Knockdown of long non-coding RNA NEAT1 relieves inflammation of ulcerative colitis by regulating the miR-603/FGF9 pathway

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Abstract. Ulcerative colitis (UC) is a significant threat to human life. Hence, there is an urgent requirement to understand the mechanism of UC progression and to develop novel therapeutic interventions for the treatment of UC. The present study aimed to evaluate the potential significance of long non-coding RNA (lncRNA) nuclear enriched abundant transcript 1 (NEAT1) in the progression of UC. NEAT1 expression was detected in colonic mucosa samples from patients with UC and healthy individuals. Fetal human cells (FHCs) were treated with different concentrations of lipopolysaccharides (LPS) to induce UC-caused inflammatory injury, and the effects of NEAT1 knockdown were investigated on cytokines production, cell apoptosis and viability. Furthermore, the correlation and regulation between NEAT1 and microRNA (miRNA/miR)-603 and the fibroblast growth factor 9 (FGF9) pathway were investigated. The results demonstrated that NEAT1 expression was upregulated in the colonic mucosa tissues of patients with UC. In addition, significant cell injury was observed in FHCs treated with different concentrations of LPS, with decreased cell viability, and increased apoptosis and inflammatory cytokines production. Conversely, NEAT1 knockdown significantly reduced LPS-induced cell injury in FHCs, which was achieved through negative regulation of miR-603 expression. Furthermore, FGF9 was negatively regulated by miR-603, and thus, FGF9 was identified as a potential target of miR-603. Notably, FGF9 knockdown reversed the suppressing effects of miR-603 on LPS-induced injury in FHCs. Taken together, the results of the present study suggest that NEAT1 contributes to the development of UC by regulating the miR-603/FGF9 pathway.

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Introduction

Inflammatory bowel disease (IBD) is characterized by an array of symptoms that cause inflammation of the gastrointestinal tract and affect millions of people annually (1). The most prevalent forms of IBD are Crohn's disease and ulcerative colitis (UC), which are considered recurring illnesses that cause serious health issues (2,3). UC is an idiopathic condition of colonic mucosa characterized by symptoms of severe abdominal pain and bloody diarrhea (4). The mucosal ulceration can proximally extend from rectum and thus affects the entire colon (5). It is important to determine the pathogenesis of UC and as its incidence continues to increase worldwide (6). Recent advancements in UC research have unveiled the role of long non-coding (lnc)RNAs in controlling gene expression and epigenetics (7,8). lncRNAs are ~200 nucleotides in length and commonly lack open reading frames that are translated into proteins (9). Aberrant expression of lncRNAs has been reported in several diseases, and their differential expression has been reported in various inflammatory conditions, including rheumatoid arthritis, osteoarthritis and asthma (10,11). However, atypical levels of lncRNAs in UC have not yet been investigated.

Inflammatory diseases have been functionally characterized by upregulated levels of various inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-23, IL-12 and interferon-AS1 (IFNG-AS1), which are regulated by lncRNAs (12,13). For example, overexpression of lncRNA H19 directly targets the intestinal epithelial barrier function, resulting in the development of UC (14). Similarly, IFNG-AS1 has been reported to regulate interferon-γ inflammatory responses and contributes to UC progression (15). A recent

study reported that ANRIL suppression may inhibit the development of UC by regulating the miR-323b-5p/toll-like receptor 4/myeloid differentiation primary response 88/NF-κB pathway (16). This indicates that lncRNAs can regulate the progress of UC through a specific mechanism.

lncRNA nuclear enriched abundant transcript 1 (NEAT1) plays a crucial role in the formation of paraspeckles (17). NEAT1 expression is upregulated in liver diseases and different types of cancer (18). Recently, its aberrant expression has been reported in hepatocellular carcinoma and fibrosis of pulmonary epithelial cells (19,20). However, its role in UC remains unclear. Thus, it is important to determine the underlying molecular mechanisms of lncRNA NEAT1 in the development and progression of UC.

The present study aimed to investigate the role of NEAT1 in UC development and determine its functional mechanism on lipopolysaccharide (LPS)-induced injury in FHCs.

Materials and methods

Tissue samples. The clinicopathological characteristics and medical status of all patients are presented in Table I. Tissue samples were collected from 30 patients with UC (17 males and 13 females; age range, 39-67 years; mean age, 56.1±7.8 years) and 30 healthy individuals (19 males and 11 females; age range, 42-66 years; mean age, 55.3±6.6 years) at Shanghai Tenth People's Hospital, Clinical Medical College of Nanjing Medical University from June 2017 to October 2019. Patients were included in the current study if they had: No other obvious complications and no therapeutic intervention applied prior to enrolment. The exclusion criteria were as follows: i) Patients transferred from other hospitals and ii) patients who were diagnosed with multiple clinical disorders, such as bacterial dysentery and intestinal tuberculosis. Following pathological analysis, a colonic mucosa pinch biopsy of the sigmoid colon was performed on all patients and the samples were immediately snap-frozen in liquid nitrogen at -80˚C until subsequent experimentation. The present study was approved by the Ethics Committee of the Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University (Jiangsu, China; approval no. IR-B-2019-11-14) and written informed consent was provided by all participants prior to the study start.

