ± SD. *P<0.05; **P<0.01; ***P<0.001. MUC2: Mucin 2; IBD: inflammatory bowel disease.

4A). ANRIL levels in LPS-treated HCoEpiC also decreased significantly, and increased in the RSV group, consistent with the results in the animal model (**Figure 4B**). Since previous studies have shown that ANRIL can negatively regulate miR-34a, we analyzed the changes of miR-34a level in HCoEpiC treated with RSV by qRT-PCR. The results indicated that the expression of miR-34a decreased after LPS treatment, while RSV treatment restored the miR-34a level in intestinal epithelial cells (**Figure 4C**).

Resveratrol promotes MUC2 to repair IBD in mice

Mucin secreted by intestinal epithelial cells is the intestinal tract's strong barrier of defense against foreign invasion. MUC2 is the major mucin of the gastrointestinal tract with GALNT7 as the enzyme for the syntheses of MUC2. Studies have shown that the expression of GALNT7 is negatively regulated by miR-34a. In the current study, qRT-PCR analysis of mRNA levels of MUC2 and its synthesis-related enzyme GALNT7 in colon tissues of mice indicated that MUC2 and GALNT7 in the DSS group were significantly reduced, while the levels of both were recovered after RSV treatment (**Figure 5A**, **5B**). Western blot result also showed that the protein expression level of GALNT7 decreased in the DSS group and recovered in the RSV group (**Figure 5C**).

Resveratrol promotes MUC2 in HCoEpiC

After establishing an in vitro model of colitis using HCoEpiC and treating it with RSV, RNA and protein were extracted. QRT-PCR results showed that the mRNA levels of MUC2 and its

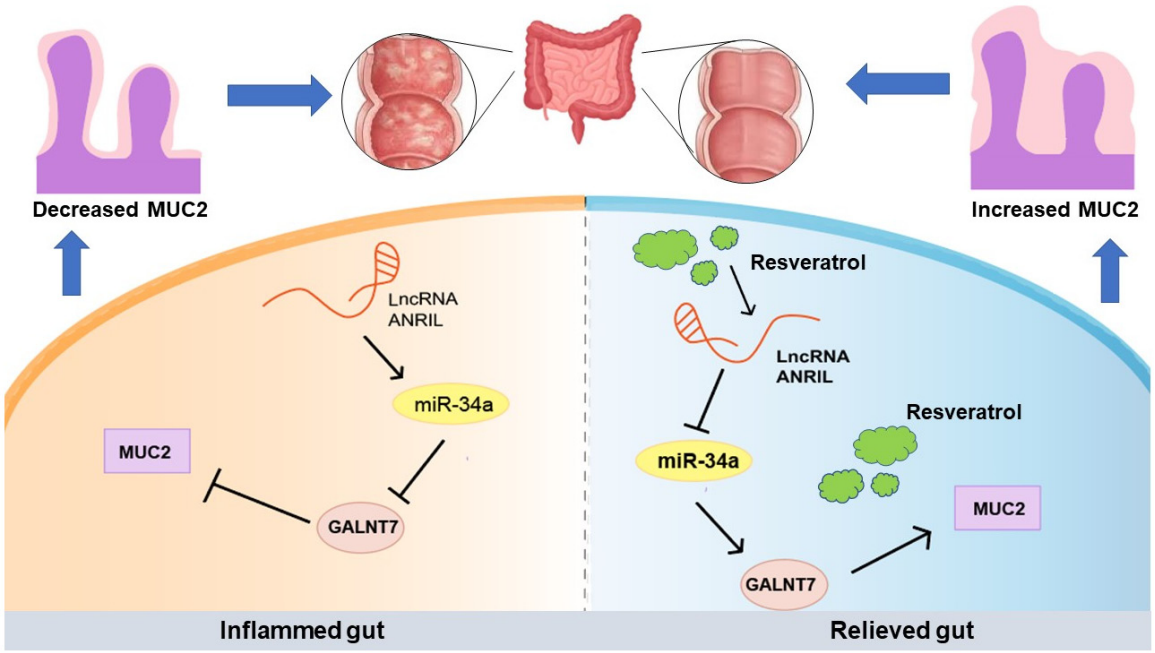

Figure 7. Resveratrol attenuates inflammation bowel diseases by promoting MUC2 synthesis via the ANRIL-miR-34a axis. MUC2: Mucin 2.

synthesis-related enzyme GALNT7 were significantly reduced in the LPS-induced inflammation group, while the levels were restored in the RSV group (**Figure 6A**, **6B**). Western blot analysis of GALNT7 protein expression level was consistent with qRT-PCR results (**Figure 6C**). These results suggest that RSV can promote the secretion of MUC2 in intestinal epithelial cells through lncRNA and thus, enhance mucosal barrier repair in IBD.

