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ORIGINAL ARTICLE

Inhibiting microRNA-7 Expression Exhibited a Protective Effect on Intestinal Mucosal Injury in TNBS-Induced Inflammatory Bowel Disease Animal Model

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Abstract— This study aimed to explore the expression and correlation of microRNA-7 (miR-7) and trefoil factor 3 (TFF3) genes and proteins in inflammatory bowel disease (IBD) mouse models and to elucidate the effect of miR-7 inhibition in the intestinal mucosa in IBD models. A TNBS-induced IBD mouse model was established. Changes in intestinal inflammation were observed by HE staining, and the expression levels of miR-7 and TFF3 were detected by RT-PCR. After miRNA-antagomir injection, the degree of colonic tissue damage and the expression levels of miR-7 and TFF3 in intestinal tissues were compared. TNBS-induced IBD mice showed significant weight loss, significantly decreased disease activity index (DAI), and a significantly increased pathological damage score. miR-7 was highly expressed in the colon tissue of IBD mice, and TFF3 was downregulated. Inhibition of the expression of miR-7 improved the stool characteristics and fecal occult blood (OB) of IBD mice, significantly increased the expression of TFF3 protein, and decreased the pathological damage scores. In the IBD mouse model, miR-7 posttranscriptionally regulates TFF3. The inhibition of miR-7 expression improves some clinical manifestations of IBD mice, reduces the pathological damage of the intestinal mucosa, and shows a protective effect in IBD.

KEY WORDS: microRNA-7; trefoil factor 3; inflammatory bowel disease; miRNA antagomir.

INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a recurrent chronic nonspecific bowel inflammation with a complex etiology. Approximately 25% of IBD patients develop the condition during adolescence (< 16 years), and studies have shown that the incidence of IBD in children is increasing annually [1]. IBD has chronic and prolonged clinical features and recurrent episodes. There is a lack of early monitoring methods and effective

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prevention and treatment measures, which seriously affects the growth and quality of life of patients. At present, the pathogenesis of IBD remains unclear but is generally believed to be related to genetic susceptibility, infection, environmental factors, intestinal flora imbalance, and immune dysfunction. Intestinal mucosal damage caused by an excessive intestinal mucosal immune response plays an important role in the pathogenesis of IBD. Therefore, the exploration of effective drugs to maintain and repair the intestinal mucosal barrier might provide new ideas for the treatment of IBD.

Trefoil factor family 3 (TFF3) belongs to the family of trefoil peptides. It is a small molecule polypeptide secreted by the gossip cells of the ileum and is highly expressed in the intestinal mucosa. It is known as one of the "super protection factors of the intestines" [2]. It is of great value in the repair of intestinal mucosal damage, interacting with mucin glycoproteins to maintain the stability of the mucus barrier.

miRNA is a noncoding single-stranded RNA consisting of 19–25 nucleotides. It has been found to be involved in many life processes such as cell proliferation, differentiation, apoptosis, signal transduction, and immune regulation at the posttranscriptional level [3–5]. Several studies have shown that miRNAs are closely related to immune system diseases such as systemic lupus erythematosus, rheumatoid arthritis, and inflammatory bowel disease [6].

Our previous studies [7] revealed that the expression of miR-7 in the lesions of children with IBD is higher than that in normal tissues. At the cellular level, miR-7 can regulate the posttranscriptional translation of TFF3, and miR-7 can regulate the proliferation and migration of LS174T by targeting trefoil factor 3 by inhibiting the phosphoinositide 3-kinase/Akt signaling pathway. This study attempted to investigate the expression and correlation of miR-7 and TFF3 genes and proteins in IBD mouse models and to observe the effect of inhibiting the expression of miR-7 in an IBD mouse model.

EXPERIMENTAL METHOD

Establishment of the TNBS-Induced Animal Model of IBD and Grouping

In this study, 100 male Balb/c mice aged 21 days were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (license number: SCXK (Shen) 2015-0001). The mice were raised in the Experimental Animal Department of Shengjing Hospital affiliated with China Medical University (license number: SYXK (Shen) 2010-0008) in an environment with a temperature of 25 °C, 45–65% relative humidity and a 12-h light-dark cycle; the mice were caged in a room with no specific pathogens, had free access to chow and water, and were allowed to adapt to the environment for 1 week after arrival. The establishment of the TNBS-induced animal model of IBD was based on the method described previously [8]. The study was approved by the ethics committee of the Affiliated Shengjing Hospital of China Medical University (approval no. 2015PS281K, Shenyang, China).

Balb/c mice were randomly divided into five groups: (1) NS group (normal control group, $n = 20$); (2) control group (ethanol group $n = 20$); (3) TNBS group ($n = 20$); (4) miR inhibition group ($n = 20$), miR-7 antagomir freeze-dried powder was dissolved in saline (0.9%, 200 μl), miR-7 antagomir (100 nmol/kg) was injected into the tail vein 2 h after perfusion of the TNBS solution, and intestinal tissue was collected after 7 days; and (5) rTFF3 intervention group ($n = 20$), rTFF3 (5 g/l, 0.1 ml/only) was injected intraperitoneally on day 2 after modeling, and samples were collected after 5 consecutive days. During the modeling period, a normal diet and drinking water were given. No significant difference in body weight was observed between the groups ($P > 0.05$). On days 1, 3, 5, and after modeling, colon tissue was collected to prepare pathological sections for confirming the presence of colitis and for the next experiments.