Cell culture and treatment. UC is characterized by abnormal colonic epithelial cells (21). Thus, colonic epithelial FHCs treated with LPS were used as a cell model of UC, as previously described (16,22). To induce cell injury, FHCs were purchased from the American Type Culture Collection and maintained in an equal proportion of (1:1) DMEM (Gibco; Thermo Fisher Scientific, Inc.) and Ham F-12 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10 ng/ml cholera toxin (Calbiochem; Merck KGaA), 25 mmol/l HEPES [N-(2-hydroxyeth-yl) piperazine-N0 (2-ethanesulfonic acid)], 0.005 mg/ml transferrin, 100 ng/ml hydrocortisone (all from Sigma-Aldrich; Merck KGaA), 0.005 mg/ml insulin and 10% fetal bovine serum (GE Healthcare). Cell injury was induced following treatment of FHCs with 0, 5, 10 and 20 ng/ml LPS (Beijing Solarbio Science & Technology, Co., Ltd.) in accordance with a previously described method (20).

Cell transfection. MicroRNA (miRNA/miR)-603 mimics/inhibitors (50 nM, respectively), negative control miRNA mimics/inhibitor (miR-NC mimics/miR-NC inhibitor), small interfering (si)-NEAT1 and si-NC were purchased from Shanghai GeneChem Co., Ltd. The overexpression vector pcDNA-fibroblast growth factor 9 (FGF9) was constructed by inserting an amplified FGF9 at the HindIII and EcoRI sites of the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.), and an empty vector was used as a control. Sequences of the constructed plasmids were confirmed via Sanger sequencing (Sangon Biotech Co., Ltd.).

For cell transfection, FHCs were seeded into 6-well plates and cultured until they reached 60-70% confluence. Subsequently, cells were transfected with 4 μg overexpression vector using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 15 min, according to the manufacturer's instructions (11). The following sequences were used: si-NEAT1, 5'-GGAGUCAUGCCUUAUACA ATT-3'; si-NC, 5'-CAACAAGATGAAGAGCACCAA-3'; miR-603 mimics, 5'-CACACACUGCAAUUACUUUUGC-3'; miR-NC mimics, 5'-UUCUCCGAACGUGUCACGUTT-3'; mi-603 inhibitor, 5'-GCAAAAGUAAUUGCAGUGUGUG-3' and miR-NC inhibitor, 5'-CGAACGUGUCACGUTT-3'. Cells were collected 48 h post-transfection and used for subsequent experimentation 72 h post-transfection.

MTT bioassay. For the MTT bioassay, FHCs were seeded into 96-well plates at a density of 10,000 cells/well for further treatment. Cells were treated with 500 mg/ml of MTT reagent (Sigma-Aldrich; Merck KGaA) and incubated for 3 h at 37˚C. Following the MTT incubation, the purple formazan crystals were dissolved using 200 ml dimethyl sulfoxide solution (Sigma-Aldrich; Merck KGaA) and absorbance was measured at a wavelength of 490 nm, using a microplate reader (Bio-Rad Laboratories, Inc.) (23).

Apoptosis analysis. For apoptosis analysis, FHCs were seeded into 96-well plates at a density of 10,000 cells/well for further treatment. Cells were subsequently stained with propidium iodide (PI) and FITC-Annexin V, using the FITC-Annexin V Apoptosis Detection kit (BD Biosciences) for 30 min at 37˚C. Apoptotic cells were subsequently analyzed using a BD FACS Calibur flow cytometer (BD Biosciences) and FlowJo 7.6.1 software (Tree Star) (24).

Analysis of inflammatory cytokines. The number of inflammatory cytokines, including IL-1β (cat. no. E-EL-H0109c), TNF-a (cat. no. E-EL-H0149c), IL-6 (cat. no. E-EL-H0192c) and IL-8 (cat. no. E-EL-H6008), that were released following infection was quantified using ELISA (Elascience Biotechnology, Inc.) in LPS induced FHCs, according to the manufacturer's protocol.

Bioinformatics analysis. Bioinformatics analysis was performed using StarBase v2.0 and TargetScan (http://starbase.sysu.edu.cn/), which revealed that the miR-603 binding sites in NEAT1 and FGF9 are potential targets for miR-603.

Dual-luciferase reporter assay. NEAT1 and FGF9 wild-type (WT) (NEAT1-WT and FGF9-WT) and mutant

Table I. Characteristics of healthy individuals and patients with UC.

Characteristic	Healthy	UC
Number of patients	30	30
Age (mean ± SD), years	55.3±6.6	56.1±7.8
Age range, years	48-62	46-64
Sex (male vs. female)	19 vs. 11	17 vs. 13
Duration of symptoms, months	NA	14
Medication		
5-ASA	0	30
Antibiotics	0	5
Steroids	0	7
Immunomodulators	0	2
Biologics	0	1

UC, ulcerative colitis; SD, standard deviation; NA, not available.

type (NEAT1-MUT and FGF9-Mut) reporter vectors were constructed by Beijing Transgen Biotech Co., Ltd. The reporter plasmids (GP-mirGLO) were co-transfected into FHCs with either miR-603 mimics or NC, using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection for 36 h at 37˚C, cells were lysed and relative luciferase activities were detected using a dual-luciferase reporter system (Promega Corporation) following 36 h of incubation. Relative luciferase activity was normalized to that of *Renilla* luciferase as previously described (25).

