**Fusobacterium nucleatum** induces excess methyltransferase-like 3-mediated microRNA-4717-3p maturation to promote colorectal cancer cell proliferation

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**Abstract**

*Fusobacterium nucleatum* infection plays vital roles in colorectal cancer (CRC) progression. Overexpression of microRNA-4717-3p (miR-4717) was reported to be up-regulated in *F. nucleatum* positive CRC tissues, however, the underlying mechanism is unknown. In this study, we found that miR-4717 promoted CRC cell proliferation in vitro and growth of CRC in vivo following *F. nucleatum* infection. MicroRNA-4717 suppressed the expression of mitogen-activated protein kinase kinase 4 (MAP2K4), a tumor suppressor, by directly targeting its 3′-UTR. Furthermore, we confirmed that methyltransferase-like 3 (METTL3)-dependent m6A methylation could methylate primary (pri)-miR-4717, which further promoted the maturation of pri-miR-4717, and METTL3 positively regulated CRC cell proliferation through miR-4717/MAP2K4 pathways. In conclusion, *F. nucleatum*-induced miR-4717 excessive maturation through METTL3-dependent m6A modification promotes CRC cell proliferation, which provides a potential therapeutic target and diagnostic biomarker for CRC.

**Keywords**
colorectal cancer, *Fusobacterium nucleatum*, MAP2K4, METTL3, miR-4717-3p
1 | INTRODUCTION

Colorectal cancer is the third most common form of cancer and the second most prominent cancer-related cause of mortality globally. Colorectal cancer is thought to develop and progress through the confluence of gene–environment interactions. Just 10%–30% of the risk of CRC is thought to be attributable to genetic factors, with environmental variables being linked to the incidence of sporadic CRC cases. Fusobacterium nucleatum, a Gram-negative anaerobic pathogen present in the digestive tract, has been identified as an important risk factor tied to CRC onset and progression. The specific mechanisms whereby F. nucleatum-related CRC develops, however, remain to be fully clarified.

MicroRNAs are noncoding RNAs that regulate gene expression at the epigenetic level, and they are frequently dysregulated in oncogenic contexts. Recent evidence suggests that miRNAs can serve as one component of the cross-talk between bacteria and host cells, thereby influencing CRC development. Yang et al., for example, reported that F. nucleatum was able to specifically induce the upregulation of miR-21 in CRC tissues, with miR-21 subsequently suppressing RAS1 expression such that MAPK activity and associated proliferative activity were enhanced. Fusobacterium nucleatum-infected tumor cells have also been reported to release exosomes containing high levels of miR-1246/92b-3p/27a-3p, with these miRNAs suppressing glycogen synthase kinase 3β activity and promoting Wnt/β-catenin pathway activity in a manner conducive to the enhanced migration of CRC cells in an in vitro context. In previous reports, we have shown that miR-4717 is markedly upregulated in F. nucleatum-positive CRC tissues. The specific role played by miR-4717 in F. nucleatum-associated CRC progression, however, has yet to be established.

N6-methyladenosine was recently identified as a dynamic epigenetic modifier that plays key roles in controlling patterns of epigenetic gene expression in eukaryotes. Methyltransferases including METTL3, METTL14, WTAP, and KIAA1429 (also known as VIRMA) are responsible for the m6A modification of specific targets, whereas these modifications can be reversed by demethylases including ALKBHS and FTO. Certain YTH domain-containing m6A binding proteins such as YTHDF1, YTHDF2, YTHDF3, and YTHDC1 have additionally been identified as readers capable of influencing the translation or degradation of RNAs bearing m6A modifications. A growing body of evidence suggests that m6A modifications can promote pri-miRNA maturation in pancreatic, breast, and CRC cells. However, there has been little study to date regarding how m6A modifications influence F. nucleatum-associated CRC progression to date.

Herein, we explore the functional importance of miR-4717 and METTL3 in F. nucleatum-associated CRC, ultimately highlighting a mechanism whereby this bacterium can promote CRC progression by inducing the enhanced METTL3-dependent m6A modification-mediated maturation of miR-4717.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

All tumor and paired paracancerous tissue samples used in this study were obtained from individuals diagnosed with CRC undergoing treatment at the Third Affiliated Hospital of Chongqing Medical University. In total, 25 pairs of F. nucleatum positive CRC samples (Fn+ CRC) and paired paracancerous samples (Fn- control) were collected, together with 25 pairs of F. nucleatum negative CRC samples (Fn- CRC) and paired paracancerous samples (Fn- control) (Table S1). After collection, samples were snap-frozen for future analysis. The present study was approved by the Ethics Committee of the Third Affiliated Hospital to Chongqing Medical University (2018-17), with all subjects having provided written informed consent prior to sample collection.

2.2 | Fusobacterium nucleatum quantification

Briefly, a QIAamp DNA Mini Kit (51,306; Qiagen) was used to extract DNA from CRC tissues, with F. nucleatum then being detected by RT-PCR using TB Green Premix Ex Taq II (RR820A; Takara Bio) and a CFX96TM real-time instrument (C1000TM Thermal Cycler; Bio-Rad) using the following thermocycler settings: 95°C for 30 min; 40 cycles of 95°C for 10 s, 57°C for 10 s, and 72°C for 20 s; and 72°C for 2 min. Cycle threshold values were utilized to quantify F. nucleatum abundance; Ct values <35.96 were considered positive. The customized F. nucleatum (NusG) primers used herein were synthesized by Sangon Biotech and are listed in Table S2.