Clinical Assessment

The body weight changes of young mice were measured every day during the experiment, and the activity, mental state, diet, and drinking water of the young mice were recorded. The occult blood (OB) was observed by OB test, and the stool traits and bloody stools were observed to evaluate the disease activity index (DAI) as DAI = (weight loss score + stool trait score + blood in the stool)/3.

Colonic Pathological Damage Assessment

The pathological injury score was measured under a high-power field (200×), and more than five visual fields were randomly selected for evaluation. The tissue sections were scored in terms of severity of inflammation, depth of inflammatory invasion, and degree of crypt injury. Score = (inflammation severity + depth of invasion + degree of crypt damage) × damage range. Specific scoring standards were previously described [9].

Real-Time RT-PCR

TRIzol was utilized for the extraction of total RNA from the tissues, and reverse transcription was performed using a PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan). qPCR was conducted using SYBR Premix Ex Taq II (Takara Bio Inc.) and a Bio-Rad iQ5 Real-Time system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers were as follows: β-actin mouse forward, 5′-GTGACGTTGACATCCGTAAAGA-3′ and reverse, 5′-GCCGGACTCATCGTACTCC-3′; TFF3-mouse forward, 5′-CCTGGTTGCTGGGTCCTCTG-3′ and reverse, 5′-GCCACGGTTGTTACACTGCTC-3′. The primers for U6 and miR-7 (mouse) were designed and synthesized by Guangzhou Ruibo Biotechnology Co., Ltd., using special chemical modifications and end-labeling techniques. Primer sequences are not provided for copyright reasons. The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Immunohistochemical Staining

Immunohistochemical staining was performed using a two-step immunohistochemistry kit (SP-9001, ZSGB-BIO, Beijing, China). Sections (with a thickness of 3.5-μms) were prepared and subjected to the immunoperoxidase method as described previously [8]. The slides were incubated with 1:400 diluted specific primary antibody TFF3 (PA5-21081; Thermo Fisher, MA, USA). The secondary antibody was biotin-labeled goat anti-rabbit IgG (1:50 dilution; SA00001-2; Proteintech, Chicago, IL, USA). The optical densities (OD) of TFF3 protein in colon from slides were determined by densitometric scanning using an Eclipse Ci Plus optical microscope (Nikon Corporation, Tokyo, Japan). PBS was substituted for the primary antibody as a negative control. The OD values are expressed as the means ± standard deviations.

Western Blot

Tissues were lysed with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). Equal quantities of protein (100 μg) were separated by 12% SDS-PAGE, and the proteins were blotted onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The primary TFF3 antibody (Thermo Fisher) was diluted at 1:500, and GAPDH (Wanleibio, Shenyang, China) was diluted 1:6000. The secondary antibody, horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (H+L) (SA00001-2; Proteintech, Chicago, USA), was diluted 1:5000. The procedure is described in a previously published manuscript [7].

Statistical Analysis

All experiments were repeated more than three times. The experimental data are represented by the means ± SEMs, and the statistical analysis was performed by SPSS 13.0. Comparisons between groups were analyzed by one-way ANOVA, two-way ANOVA, and Dunnett's *t* test. $P < 0.05$ was statistically significant.

RESULTS

Establishment of the TNBS-IBD Mouse Model

Both clinical manifestations and pathological changes confirmed the successful establishment of an IBD mouse model using TNBS. The control mice showed normal feeding of water, had clean hair, moved normally, and exhibited normal urine, and normal fecal traits (yellow-brown and oval). The TNBS group of young mice showed mucus-like bloody stools or loose stools, perianal contamination, less animal activity, loss of appetite, fatigue, and laziness, and these mice preferred to stay together. As shown in Fig. 1, the weight gain began to appear on day 3 after modeling in the TNBS group, but the growth rate was significantly lower than in those of the NS and ethanol groups (Fig. 1a, $P < 0.05$, the difference was statistically significant). With the appearance of weight loss and bloody stool symptoms, the DAI of the young IBD mice increased sharply. With the passing of time, the clinical symptoms of the TNBS group gradually eased, and the DAI index began to decline. The DAI scores of the NS and Ethanol groups were lower than those of the TNBS group. The DAI of the three groups of mice was essentially the same on day 7 after modeling (Fig. 1b, $P < 0.05$, the difference was statistically significant). Furthermore, the histopathological changes of colon tissue form IBD mice were detected. Intestinal tissue damage was not observed in the NS group. The intestinal epithelium was intact, the crypt structure was normal, and scattered inflammatory cells were visible in the lamina propria. In the TNBS group, the colons of mice showed obvious transmural inflammation, and the intestinal epithelium and crypt structure were destroyed or had even disappeared. A large number of inflammatory cells infiltrated into the submucosa, forming inflammatory masses, thickening the intestinal wall, and causing obvious congestion and edema. The histopathological scores in