Reverse transcription-quantitative (RT-q) PCR. Cellular RNA was extracted from FHC cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the Reverse Transcription (Applied Biosystems; Thermo Fisher Scientific, Inc.) and Bio-Rad select cDNA synthesis (Qiagen, Inc.) kits for 60 min at 37˚C. Relative NEAT1 and FGF9 expression levels were quantified using the Takara relative fluorescence quantification kit (One Step TB Green® PrimeScript™ RT-PCR kit, Takara Bio, Inc.). The following primer sequences were used for qPCR: NEAT1 forward, 5'-CCTAGCATGTTTGACAGG CG-3' and reverse, 5'-TGCCACCTGGAAAATAAAGCG-3'; FGF9 forward, 5'-GAAAGACCACAGCCGATTTG-3' and reverse, 5'-TTCATCCCGAGGTAGAGTCC-3'; and GAPDH forward, 5'-CCCACTCCTCCACCTTTGAC-3' and reverse, 5'-CATACCAGGAAATGAGCTTGACAA-3'; miR-603 forward, 5'-CAGCACACACTGCAATTAC-3' and reverse, 5'-GTCCAGTTTTTTTTTTTTTTTGCAA-3'; U6 forward, 5'-GCGCGTCGTGAAGCGTTC-3' and reverse, 5'-GTG CAGGGTCCGAGGT-3'.miR-603 expression was quantified by using TaqMan Universal Master Mix II with TaqMan microRNA assays, in which U6 served as the internal control. The thermocycling conditions were as follows: Pre-denaturation at 95˚C for 1 min, followed by 35 cycles of denaturation for 10 sec, 60˚C for 20 sec, 72˚C for 10 sec (25). The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (26).

Western blotting. Total cellular proteins from LPS-induced FHCs cells were extracted after FHC lysates were generated using RIPA buffer (Santa Cruz Biotechnology, Inc.) for 30 min. Protein concentration was then determined using a Nanodrop Nd-1000 UV-Vis Spectrophotometer (Nanodrop Technologies; Thermo Fisher Scientific, Inc.). Next, 30 μg protein samples with equal amounts were resolved using 10% SDS-PAGE, transferred onto PVDF membranes (MilliporeSigma) and blocked with 5% non-fat skim milk/TBST at room temperature for 2 h. Membranes were incubated with primary antibodies (all, 1:1,000) against β-actin (cat. no. ab8226), Bax (cat. no. ab32503), caspase 3 (cat. no. ab32351), caspase 9 (cat. no. ab32539) and FGF9 (cat. no. ab206408) purchased from Abcam overnight at 4˚C. Following the primary incubation, membranes were incubated with secondary HRP-conjugated rabbit IgG antibodies (cat. no. ab6702; 1:2,000; Abcam) (20), and protein bands were visualized using chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.). Quantity One software (version 4.3.0; Bio-Rad Laboratories, Inc.) was used for densitometric analysis.

Statistical analysis. Data were presented as the mean ± SD and analyzed using SPSS 22.0 software (IBM Corp.). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. Paired and unpaired Student's t-test were used to compare differences between two groups, while one-way ANOVA with Tukey's post hoc test was used to compare differences between multiple groups. Pearson's correlation analysis was performed to assess the correlation between NEAT1 and miR-603 expression. $P<0.05$ was considered to indicate a statistically significant difference.

Results

NEAT1 expression is upregulated in patients with UC. RT-qPCR analysis was performed to detect relative NEAT1 expression in UC tissues of patients and healthy individuals. The results demonstrated that NEAT1 expression was significantly upregulated in patients with UC, suggesting its potential role in UC development ($P<0.01$; Fig. 1A). FHCs were treated with different concentrations of LPS (0, 5, 10 and 20 ng/ml) for 2 h to stimulate FHCs. RT-qPCR analysis demonstrated that NEAT1 expression significantly increased in relation to increased LPS concentration ($P<0.01$; Fig. 1B). Therefore, 20 ng/ml LPS was used to induce cells for further study. RT-qPCR analysis was also performed to detect NEAT1 expression at different time points (0, 1, 2 and 4 h) following treatment with 20 ng/ml LPS. The results demonstrated that NEAT1 expression significantly increased overtime following treatment with LPS, suggesting the potential involvement of NEAT1 in the development of UC ($P<0.01$; Fig. 1C). The results demonstrated that NEAT1 played an indispensable role in UC.

