Fusobacterium nucleatum promotes the progression of colorectal cancer by interacting with E-cadherin

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Abstract. Increasing evidence suggests that *Fusobacterium nucleatum* is involved in colorectal carcinogenesis. Previous studies have explored whether *F. nucleatum* may trigger colonic epithelial-mesenchymal transition. The results of the present study demonstrated that *F. nucleatum* enhances the proliferation and invasion of NCM460 cells compared with that of normal control and DH5α cells. Furthermore, *F. nucleatum* significantly increased the phosphorylation of p65 (a subunit of nuclear factor-κB), as well as the expression of interleukin (IL)-6, IL-1β and matrix metalloproteinase (MMP)-13. Additionally, *F. nucleatum* infection did not affect the expression levels of epithelial (E-)cadherin and β-catenin. E-cadherin knockdown in NCM460 cells did not induce the activation of inflammatory responses in response to *F. nucleatum* infection, whereas it increased inflammation in response to β-catenin silencing. *F. nucleatum* infection could not increase the proportion of cells at S phase when E-cadherin was silenced. Nevertheless, *F. nucleatum* infection enhanced the proportion of NCM460 cells at S phase when transfected with small interfering RNAs to knock down β-catenin expression. In conclusion, the results of the present study demonstrated that *F. nucleatum* infection interacted with E-cadherin instead of β-catenin, which in turn enhances the malignant phenotype of colorectal cancer cells.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer in men and the second most common type of cancer in women worldwide (1). Despite advances in the diagnosis and treatment of CRC in the last few years, the overall prognosis remains poor. It has been reported that tumor metastasis serves a function in the diagnosis and therapy of CRC (2). Elucidation of the underlying molecular mechanism of metastasis in CRC may lead to novel treatment strategies.

Epithelial-mesenchymal transition (EMT) is an important biological process for tumor polarized epithelial cells to acquire mesenchymal cell phenotypes (3). In the progression of CRC, the typical characteristics of EMT include the loss of epithelial (E-)cadherin, which is usually accompanied by dysregulation of the Wnt signaling pathway (4,5). In the canonical Wnt signaling pathway, β-catenin regulates the expression of E-cadherin and the induction of EMT (6,7). β-catenin and E-cadherin usually exist in the form of an E-cadherin-β-catenin complex at the membrane (8,9). However, as tumors progress, this complex dissociates and is translocated into the nucleus, thereby triggering the downstream signaling pathways (8,9). Despite the known function of the E-cadherin-β-catenin complex in cell-cell association, little is understood concerning the molecular mechanisms underlying tumor invasion and metastasis.

Increasing evidence suggests that *Fusobacterium nucleatum* is involved in colorectal carcinogenesis. Previous studies have indicated that *F. nucleatum* is present in human colorectal adenomas and carcinomas (10,11). Additionally, *F. nucleatum* is positively associated with advanced tumor stage and poor prognosis in CRC (12-14). Recent studies have identified that *F. nucleatum* adheres to and invades epithelial cells mainly through the virulence factors, including *Fusobacterium* adhesin A (FadA), *Fusobacterium* autotransporter protein 2 and fusobacterial outer membrane protein A (15-17). Nevertheless, it is remains unclear whether *F. nucleatum* triggers the colonic EMT process.

The aim of the present study was to identify the molecular mechanism underlying *F. nucleatum*-mediated EMT process, and to additionally elucidate the role of *F. nucleatum*-mediated EMT in patients with CRC.

Materials and methods

Bacterial strain and culture conditions. *F. nucleatum* strain was purchased from the American Type Culture Collection (ATCC 25586; Manassas, VA, USA). *F. nucleatum* was cultured

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in Columbia blood agar supplemented with 5 μg/ml hemin, 5% defibrinated sheep blood (5%) and 1 μg/ml vitamin K1 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in an anaerobic glove box containing 85% N_2, 10% H_2 and 5% CO_2 at 37˚C. *Escherichia coli* strain DH5α (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was propagated in Luria-Bertani medium (Difco; BD Biosciences, Franklin Lakes, NJ, USA) aerobically at 37˚C.