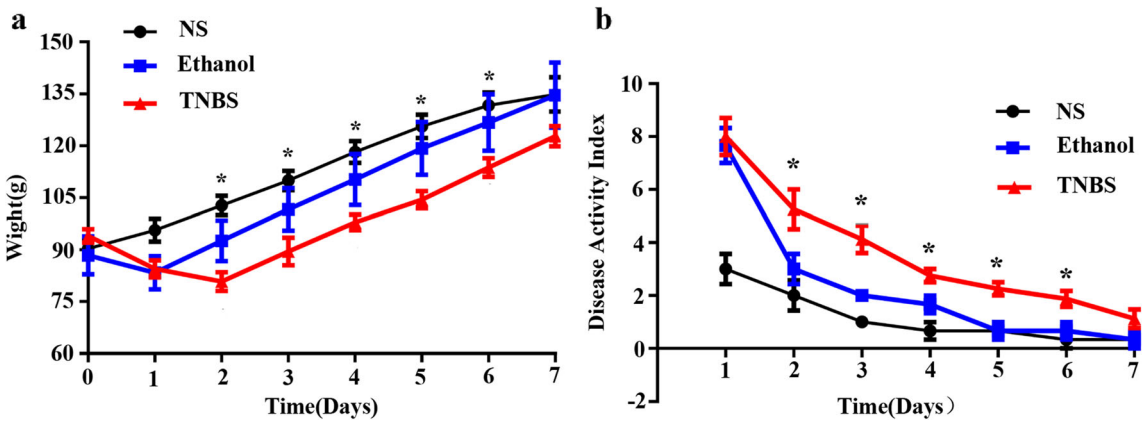

Fig. 1. Clinical manifestation evaluation of IBD mice induced by TNBS. **a** Trend of the body weight changes in young IBD mice. Weight gain began to be observed on day 3, but the growth rate of the TNBS group was significantly lower than those of the NS and ethanol groups, $*P < 0.05$. **b** Trend of DAI in young IBD mice. The DAI scores of the NS and ethanol groups were significantly lower than those of the TNBS group starting on day 2 after modeling, $*P < 0.05$. The DAIs of the three groups of mice were essentially the same on day 7 after modeling.

TNBS group were significantly different from those of the NS and ethanol group (Fig. 2, $P < 0.01$).

Colonic tissue inflammation on day 3 after modeling was the most severe, with statistical significance (Fig. 3, $P < 0.05$). The intestinal mucosa ulcer was essentially healed on day 7 after modeling.

Protective Effect of the miR-7 Antagomir in IBD Mice

Improvement of the Clinical Manifestation of the IBD Mice Model After Injection of the miR-7 Antagomir

Compared with the TNBS group, the body weight of the miR-7 group did not change significantly, but the stool traits and bloody stools improved; the stool traits were mostly soft stools, and the fecal occult blood test score was significantly reduced (Table 1).

Colon Histological Changes After Injection of the miR-7 Antagomir

First, as shown in Fig. 4, in the control group, the colonic mucosal folds had a clear texture, the intestinal wall was evenly thin, and the boundary with the surrounding tissue was clear. The TNBS group showed obvious intestinal congestion and edema in the colonic lesions and distorted intestines; in addition, some of the intestines were narrow, the intestinal wall was thickened, and the intestinal fistulas adhered to each other or to the surrounding tissues. Scattered erosions and bleeding points were detected in the intestinal lumen. There are certain degrees of improvement of the colon in the miR-7 antagomir group, and these improvements included a reduction in the severity and extent of intestinal damage and reductions in the degrees of colon congestion and edema. Furthermore, the histopathological changes were observed. As shown in Fig. 5, the colons of the mice in the TNBS group showed obvious intestinal inflammation. The structure of the intestinal epithelium and crypts was destroyed or had even disappeared. A large number of inflammatory cells had infiltrated, and the lesions reached the mucosa and submucosa. However, in the miR inhibition group, the intestinal epithelium was almost intact, the crypt structure was slightly damaged, and this effect was accompanied by a large number of goblet cells with hyperplasia. In addition, fewer intestinal mucosal epithelial cells were missing, indicating scattered inflammatory cell infiltration. In the rTFF3 intervention group, the intestinal epithelium had returned to normal, and scattered inflammatory cell aggregation and crypt damage were observed.

The pathological scores showed that the TNBS group had the highest pathological score and the most severe inflammatory injury. The pathological scores of the miR inhibition and rTFF3 intervention groups were lower than those of the TNBS group, and a significant difference was found between the two groups ($P < 0.05$). These results suggest that the miR-7 antagomir has the same effect as rTFF3, which can alleviate the damage of TNBS to the intestinal mucosa and promote the secretion of goblet cells and recovery of the intestinal mucosal barrier. Combined with previous experiments, these findings suggest that miR-7 might regulate TFF3 expression and affect intestinal barrier function.