NEAT1 knockdown induces cell viability, and suppresses cell apoptosis and the production of cytokines in LPS-stimulated FHCs. The effects of NEAT1 knockdown on FHCs were evaluated via transfection with si-lncRNA NEAT1 plasmid. Knockdown efficiency of si-NEAT1 plasmid was assessed 48 h post-transfection, which demonstrated that NEAT1 expression significantly decreased in the transfected cells

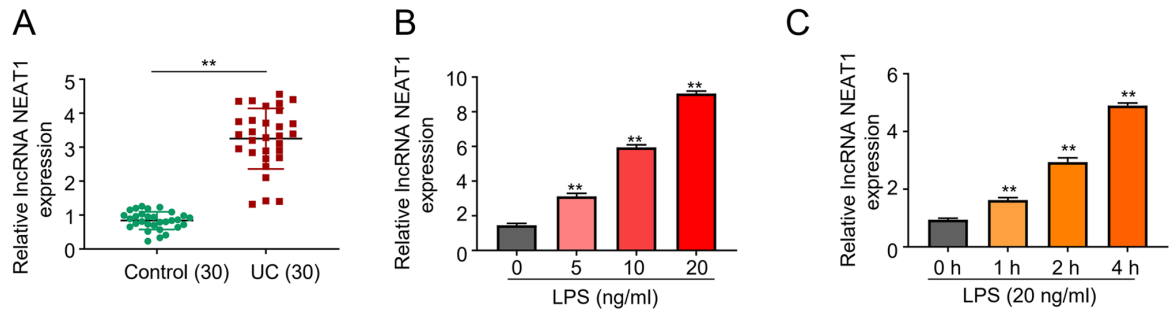

Figure 1. Relative NEAT1 expression in colonic mucosa tissues from patients with UC and healthy individuals, and LPS induced injury in FHCs. (A) The expression of lncRNA NEAT1 in tissues from patients with UC patients and healthy individuals. (B) Relative expression of lncRNA NEAT1 in FHCs treated with different concentrations of LPS. (C) Relative expression of lncRNA NEAT1 in FHCs treated with LPS at different time points. All experiments were performed in triplicate. **P<0.01 vs. the 0 ng/ml LPS group. NEAT1, nuclear enriched abundant transcript 1; UC, ulcerative colitis; LPS, lipopolysaccharide; FHCs, fetal human cells.

compared with the control group (P<0.01; Fig. 2A). si-NEAT1 transfected FHCs were treated with 20 ng/ml LPS for 2 h. The MTT bioassay was performed to assess the viability of the control, LPS, LPS+ si-NC and LPS + si-NEAT1 groups, revealing that si-NEAT1 significantly recovered the decreased cell activity induced by LPS (P<0.01; Fig. 2B). In addition, LPS treated FHCs transfected with si-NEAT1 significantly attenuated apoptosis levels compared with the other groups (P<0.01; Fig. 2C). Similarly, ELISA emphasized the increased production of inflammatory cytokines, IL-1β, IL-6, IL-8 and TNFα, in LPS treated cells. However, NEAT1 knockdown in the LPS + si-NEAT1 group significantly inhibited cell apoptosis and restored cell viability (P<0.01; Fig. 2D). Western blot analysis demonstrated the notable downregulation of apoptosis regulator, including Bax and cleaved caspase 3 and caspase 9, in FHCs transfected with LPS + si-NEAT1, which was previously upregulated following treatment with LPS (Fig. 2E). The results revealed that NEAT1 knockdown increased cell viability, decreased cell apoptosis and inhibited the production of cytokines in LPS-stimulated FHCs.

NEAT1 knockdown alleviates LPS-induced cell injury by upregulating miR-603 expression. Bioinformatics analysis revealed that NEAT1 is a potential target of miR-603, which was further confirmed by generating NEAT1 mutant (Fig. 3A). RT-qPCR analysis was performed to detect miR-603 expression in si-NEAT1 transfected FHCs. The results demonstrated that miR-603 expression increased in the si-NEAT1 group compared with the control group (P<0.01; Fig. 3B).

The dual-luciferase activity of miR-603 mimics was significantly lower in cells co-transfected with miR-603 mimics + NEAT1-WT compared with miR-NC + NEAT1-WT (P<0.01; Fig. 3C). Notably, no significant difference was observed between NEAT1 mutant co-transfected with miR-603 mimics or miR-NC (P<0.01; Fig. 3C). The results demonstrated that miR-603 expression was upregulated in healthy individuals compared with patients with UC (P<0.01; Fig. 3D). In addition, Pearson's correlation analysis demonstrated that NEAT1 expression was negatively correlated with miR-603 expression (Fig. 3E). Notably, miR-603 expression significantly decreased in FHCs treated with LPS, in a concentration-dependent manner (P<0.01; Fig. 3F). Similarly, incubation with LPS significantly decreased miR-603 expression in a time-dependent manner (P<0.01; Fig. 3G). The present study also investigated miR-603 expression in FHCs transfected with miR-603 mimics and miR-603 inhibitor via RT-qPCR analysis (Fig. S1). The results demonstrated that miR-603 expression increased in cells transfected with miR-603 mimics, the effects of which were reversed following transfection with miR-603 inhibitor. The results demonstrated that NEAT1 knockdown alleviated LPS-induced cell injury by upregulating miR-603 expression levels.