Cell culture. The human normal colon epithelial cell line NCM460 was obtained from Incell Corporation, LLC (San Antonio, TX, USA). NCM460 cells were cultured in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) at 37˚C in a humidified atmosphere containing 5% CO_2.

Wound healing assays. NCM460 cells ($1x10^6$ cells/well) were seeded into a 6-well plate to form a confluent monolayer and cells were wounded using a pipette tip. Cells were incubated with *F. nucleatum* or *E. coli* DH5α at a multiplicity of infection (MOI) of 1,000:1. Images were captured at 6, 24, 48 and 72 h. The wound size of each well was calculated as the distance between the edges. The images were captured under a light microscope (magnification, x40; XDS-500D; Shanghai Caikon Optical Instrument Co., Ltd., Shanghai, China).

Cell proliferation assays. NCM460 cells were seeded in 24-well plates at a density of $1x10^4$ cells/well with 1 ml complete RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences). Cells were incubated with *F. nucleatum* or *E. coli* DH5α at an MOI of 1,000:1. Cells treated with PBS were used as a negative control. Cell numbers were calculated using a hemocytometer and cell proliferation was analyzed using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol.

Cell cycle assays. NCM460 cells (~$1x106$) cells were trypsinized at room temperature for 2 min, washed twice with PBS and fixed in 70% ice-cold ethanol for 1 h at room temperature. The samples were centrifuged at 300 x g for 5 min at 4˚C, the ethanol was removed and they were exposed to 100 mg/ml RNaseA (Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C. Cellular DNA was stained with propidium iodide (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Cell-cycle distributions were determined by flow cytometry using a BD FACSCalibur system (SKU#: 8044-30-1001, BD Biosciences, Franklin Lakes, NJ, USA) and data was analyzed using the ModFit software (version 4.1; Verity Software House, Inc., Topsham, ME, USA).

Western blot analysis. NCM460 cells were lyzed using radioimmunoprecipitation buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). A BCA kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. Equal quantities of protein (15 μg) were separated by SDS-PAGE (10% gels). Electrophoresed proteins were transferred onto nitrocellulose membranes. The membranes were then blocked with 5% fat-free milk suspended in TBS-T buffer for 2 h at room temperature and incubated with the following primary antibodies at 4˚C overnight: Anti-phospho-p65, a subunit of nuclear factor-κB (NF-κB) (cat. no. ab86299, Abcam, Cambridge, MA, USA), anti-interleukin (IL)-6 (cat. no. ab6672, Abcam), anti-IL-1β (cat. no. ab156791, Abcam), anti-matrix metalloproteinase (MMP)-13 (cat. no. ab39012, Abcam), anti-E-cadherin (cat. no. ab1416, Abcam), anti-β-catenin (cat. no. ab32572, Abcam) and anti-GAPDH (cat. no. ab8245, Abcam). Following several washes with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:5,000; cat. no. ZB-2306, Zhongshan Gold Bridge Biological Technology Co., Beijing, China) for 2 h at room temperature and then washed. The proteins were detected using enhanced chemiluminescence, according to the manufacturer's protocol (Merck KGaA). ImageJ 1.8.0 (National Institutes of Health, Bethesda, MD, USA) was applied to quantify the relative protein levels. GAPDH was used as an internal control.