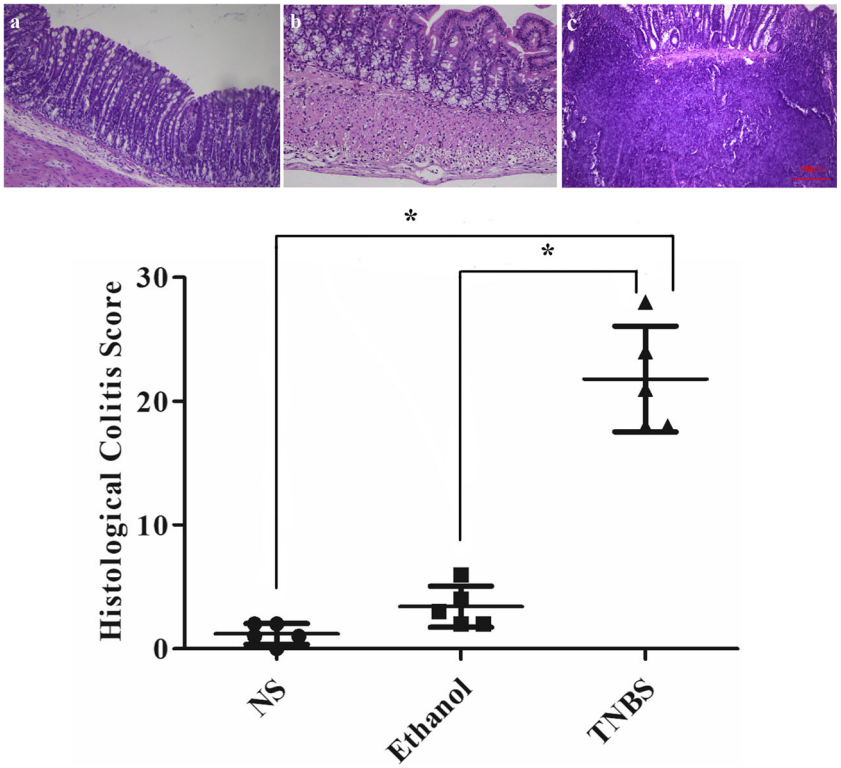

Fig. 2. Histopathological manifestations in the TNBS-induced IBD mouse model (HE × 100). **a** NS group. **b** Ethanol group. **c** TNBS group. In the TNBS group, obvious signs of transmural inflammation were detected, and the intestinal epithelium and crypt structure were destroyed. The histopathological scores of the TNBS group were significantly higher than those of the NS and ethanol group, $*P < 0.01$. No significant difference between the NS and ethanol groups.

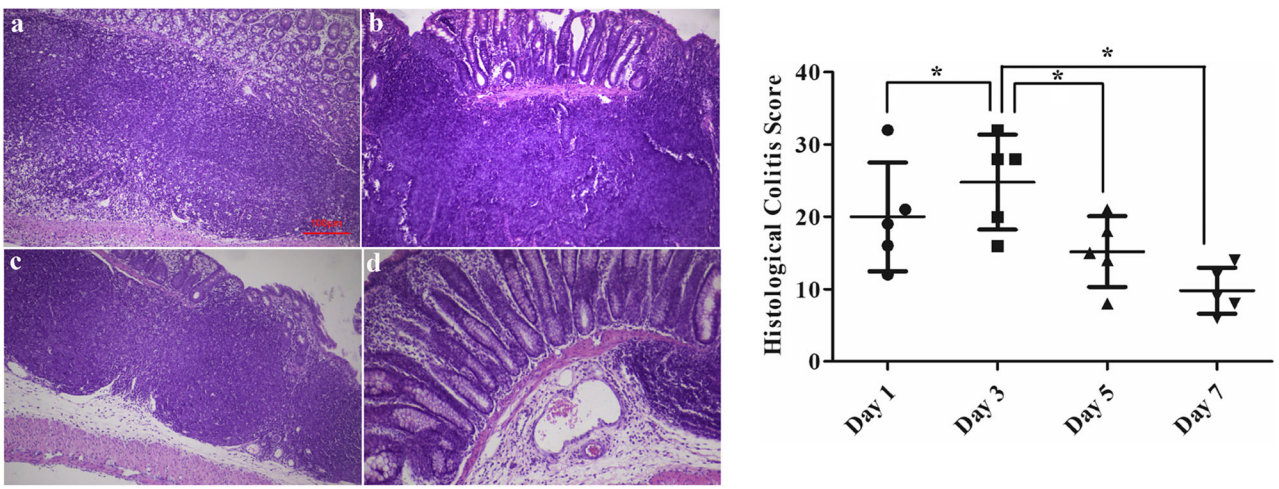

Fig. 3. Time-course analyses of colon tissue pathological changes in young IBD mice (HE × 100). **a** Day 1 after modeling. **b** Day 3 after modeling. **c** Day 5 after modeling. **d** Day 7 after modeling. On day 3, the injury reached a peak and gradually improved, and the intestinal mucosa ulcer was essentially healed on the 7th day after modeling, $*P < 0.05$.

Table 1. Number of Fecal OB Positive Mice of TNBS and miR-7 Antagomir Groups

OB score	TNBS group (*N*)	miR-7 antagomir group (*N*)
3+	4	2
2+	3	2
1+	1	4

Protective Effect of miR-7 on the Colon Mucosa Through Regulation of TFF3 Expression

miR-7 Regulates the mRNA Expression of TFF3 in the Colon Tissue of IBD Mice

RT-PCR was used to detect the expression levels of the miR-7 and TFF3 genes. As shown in Fig. 6a and b, the expression level of miR-7 in the intestinal tissues of young mice in the TNBS group gradually decreased after model establishment. Initially, the mRNA expression level of TFF3 decreased until the lowest expression was found on day 3 after modeling, and then gradually increased, resulting in a significant difference ($P < 0.05$).