miR-603 negatively regulates FGF9 expression and FGF9 is a target of miR-603. Potential binding sites of miR-603 in FGF9 (3'-untranslated region) were identified using the TargetScan online database (Fig. 4A). Co-transfection with miR-603 mimics and FGF9-WT significantly decreased luciferase activity, while no significant difference was observed in luciferase activity following co-transfection with miR-603 mimics and FGF9-MUT, confirming that FGF9 is a target of miR-603 (P<0.01; Fig. 4B). Relative FGF9 expression was based on the transfection of FHCs with LPS, LPS + si-NEAT1, LPS + si-NEAT1 + miR-603 inhibitor and LPS + si-NEAT1 + FGF9. The results suggest that NEAT1 acts as a sponge for FGF9 and treatment with LPS + si-NEAT1 significantly decreased FGF9 expression compared with the other treatments (P<0.01; Fig. 4C). Similarly, western blot analysis demonstrated that treatment with LPS + si-NEAT1 notably decreased FGF9 expression (Fig. 4C). The results of the MTT bioassay demonstrated that cell viability was significantly enhanced in cells in the control and LPS + si-NEAT1 groups compared with the other groups (LPS, LPS + si-NEAT1 + miR-603 inhibitor and LPS + si-NEAT1 + FGF9 groups (P<0.01; Fig. 4D). The levels of inflammatory cytokines, including IL-1β, IL-6, IL-8 and TNFα, significantly increased in LPS treated FHCs, but significantly decreased following treatment with LPS + si-NEAT1 (P<0.01; Fig. 4E). The results revealed that lncRNA NEAT1 knockdown relieved UC inflammation by regulating the miR-603/FGF9 pathway.

Discussion

Despite recent efforts, the treatment and diagnosis of IBD remains a challenge in clinical studies (27,28). Thus, recent studies have aimed to investigate the underlying