RNA extraction and quantitative polymerase chain reaction (qPCR). Total RNA was extracted from NCM460 cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The concentration and the purity of the RNA samples were assayed by absorbent density analysis using an optical density (OD) ratio of 260/280 nm. A total of 2 μg RNA was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR amplifications were performed in a 10-μl reaction system containing 5 μl SYBR Green Supermix (Tarkara, Japan) on Bio-Rad iQ5 Optical System (Bio-Rad Laboratories, Inc., Hercules, California, USA), 0.4 μl forward primer, 0.4 μl reverse primer, 2.2 μl double-distilled water and 2 μl template cDNA. The following thermal cycling conditions were used for the qPCR: Initial denaturation at 95˚C for 10 min, 40 cycles of 95˚C for 15 sec and a final extension at 60˚C for 1 min. mRNA levels were determined using the $2^{-\Delta\Delta Cq}$ method (18). The data was analyzed using Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Inc.). GAPDH was used as a reference gene. The primers used in the current studies were as follows: TNFα-forward: CTGGGCAGGTCTACTTTGGG; TNFα-reverse: CTGGAG GCCCCAGTTTGAAT; IL6 foward: AGTGAGGAACAA GCCAGAGC; IL6 reverse: AGCTGCGCAGAATGAGAT GA; IL-1β foward: CTTAAAGCCCGCCTGACAGA; IL-1β reverse: ACACTGCTACTTCTTGCCCC; MMP-13 foward: AGGCCATGGCATCCCTTTTT; MMP-13 reverse: AGC ACCCTCCCCAAGTATCA; GAPDH foward: GAGAAG GCTGGGGCTCATTT; GAPDH reverse: AGTGATGGC ATGGACTGTGG.

Transient transfection. Cells were transfected with small interfering RNA (siRNA) targeting E-cadherin (si-E-cadherin) or β-catenin (si-β-catenin), or with negative control siRNA (NC; 5'-ACUAGUCGAUCUAUGUGUGAUATT-3') (Shanghai GenePharma Co., Ltd., Shanghai, China) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at the indicated concentrations, according to the manufacturer's protocol. In brief, NCM460 cells ($1x10^6$ cells/well) were seeded in a six well plate with 2 ml RPMI-1640 medium. At 60% confluence,
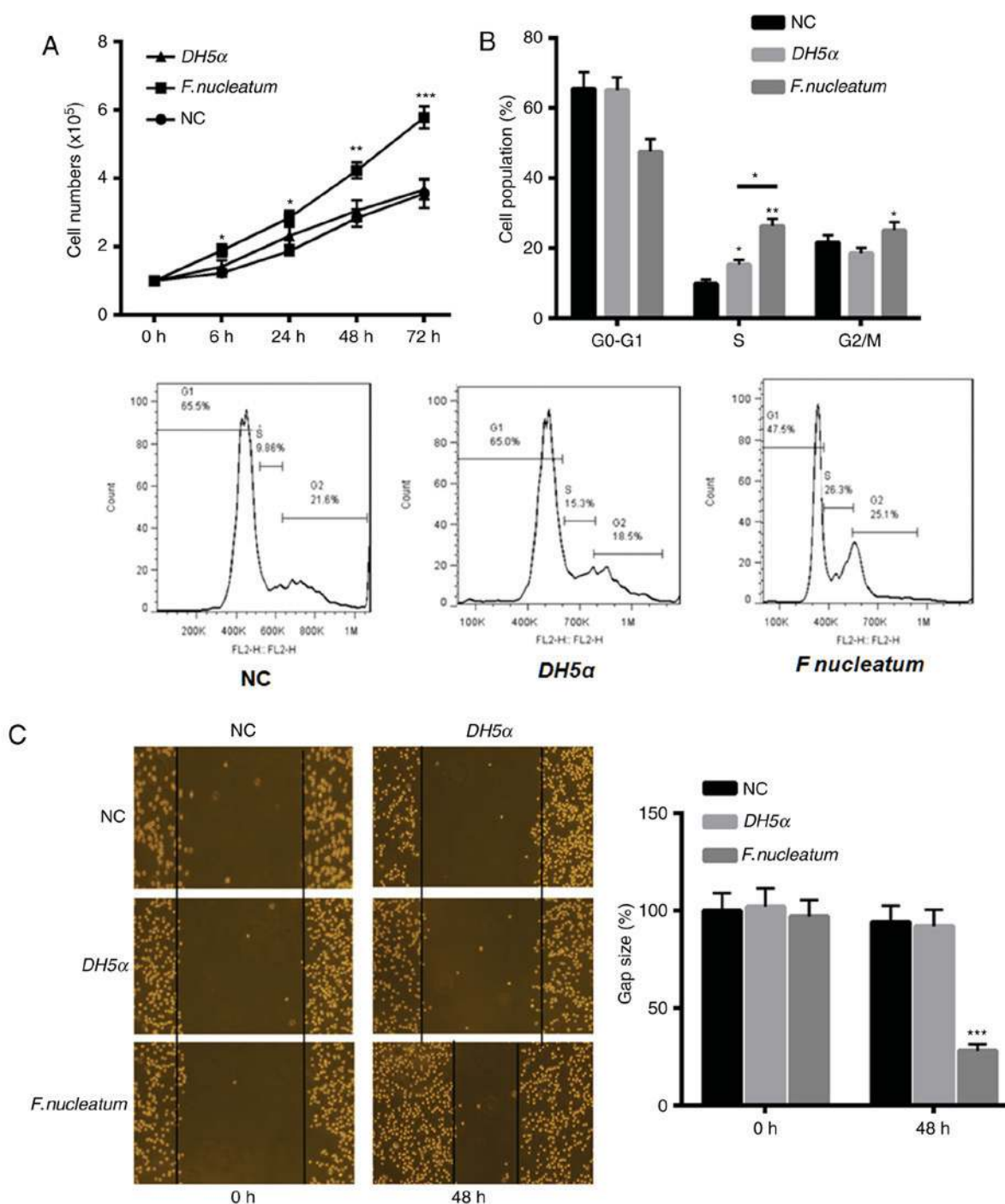
Figure 1. *F. nucleatum* enhances the proliferation and invasion of NCM460 cells. (A) *F. nucleatum* treatment significantly increased the proliferation of NCM460 cells at 24, 48 and 72 h. (B) Cell cycle analysis indicated that *F. nucleatum* increased the proportion of cells at S and G2/M phase. (C) *F. nucleatum* enhanced the invasive ability of NCM460 cells at 48 h compared with the normal control and DH5α groups. *P<0.05, **P<0.01, ***P<0.001 vs. control. NC, negative control group.