As shown in Fig. 6d, miR-7 was highly expressed in the colon tissue of IBD mice. After the miR-7 antagomir inhibited the expression of miR-7, the expression level of miR-7 in the miR-7 inhibitor group was significantly lower than that in the TNBS group, and the difference was statistically significant ($P < 0.01$). These results suggest that miR-7 antagomir can inhibit the expression of miR-7 in mice with colitis. The expression level of miR-7 in the rTFF3 group was not significantly different from that in the TNBS group ($P > 0.05$), which suggests that TFF3 protein can not regulate the expression of the miR-7 gene. Figure 6 c shows the expression level of TFF3 in the colon tissue of young IBD mice on day 7 after modeling, and the TNBS group showed no significant difference in TFF3 expression compared with the NS group ($P > 0.05$). The mRNA expression level of TFF3 in the miR inhibitor group was significantly higher than those in the NS and TNBS groups, with a statistically significant difference (Fig. 6d, $P < 0.05$). The results confirmed that miR-7 is an upstream regulator of TFF3 and that miR-7 antagomir can promote the expression of TFF3 while inhibiting the expression of the miR-7 gene.

miR-7 Regulates the Protein Expression of TFF3 in the Colon Tissue of IBD Mice

Colon tissue was collected on day 7 after modeling. After immunohistochemical staining, the positive expression of TFF3 protein was visible as brownish yellow particles, which were mostly located in the cytoplasm. As shown in Fig. 7, compared with the NS group, a small amount of brown-yellow staining was observed in the TNBS group, and the positive coloration was significantly decreased ($P < 0.05$), which suggests that the expression of TFF3 protein was decreased in the colon tissue of young IBD mice. The expression of TFF3 protein in the miR inhibitor group was higher than that in the TNBS group ($P < 0.05$), which suggests that miR-7 antagomir can

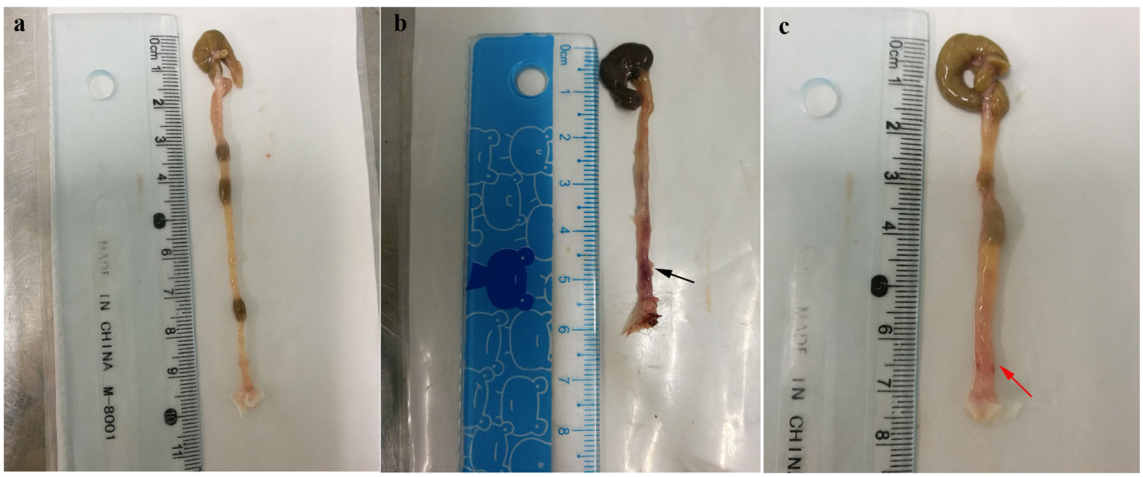

Fig. 4. Manifestations of colon gross specimens after injection of the miR-7 antagomir. **a** NS group. **b** TNBS group. **c** miR inhibition group. The TNBS group exhibited obvious colon intestinal congestion and edema (as shown by the black arrow) and scattered erosions and bleeding points in the intestinal lumen. The miR inhibition group showed reductions in the severity and extent of intestinal damage and reductions in the degrees of colon congestion and edema (as shown by the red arrow).