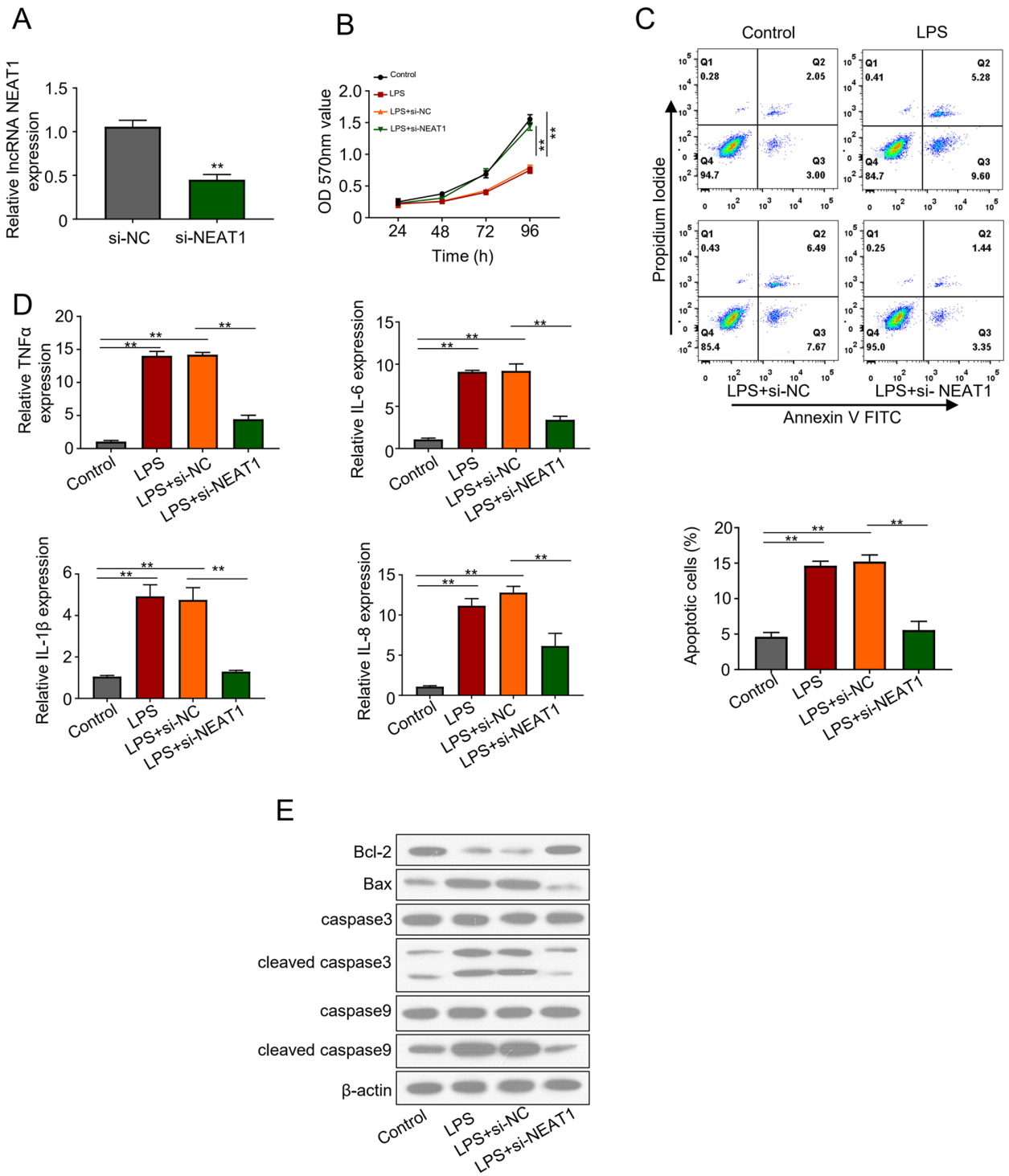

Figure 2. NEAT1 knockdown promotes cell viability, and inhibits cell apoptosis and the production of cytokines in LPS-stimulated FHCs. (A) Relative lncRNA NEAT1 expression in si-NC and si-NEAT1 transfected FHCs. (B) The MTT bioassay was performed to assess cell viability. (C) Flow cytometric analysis was performed to detect apoptosis in different groups of LPS-treated FHCs. (D) ELISA was performed to detect the levels of different inflammatory cytokines in LPS-treated FHCs. (E) Western blot analysis was performed to detect the protein expression levels of different apoptosis related proteins. All experiments were performed in triplicate. **P<0.01 vs. si-NC. NEAT1, nuclear enriched abundant transcript 1; LPS, lipopolysaccharide; FHCs, fetal human cells; si, small interfering; NC, negative control; TNF, tumor necrosis factor; IL, interleukin; OD, optical density.

molecular mechanisms of IBD development and disease progression (29-31). lncRNAs are critical diagnostic and therapeutic biomarkers, which act as competitive endogenous RNAs (32). These competitive endogenous RNAs regulate gene expression and sponge miRNAs (33,34). Recently, the role and differential expression of lncRNAs have been investigated in the development of UC and IBD (10,35). A total of 455 differentially expressed lncRNAs were detected in patients with UC compared with the control group, and BC012900 was demonstrated to modulate epithelial cell apoptosis (36). Similarly, the potential of differentially expressed lncRNA IFNG-AS1 was validated in IBD pathophysiology (15).