50 nM si-E-cadherin or 50 nM si-β-catenin or 50 nM NC was mixed with Lipofectamine® 2000 at room temperature for 20 min. Then, the mixture was added into each well at a final concentration of 20 nM for 48 h. Then, the cells were collected for further analysis.

Immunofluorescence. NCM460 cells were cultured in a 6-well plate with glass coverslips and were fixed in 4% para-formaldehyde for 30 min at room temperature. The samples were washed three times in PBS for 5 min. Following

washing with PBS three times for 5 min, the slides were blocked with 8% bovine serum albumin (Sigma-Aldrich; Merck KGaA) at room temperature for 2 h The cover-slips were incubated with antibodies against E-cadherin (1:50; cat. no. ab1416, Abcam) and β-catenin (1:50; cat. no. ab32572, Abcam) in a humidified chamber overnight at 4°C. After that the slides were washed with PBS for three times and incubated with tetramethylrhodamine-conjugated anti-rabbit immunoglobulin G (1:500; cat. no. ZDR5209, Zhongshan Gold Bridge Co.) and with DAPI (1:1,000;
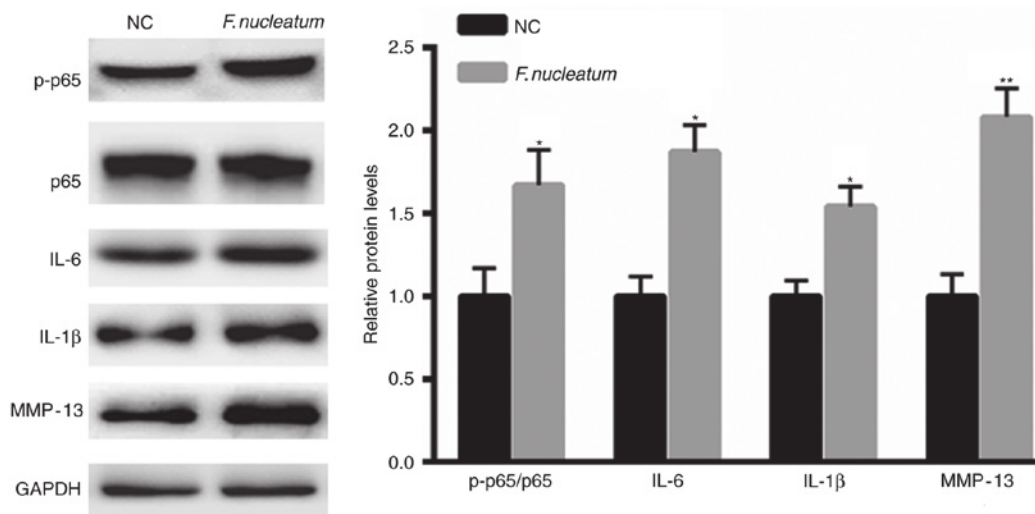
Figure 2. Western blot analysis reveals that *F. nucleatum* promotes the activation of NF-κB signaling. Western blot analysis demonstrated that *F. nucleatum* increased the expression levels of p-p65, p65, IL-6, IL-1β and MMP-13. GAPDH was used as an endogenous control. *P<0.05, **P<0.01 vs. control. NF-κB, nuclear factor-κB; IL, interleukin; MMP, matrix metalloproteinase; p-p65, phospho-p65; NC, negative control group.
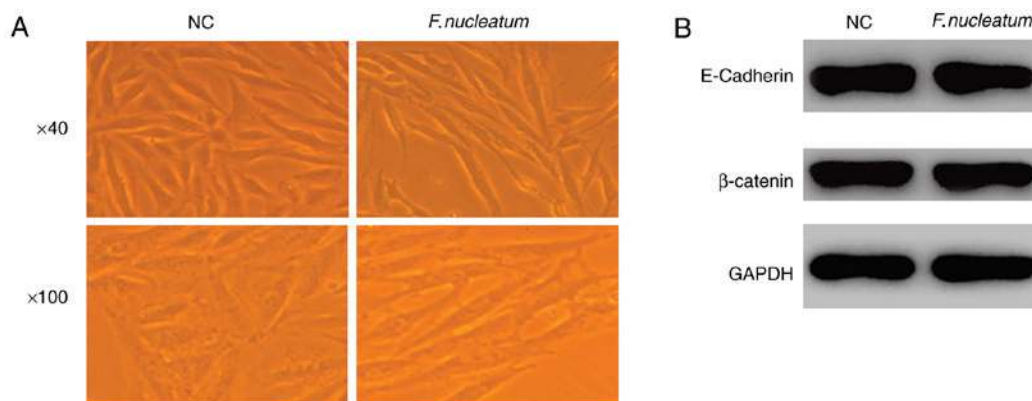

Figure 3. *F. nucleatum* promotes EMT without affecting the expression of E-cadherin. (A) At 48 h marked *F. nucleatum* infection induces mesenchymal transition from epithelial cells as observed using microscopy (magnification, x40 and x100). (B) *F. nucleatum* infection did not alter the expression levels of E-cadherin, but increased the expression of β-catenin as demonstrated by western blot analysis. GAPDH was used as an endogenous control. *P<0.05, **P<0.01 vs. control. NC, negative control group.

cat. no. C0060, Solarbio Science & Technology Co., Ltd.) for 20 min at room temperature. Following incubation with the secondary antibody, the slides were washed three times with PBS in the dark and the coverslips were mounted with a mounting medium and coated on glass slides. The slides were sealed at room temperature for ~1 h in the dark and fluorescence intensity was examined using a fluorescence microscope.