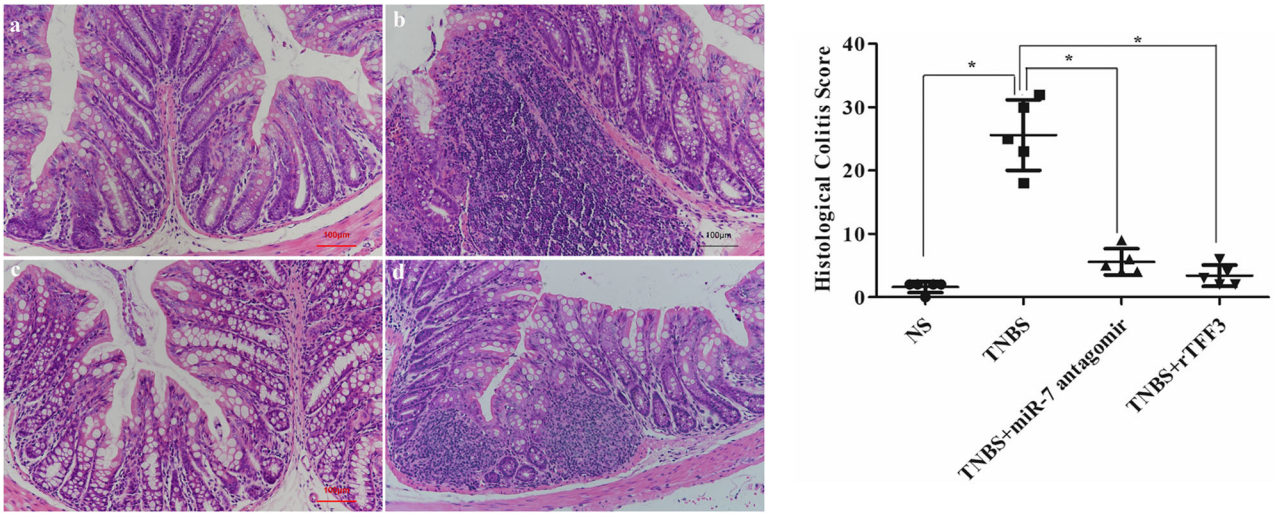

Fig. 5. Protective effect of the miR-7 antagomir in IBD mice (HE × 100). **a** NS group. **b** TNBS group. **c** miR inhibition group. **d** rTFF3 intervention group. The histopathological scores of the TNBS group were significantly higher than those of the NS group, and the histopathological scores of the miR inhibition group were significantly decreased compared with those of the TNBS group. rTFF3 intervention reduced the injury of TNBS. $*P < 0.05$.

promote the secretion of TFF3 protein in goblet cells at the posttranscriptional level and can increase the expression of TFF3 protein. Western blot analysis was also performed to determine the gray values of the TFF3 target and GAPDH bands, and the ratio of the gray values of the target band to that of the GAPDH bands was used to analyze the protein expression. The results showed that the expression of TFF3 protein in the miR inhibitor group was significantly higher than that in the TNBS group (Fig. 8, $P < 0.05$), which suggests that the miR-7 antagomir can promote the expression of TFF3 protein in colon tissue after transcription and that TFF3 plays a protective role against intestinal inflammation.

Overall, we can infer that the miR-7 antagomir can inhibit the expression of the miR-7 gene, upregulate the expression of the TFF3 gene, and thereby promote the expression of TFF3 protein in the intestine, which exerts a protective effect on the intestinal mucosa of IBD mice.

DISCUSSION

The intestinal mucosal barrier is essential for preventing the invasion of foreign antigens into the body and maintaining environmental stability in the body. Once the integrity of the barrier is destroyed, the permeability of the intestinal tract to macromolecules increases, and potentially antigenic proteins enter the body; genetically susceptible people will have a variety of intestine-related diseases [10].

The incidence of IBD is increasing annually [11], but the etiology of IBD remains unclear, and its pathogenesis is complicated. The formulation of a reasonable and effective diagnosis and treatment plan is difficult. In recent years, biologics, nutritional therapy, stem cell transplantation, and fecal transplantation have been entertained as possible therapies, and some progress has been made in the clinical diagnosis and treatment of IBD. The discovery of microRNAs also provides new hope for the identification of new targets for IBD treatment.

miRNAs are a class of endogenous noncoding small RNAs that are 18 to 22 nucleotides in length and that are evolutionarily conserved and can regulate the expression of target genes at the posttranscriptional level [4]. miR-7 is a common tumor suppressor that affects multiple life processes, such as cell migration, proliferation, and apoptosis. Fang et al. found that miR-7 inhibits tumor cell growth and metabolism by targeting the PI3K/AKT pathway [12]. Xu et al. showed that miR-7 can regulate XRCC2 and inhibit tumor cell proliferation and induce apoptosis [13]. Our previous study showed that miR-7-5p can regulate the proliferation and migration of intestinal epithelial cells by targeting trefoil factor 3 by inhibiting the phosphoinositide 3-kinase/Akt signaling pathway [14].

In this study, a model of young mice with IBD enteritis was established by TNBS. Comparing body weight changes, disease activity indexes, and pathological damage scores, it was found that miR-7 was highly expressed in colon tissue during the most severe inflammation, that TFF3 expression was low, and that the two were negatively