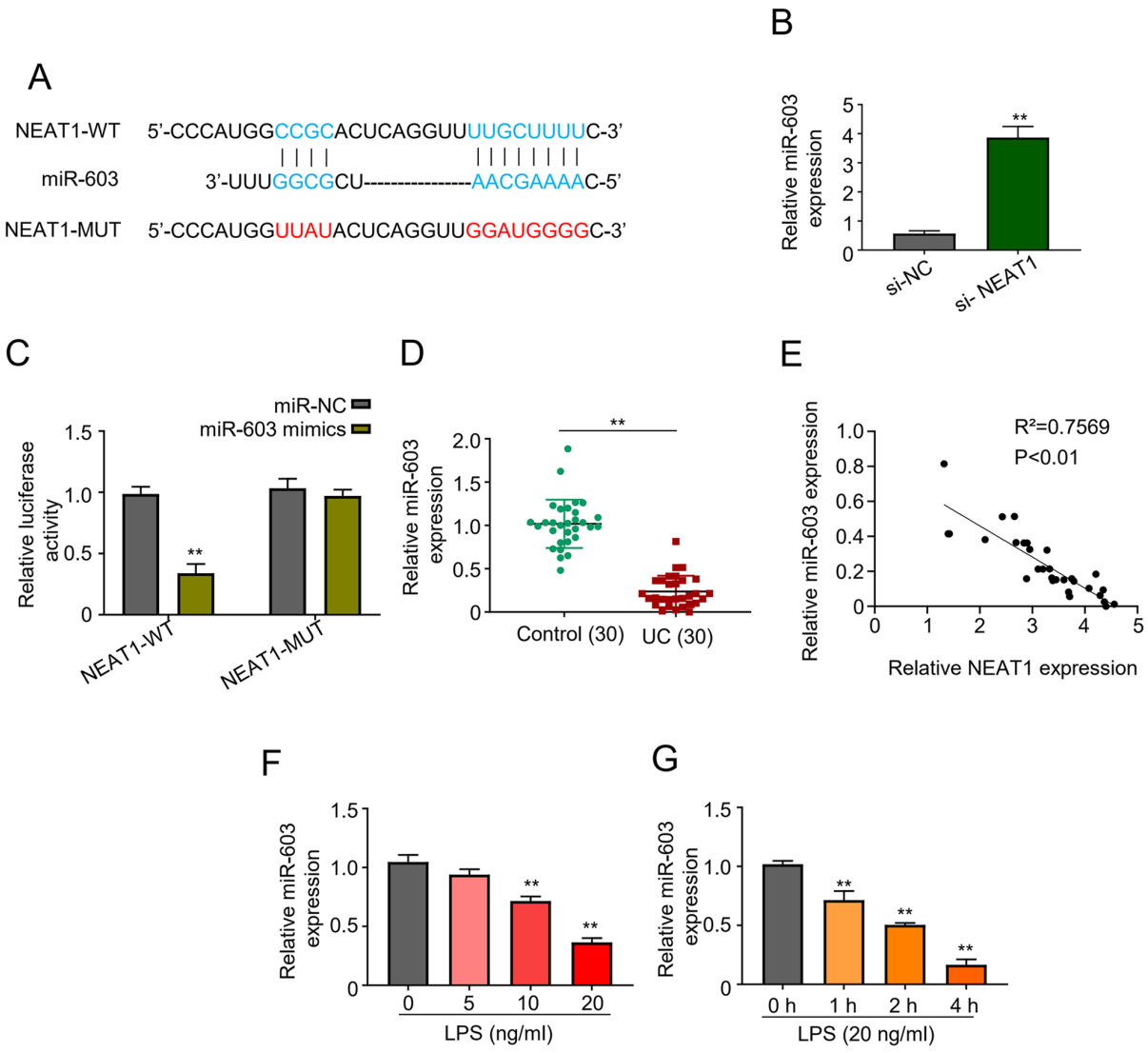

Figure 3. NEAT1 knockdown alleviates the LPS-induced cell injury by negatively regulating miR-603 expression. (A) Bioinformatics prediction of miR-603 binding sites in NEAT1. (B) Reverse transcription-quantitative PCR analysis was performed to detect miR-603 expression in the si-NC and si-NEAT1 groups. **P<0.01 vs. si-NC. (C) Relative luciferase activity of miR-NC and miR-603 mimics co-transfected with NEAT1-WT and NEAT1-MUT type. **P<0.01 vs. miR-NC. (D) Relative miR-603 expression in healthy individuals and patients with UC. (E) Pearson's correlation analysis was performed to assess the correlation between NEAT1 and miR-603 expression in patients with UC. (F) Relative miR-603 expression in LPS-treated FHCs. (G) Relative miR-603 expression in LPS-treated FHCs at different time points. All experiments were performed in triplicate; **P<0.01 vs. 0 ng/ml LPS. NEAT1, nuclear enriched abundant transcript 1; LPS, lipopolysaccharide; miR, microRNA; NC, negative control; si, small interfering; WT, wild-type; MUT, mutant; UC, ulcerative colitis; FHCs, fetal human cells.

The present study aimed to investigate the differential expression of NEAT1 in UC progression and development. The results demonstrated that NEAT1 expression was upregulated in patients with UC compared with healthy individuals, which is consistent with the results of a study by Pan *et al* (37) in which LncRNA NEAT1 mediated intestinal inflammation by regulating TNFRSF1B. Mechanistically, inhibition of NEAT1 significantly decreased injury in FHCs treated with LPS. NEAT1 also plays a role in inflammation by regulating different signaling pathways. For example, NEAT1 ameliorates LPS-induced inflammation in MG63 cells by activating autophagy and suppressing the NLRP3 inflammasome (38). Furthermore, NEAT1 acts as a key regulator of cell apoptosis and the inflammatory response via the miR-944/TRIM37 axis in acute lung injury (39). Furthermore, FGF9 was identified as a direct target of miR-603, and miR-603 expression was negatively correlated with FGF9. Taken together, these results suggest that high NEAT1 expression plays a key role in the progression of UC, and its association in the development of other diseases was speculated.

NEAT1 expression is upregulated in glioma, and NEAT1 knockdown is associated with inhibition of cell invasion and migration (25). In addition, NEAT1 contributes to the development of hepatocellular carcinoma by enhancing STAT3 expression and sponging miR-485 (20). Furthermore, NEAT1 has been implicated in the disease progression of breast cancer and fibrosis of pulmonary epithelial cells (19,40). Increasing evidence suggest that miR-603 may serve as a tumor suppressor to inhibit tumorigenesis by targeting several signaling enzymes. For example, miR-603 inhibits breast cancer by targeting elongation factor-2 kinase (41). Furthermore, miR-603 expression is downregulated in ovarian cancer tissues compared with