Statistical analysis. Data are presented as the mean + standard deviation for the indicated number of separate experiments. A total of three independent experiments were performed for each experiment. Differences in the quantitative data between two groups were determined using the unpaired Student's t-test. Comparisons of means among multiple groups were determined using one-way analysis of variance. All statistical analyses were performed using GraphPad Software (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). All P-values were two-tailed. P<0.05 was considered to indicate a statistically significant difference.

Results

F. nucleatum enhances the proliferation and invasion of NCM460 cells. First, the function of *F. nucleatum* in the proliferation and invasion of NCM460 cells was investigated. The results indicated that *F. nucleatum* significantly enhanced the proliferation of NCM460 cells at 24, 48 and 72 h, compared with the control and *E. coli* DH5α groups (Fig. 1A). Additionally, flow cytometric analysis of the cell cycle revealed an increased proportion of cells at S and G₂/M phase in response to *F. nucleatum* treatment (Fig. 1B). *F. nucleatum* also increased the invasive ability of NCM460 cells at 48 h compared with the control and *E. coli* DH5α groups (Fig. 1C).

F. nucleatum promotes the activation of NF-κB signaling. Increasing evidence has indicated the important function of pro-inflammatory factors during tumorigenesis (19,20). Therefore, whether *F. nucleatum* infection regulates signaling was also evaluated. *F. nucleatum* infection significantly
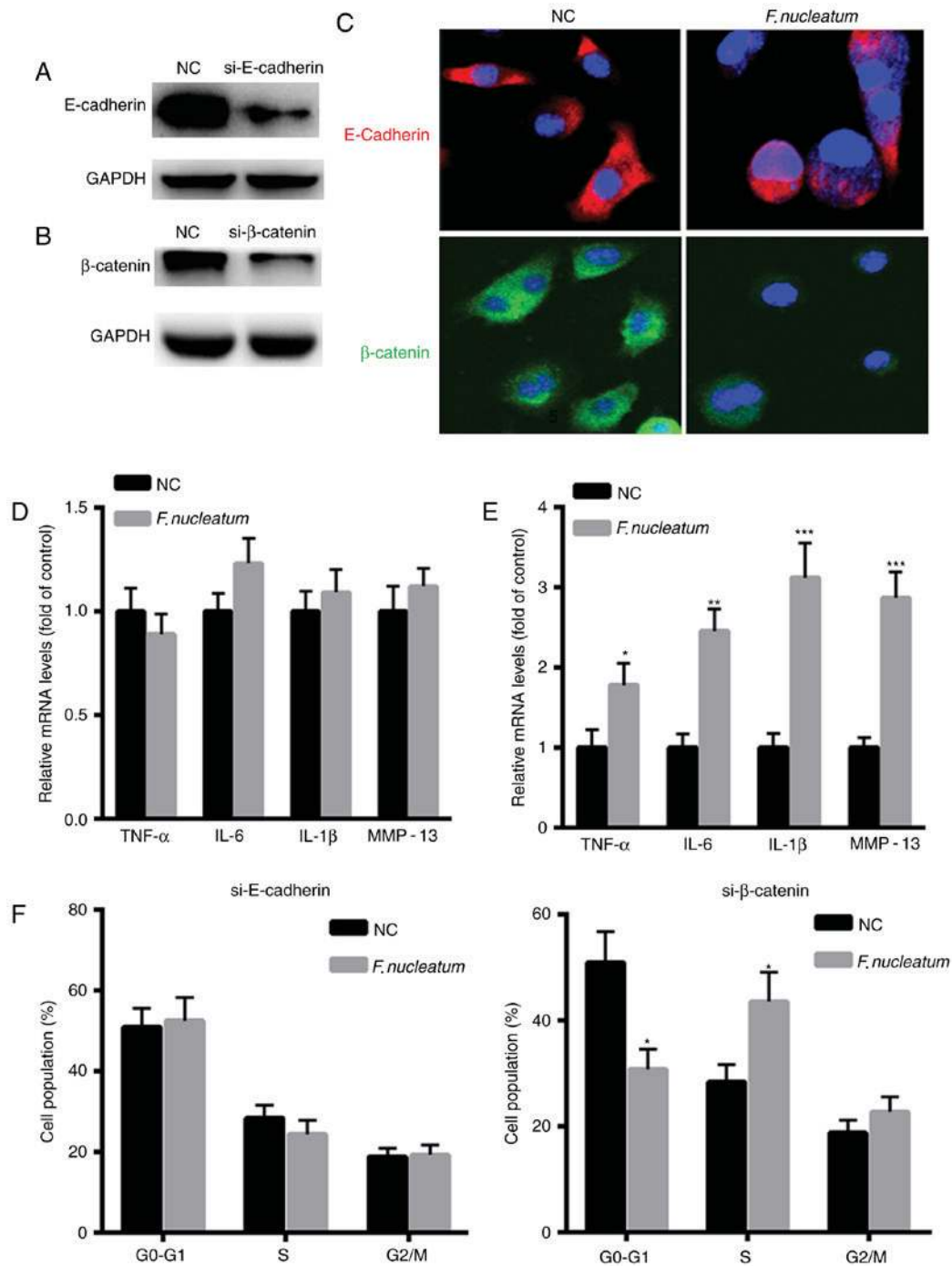
Figure 4. *F. nucleatum* promotes the malignant phenotype of NCM460 cells by interacting with E-cadherin. Western blot analysis of (A) E-cadherin or (B) β-catenin in NCM460 cells transfected with si-E-cadherin or si-β-catenin for 48 h. (C) Immunofluorescence assay revealed decreased expression of E-cadherin or β-catenin following transfection with si-E-cadherin (Red) or si-β-catenin (Green) for 48 h in NCM460 cells infected with *F. nucleatum* or control siRNA. Magnification, x400. The nucleus was stained DAPI). (D) *F. nucleatum* did not increase the mRNA levels of TNF-α, IL-6, IL-1β and MMP-13 in NCM460 cells when E-cadherin was silenced. (E) Increased expression of TNF-α, IL-6, IL-1β and MMP-13 in NCM460 cells when transfected with a si-β-catenin. (F) Flow cytometric analysis of the cell cycle following transfection with si-E-cadherin (Red) or si-β-catenin (Green) for 48 h in NCM460 cells (10^6/well) infected with F. nucleatum or control siRNA. *P<0.05, **P<0.01, ***P<0.001 vs. control. NC, negative control group; IL, interleukin; MMP, matrix metalloproteinase; TNF, tumor necrosis factor; si, siRNA.