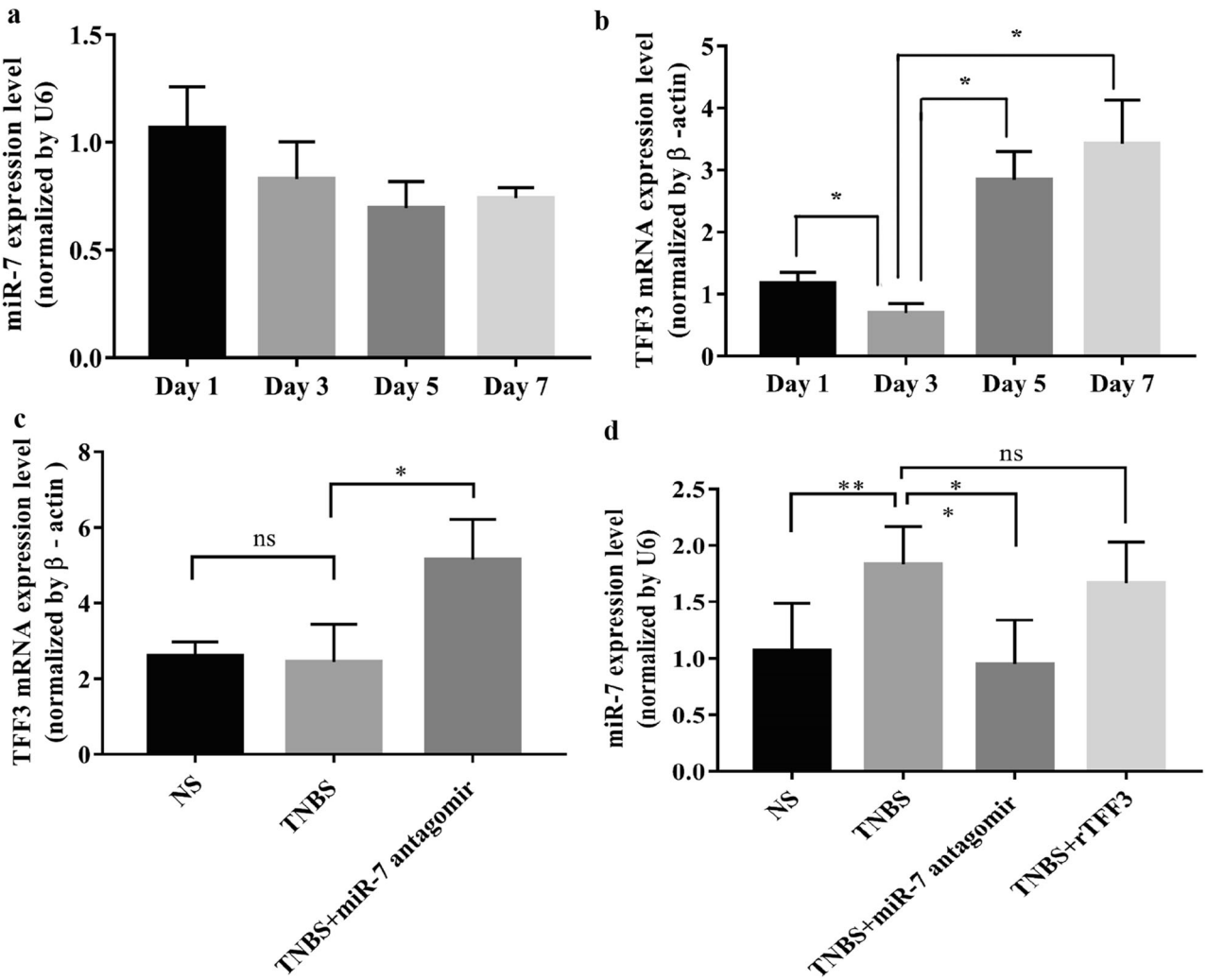

Fig. 6. Expression levels of miR-7 and TFF3 genes in the colon tissues of IBD mice. **a** The expression of miR-7 in the intestinal tissues of young mice in the TNBS group gradually decreased after modeling. **b** The mRNA expression of TFF3 reached its lowest level on day 3 after modeling, and then gradually increased. **c** and **d** miR-7 antagomir can inhibit expression of the miR-7 gene and can simultaneously promote the expression of TFF3. * means $P < 0.05$, ** $P < 0.01$. ns, not significant.

correlated. This finding is consistent with our previous research findings [7]. With the repair of the intestinal mucosa and with goblet cell hyperplasia, the expression of TFF3 is gradually upregulated, and the expression of miR-7 is downregulated. Thus, the differential changes of TFF3 and miR-7 in the model of enteritis are verified. After inhibiting the expression of miR-7, the expression of TFF3 protein was significantly increased. It can be considered that miR-7 might negatively and posttranscriptionally regulate TFF3 expression. TFF3 plays an important role in IBD [15–17] and can reduce intestinal permeability by restoring tight junction protein expression and cytoskeletal rearrangement to protect against intestinal epithelial damage. Therefore, inhibiting the expression of miR-7 might exert a protective effect in IBD.

miRNA antagomirs play very important roles in miRNA function experiments, and functional deletion studies have been performed through competitive inhibition/specific degradation of endogenous miRNAs. These drugs act on blood-rich tissues such as the intestines in the body and can last for several weeks. miRNA antagomirs can be administered by various methods such as local injection or tail vein injection [18, 19]. Huang Y et al. [20] found that miRNA antagomirs can specifically degrade endogenous miRNAs for loss-of-function studies, and miRNA antagomirs have high stability and can be