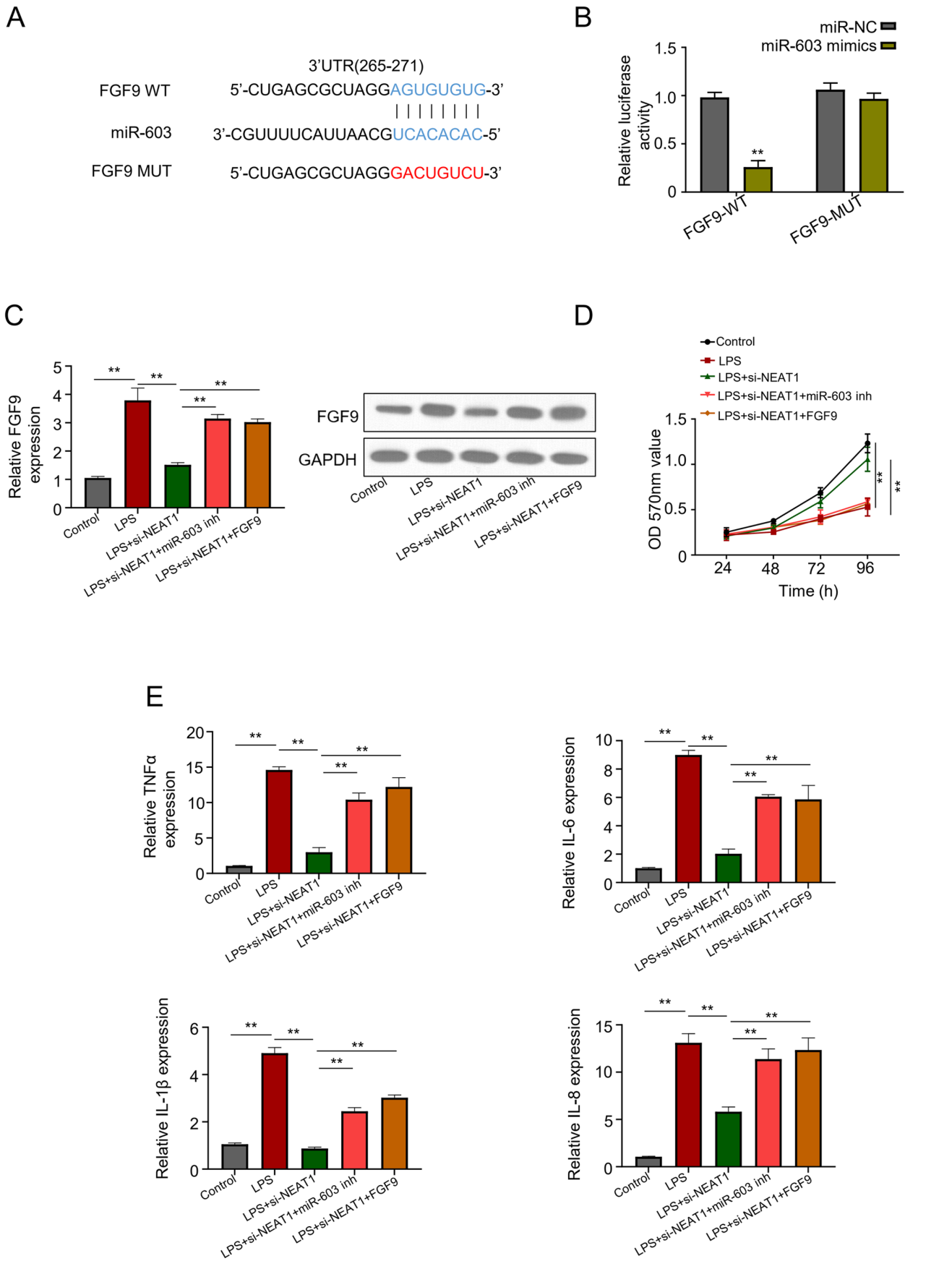

Figure 4. miR-603 negatively regulates FGF9 expression and FGF9 is a target of miR-603. (A) Bioinformatics analysis of miR-603 binding sites in FGF9. (B) Relative luciferase activity of FHCs co-transfected with miR-NC, miR-603 mimics, and FGF9-WT and FGF9-MUT. (C) Relative FGF9 expression in FHCs in the different treatment groups. (D) The MTT bioassay was performed to assess cell viability in the different treatment groups. (E) Expression levels of different cytokines in LPS-treated FHCs. All experiments were performed in triplicate. **P<0.01 vs. miR-NC. miR, microRNA; FGF9, fibroblast growth factor 9; FHCS, fetal human cells; NC, negative control; WT, wild-type; MUT, mutant; LPS, lipopolysaccharide; OD, optical density; TNF, tumor necrosis factor; IL, interleukin.

paracancerous tissues, and miR-603 targets hexokinase-2 to inhibit the malignancy of ovarian cancer (42). Genome-wide analysis demonstrated that miR-603 acts as O^6-methylguanine methyl transferase by regulating miRNAs in glioblastomas, suggesting its potential as a therapeutic target in the treatment of different tumors (27).

FGF9 is involved in several processes in the human body, including mitogenic and cell survival activities, tumor growth, tissue repair and embryonic development (43,44). The protein is associated with the modulation of Schwann cell myelination in developing nerves and creating a pro-inflammatory environment (45). In addition, inhibition of apoptosis in the PI3K/AKT signaling pathway in ischemia is associated with upregulated FGF9 expression (14,15,46). To the best of our knowledge, the present study was the first to investigate the development of UC by NEAT1 through the regulation of the miR-603/FGF9 pathway. Given the potential of FGF9 as a downstream ligand and an activator of the PI3K/AKT pathway, it can modulate several inflammatory responses. For example, FGF9 negatively regulates bone mass by inhibiting osteogenesis and promoting osteoclastogenesis via the MAPK and PI3K/AKT signaling pathway (47).

In conclusion, the results of the present study revealed that FGF9 is a direct target of miR-603 and demonstrated that NEAT1 knockdown alleviates LPS-induced cell injury. Taken together, these results suggest that NEAT1 knockdown can potentially inhibit the progression and development of UC by regulating the miR-603/FGF9 pathway, representing a promising therapeutic target for UC treatment and diagnosis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FL and ZL designed the current study. JF collected the data. LF analysed the data. HL and HZ performed the experiments. SL analyzed the data and drafted the manuscript. All the authors revised and corrected the manuscript. All authors have read and approved the final manuscript. FL and ZL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University (Jiangsu, China; approval no. IR-B-2019-11-14) and written informed consent was provided by all participants prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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