enhanced the phosphorylation levels of p65 and the expression of IL-6, IL-1β and MMP-13 (Fig. 2).

F. nucleatum promotes EMT without affecting the expression of E-cadherin and. Attachment and invasion are hallmarks of *F. nucleatum*. Therefore, the effects of *F. nucleatum* infection on the induction of EMT were also assessed. At 48 h

post-infection, epithelial cells transdifferentiated into mesenchymal-like cells as observed using microscopy (Fig. 3A). Furthermore, *F. nucleatum* infection did not alter the protein levels of E-cadherin and β-catenin as demonstrated using western blot analysis (Fig. 3B), indicating that *F. nucleatum* infection induced EMT without affecting the expression of E-cadherin.

F. nucleatum promotes malignancy of NCM460 cells by interacting with E-cadherin. To further elucidate the underlying molecular mechanism of *F. nucleatum* in malignant transformation of NCM460 cells, E-cadherin or β-catenin expression was silenced in NCM460 cells (Fig. 4A and B). An immunofluorescence assay revealed decreased expression of E-cadherin or β-catenin in NCM460 cells following transfection with si-E-cadherin or si-β-catenin for 48 h (Fig. 4C). qPCR was performed to evaluate the expression levels of inflammatory factors, including tumor necrosis factor (TNF)-α, IL-6, IL-1β and MMP-13. The results indicated that *F. nucleatum* did not increase the mRNA levels of TNF-α, IL-6, IL-1β and MMP-13 in NCM460 cells when E-cadherin was silenced (Fig. 4D). However, increased expression of TNF-α, IL-6, IL-1β and MMP-13 was identified in NCM460 cells when transfected with si-β-catenin (Fig. 4E). Additionally, *F. nucleatum* infection did not increase the proportion of cells at S phase when E-cadherin was silenced, but it increased the number of cells at S phase when NCM460 cells were transfected with si-β-catenin (Fig. 4F). These results indicated that although E-cadherin or β-catenin are required for tumor growth and inflammatory responses, E-cadherin and β-catenin are differentially regulated by *F. nucleatum* infection.

Discussion

It is estimated that >1,000,000 new cases of CRC are diagnosed every year (21,22). Therefore, it is important to understand the underlying molecular mechanisms in CRC and identify potential risk factors. Infectious agents have attracted attention since they are associated with several human malignancies (23). In the present study, the function of *F. nucleatum* in CRC progression through modulating the expression of E-cadherin/β-catenin signaling was investigated.

Previous studies have demonstrated the significant overabundance of *F. nucleatum* in colorectal adenomas and cancer. However, it remains unclear how *F. nucleatum* is involved in the CRC microenvironment (24,25). In the present study, *F. nucleatum* enhanced the growth and proliferation of NCM460 cells, suggesting that *F. nucleatum* may contribute to the development of CRC. The results of the present study also suggested that *F. nucleatum* is associated with intratumoral immune cell responses and promotes tumorigenesis by regulating the tumor microenvironment (10,26). *F. nucleatum* invades epithelial cells through the virulence factor FadA which then triggers pro-oncogenic signaling and promotes CRC development (17). The present study demonstrated that *F. nucleatum* infection activated NF-κB signaling in NCM460 cells, thereby enhancing downstream signaling pathways.

E-cadherin is a transmembrane glycoprotein, which is located at the adherens junctions and mediates calcium-dependent cell-cell adhesion (17). At the C-terminus, E-cadherin is linked to α-catenin and the actin cytoskeleton through interaction with β-catenin. E-cadherin, α-catenin and β-catenin form a complex, thus creating tight cell-cell interactions and restraining cell mobility. Dysregulation of the E-cadherin-β-catenin complex leads to the invasion and metastasis of CRC cells (13,14). Thus, the function of *F. nucleatum* in the E-cadherin-β-catenin

complex in invasion and metastasis of CRC cells was examined. The results indicated that infection by *F. nucleatum* induced EMT and did not affect the expression levels of E-cadherin and β-catenin.

The present study sought to investigate the molecular mechanism underlying *F. nucleatum*-mediated EMT. Loss of E-cadherin-mediated cell adhesion is associated with tumor invasion. Decreased β-catenin expression is associated with the loss or decrease of E-cadherin expression in invasive tumor cells (27,28). Additionally, membrane β-catenin/cytosolic expression level is decreased in primary tumors, indicating that low β-catenin expression level may be a potential marker for the metastasis and a worse outcome in breast cancer (29). The results of the present study demonstrated that *F. nucleatum* may only increase the inflammatory responses when β-catenin expression is knocked down in NCM460 cells, whereas no changes were identified when E-cadherin expression is knocked down. These results indicated that *F. nucleatum* may promote the malignant phenotype of CRC by interacting with E-cadherin.

In conclusion, to the best of our knowledge, the present study is the first to demonstrate that *F. nucleatum* infection may interact with E-cadherin, which in turn induces the malignant phenotype of CRC cells. The results of the present study may provide insights that lead to the development of novel antimicrobial targets for the treatment of CRC.

Competing interests

The authors declare that there are no competing interests.

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