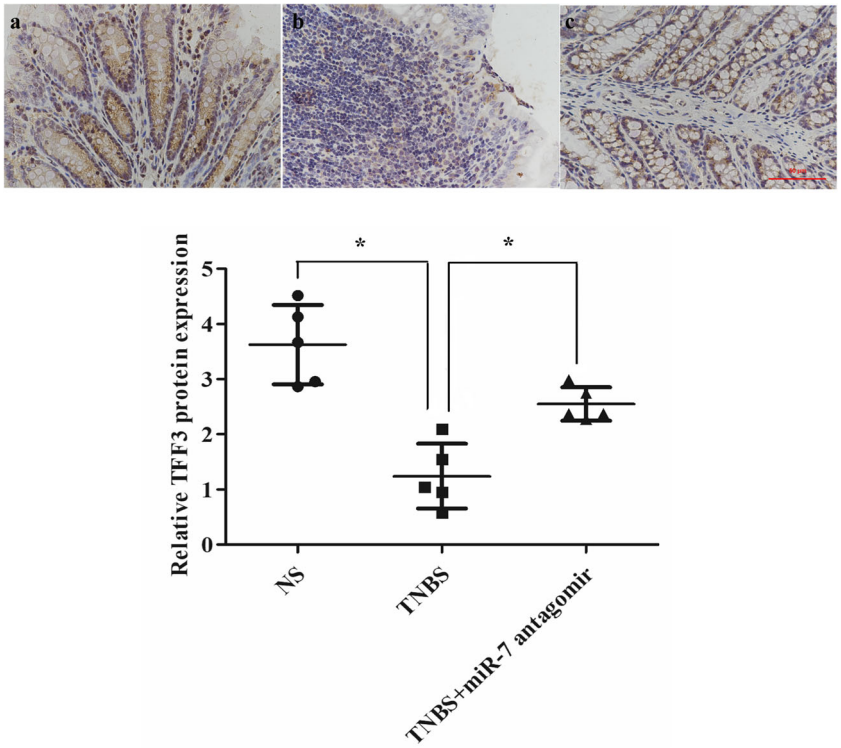

Fig. 7. Detection of decreased expression of the TFF3 protein in IBD mice by immunohistochemical staining (× 400). **a** NS group. **b** TNBS group. **c** miR inhibitor group. The expression of the TFF3 protein in colon tissue in the TNBS group was lower than that in the NS group, and the expression of the TFF3 protein in the miR inhibitor group was higher than that in the TNBS group, $*P < 0.05$.

administered by tail vein injection without transfection reagents. In the study of Liu W et al. [21], the inhibition of miR-7 function by a miR-7 antagomir prevents the nucleus pulposus cell detrimental catabolic changes in response to IL-1β. In this study, inhibition of the expression of miR-7 by a miR-7 antagomir improved the stool

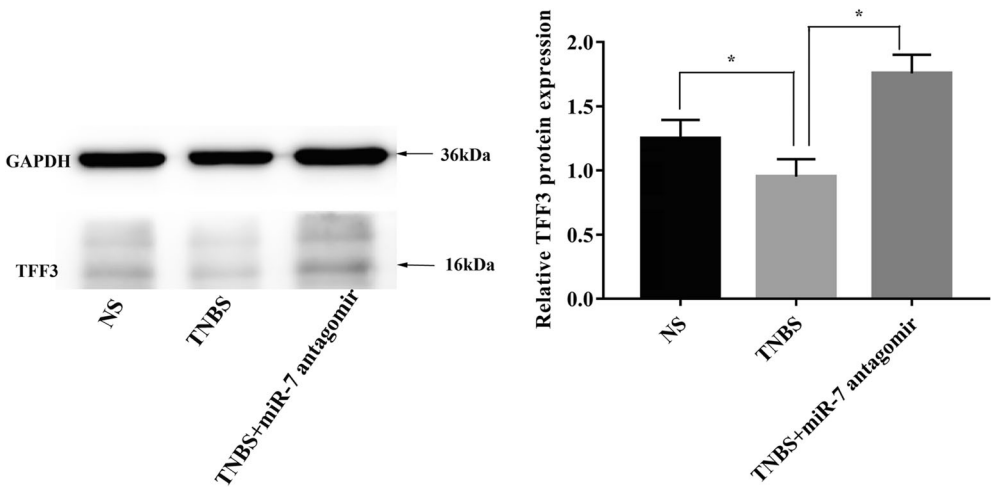

Fig. 8. Western blot analysis of the expression of TFF3 protein in IBD mouse model (Western blot). The expression of the TFF3 protein in colon tissue in the TNBS group lower than that in the NS group, and the expression of TFF3 protein in the miR inhibitor group were higher than that in the TNBS group, $*P < 0.05$.

characteristics and fecal occult blood of IBD mice and decreased the pathological damage scores. These results suggest that inhibitors of miR-7 might play a protective role in IBD mice.

In summary, miR-7 can regulate the expression of TFF3 at the posttranscriptional level, and the inhibition of miR-7 expression exerts a protective effect on intestinal mucosal injury in a TNBS-induced inflammatory bowel disease animal model. These findings provide a theoretical basis for miR-7 as a target to increase the protective role of TFF3 in the intestinal tract, and these data provide promising new ideas for treatments aiming to promote the mucosal repair of IBD. However, the specific pathways and mechanisms still require further study.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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