Discussion

IBD is a chronic inflammatory disease whose pathogenesis remains unclear. The induction and perpetuation of IBD requires the interaction of one or more influencing factors, including barrier dysfunction, microbial dysbiosis, immune over-activation, and genetic predisposition [25, 26]. In addition, the intestinal barrier plays a pivotal role in protection against IBD. Although a great deal of effort has been invested in studying the intestinal inflammatory response, our knowledge of the intestinal mucosal barrier is still limited. Therefore, to benefit IBD patients, it is necessary to protect the intestinal mucosal barrier and suppress intestinal inflammation. The mucus layer, as a main undertaker of the intestinal mucosal barrier function, is often neglected and thus lacks present studies [27-29]. lncRNA shows great potential in promoting mucin expression by inducing goblet cell proliferation or acting as competitive endogenous RNAs. For example, LASI lncRNA is associated with high mucus expression and mucus cell proliferation in the airway of severe chronic obstructive pulmonary disease [30], and MIR210HG promotes mucin-1C expression in invasive breast cancer [31].

Given that the variation of inflammation factor expression and pathological features of colitis in mice is reflective of colon function (**Figure 1**) and inflammatory response (**Figure 2**) in IBD model mice, our data indicated that RSV ameliorates IBD. The IBD cell model also indicated that RSV effectively relieves IBD (**Figure 3**). To further explore how resveratrol alleviates IBD, this investigation further revealed that ANRIL, which was down-regulated in the DSS-induced IBD model mice was to some degree, normalized after RSV treatment (**Figure 4**). This also verified the decrease of LNCRNA in IBD. We observed that an increase in miR-34a level was accompanied by a decrease in ANRIL level after LPS treatment, and RSV pretreatment could reverse these two trends (**Figure 5**). Previous study shows that miR-34a is a downstream tar-

get gene of ANRIL and is negatively regulated by miR-34a [20]. Collectively, this study shows that RSV likely ameliorates IBD by regulating the ANRIL-miR-34a axis.

Additionally, as MUC2 is known to protect the colonic epithelial cells from direct contact with bacteria, its restoration in the inflamed mucosal environment is necessary to prevent deterioration and encourage healing [32]. Moreover, GALNT7 serves as an enzyme that regulates the synthesis of MUC2 [14, 33]. Our data from both the colon of IBD mice model and in vitro HCoEpiC model revealed that MUC2 and GALNT7 were significantly decreased in IBD but were effectively increased by treatment with RSV. Furthermore, GALNT7 is previously reported to be negatively regulated by miR-34a [24], therefore the ability of RSV to downregulate miR-34a might have contributed to the increased expression of GALNT7.

In conclusion, RSV is beneficial to reliving IBD by promoting MUC2 synthesis via the ANRIL-miR-34a axis (**Figure 7**). However, some difficulties remain to be resolved in future studies to better guide the clinical application of RSV in IBD. First, RSV has low bioavailability and is easily inactivated by light. To partly overcome this challenge, Kim, et al. modified RSV by gamma irradiation which enhanced its immune tolerance and anti-inflammatory effects [9], providing a new idea for improving the bioavailability of RSV. Second, although the regulatory effect of ANRIL on miR-34a has been studied, the specific mechanism has not been fully discussed. This experiment has detected the expression of molecules, and the subsequent experiments can be used to further explore the tight connections between molecules. Finally, the downstream pathways through which RSV regulates glycosylation and perhaps other vital cellular functions via ANRIL need to be studied in depth.

Acknowledgements

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Disclosure of conflict of interest

None.

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