2.3 | Quantitative RT-PCR

TRizol (15,596,026; Thermo Fisher Scientific) was used to extract total RNA from CRC cell lines and tissues, after which a PrimeScript RT reagent kit (RR047A; Takara Bio) was used to prepare cDNA followed by qPCR analysis performed using TB Green Premix Ex Taq (RR820A; Takara Bio) and the CFX96TM real-time system (C1000TM Thermal Cycler; Bio-Rad). Thermocycler settings were: 95°C for 3 min; 40 cycles of 95°C for 15 s, 57°C for 10 s, and 72°C for 20 s. Primers used for this analysis were synthesized by Sangon Biotech and are compiled in Table S2.

To assess the expression of miR-4717-3p, the TaqMan PCR Reagent (E22007; GenePharma) was utilized, with relative expression being quantified by the 2-ΔΔCt method. Reaction settings for this analysis were: 95°C for 3 min; 39 cycles of 95°C for 12 s; and 62°C for 40 s. Primers for this analysis were synthesized by GenePharma and are compiled in Table S2.
### 2.4 Mouse xenograft tumor model experiments

In total, 30 female BALB/c nude mice (5 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal experiments were carried out in accordance with protocols approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (AMUWEC20191778).

A human CRC xenograft model system was established by infecting HCT-116 cells with *F. nucleatum* (MOI = 10) for 24 h following treatment with a microOFF mmu-miR-4717-3p antagonir (RiboBio). These cells were then subcutaneously injected deep within the left and right dorsal flanks of BALB/c nude mice (n = 5/group). These animals were housed under specific pathogen-free conditions with free access to sterilized water and food. At 3 weeks postimplantation, animals were killed and tumor weight was calculated. Tumors were then subjected to Ki-67 staining.

### 2.5 Immunohistochemical staining

Tissue sections were deparaffinized and rehydrated with an ethanol gradient, after which they were treated for 10 min with peroxidase blocking buffer, heated in citrate buffer (pH 6.0) for 25 min to facilitate antigen retrieval, blocked for 1 h at room temperature in 5% normal goat serum (in PBS), and incubated in sequence with anti-Ki-67 (ab92742, 1:200, Abcam), anti-MAP2K4 (A14781, 1:200, Abcam), and an appropriate secondary Ab. Sections were then dehydrated, cleaned, and stained with 3,3′-diaminobenzidine (D12384S; Sigma-Aldrich). Sections were sealed with neutral resin and imaged by light microscope.

### 2.6 Bacterial and cell culture

*Fusobacterium nucleatum* ATCC 25586 was obtained from ATCC. *Bacteroides fragilis* was obtained from the BeNa Culture Collection. Both were cultured in anaerobe basal broth (Oxoid) in an Anaeromat. Bacterial and cell culture

### 2.7 Western blot analysis

RIPA buffer (P0013B; Beyotime) was used to lyse cell and tissue samples on ice, after which a BCA kit (P0012S; Beyotime) was used to measure protein concentrations in isolated samples. Proteins were then separated by SDS-PAGE and transferred to PVDF membranes (A10600023; GE Healthcare Life Science). Blots were blocked using 5% nonfat milk, stained overnight with Abs specific for MAP2K4 (A14781, 1:1000; ABclonal), METTL3 (96391s, 1:1000; Cell Signaling Technology), or GAPDH (2118, 1:1000; Cell Signaling Technology) at 4°C, and probed with a secondary HRP-conjugated Ab (A25022, 1:8000; Abbkine). Protein bands were then visualized with a ChemiDoc Touch Imaging System (Bio-Rad) and radiographic film.

### 2.8 Transfection

The siRNA METTL3, pcDNA3.1(+)-METTL3, and pcDNA3.1(+)-MAP2K4 constructs were synthesized by Sangon Biotech, whereas an siRNA specific for MAP2K4 was from Santa Cruz Biotechnology (sc-35909). Cells were transfected with these different constructs or corresponding controls using Lipofectamine 3000 based on provided protocols, with METTL3 siRNA sequences being as follows: sense, 5′-GCCUUAACAUUGCCACUGAAT-3′, and anti-sense, 5′-AUCAGUGGGAUGCUAAGGCTT-3′.

### 2.9 Cell viability assay

Following transfection with NC, miR-4717 mimic/inhibitor, siRNA, overexpression plasmid, or control constructs and/or *F. nucleatum* infection (MOI = 100) for 24 h, CRC cells were added to 96-well plates (2 × 10^3/well) and analyzed with a Cell Counting Kit-8 (CK04; Dojindo). Briefly, Cell Counting Kit-8 solution was added to appropriate wells, and cells were incubated at 37°C for 1 h, after which absorbance at 450nm was measured using a microplate reader (iMark; Bio-Rad).

### 2.10 Cell proliferation assay

Following transfection with NC, miR-4717 mimic/inhibitor, siRNA, overexpression plasmid, or control constructs and/or *F. nucleatum* infection (MOI = 100) for 24 h, CRC cells were added to 6-well plates (4 × 10^3/well) and analyzed with an EdU kit (C0078S; Beyotime). Cells were incubated for 2 h with appropriately diluted EdU reagent at 37°C, after which they were fixed using 4% paraformaldehyde and imaged using immunofluorescence microscopy (EVOSTM AUTO2; Thermo Fisher Scientific), with proliferation being quantified based on provided directions.

### 2.11 Cell cycle progression analysis

Following *F. nucleatum* infection, HCT-116 cells were transfected with NC, miR-4717 mimic/inhibitor, siRNA (MAP2K4, METTL3), or overexpression (MAP2K4, METTL3) constructs, followed by culture for 24 h in a 6-well plate. A Cell Cycle and Apoptosis Analysis Kit (C1052; Beyotime) was then used to analyze these cells. Briefly, cells were harvested, stained with propidium iodide (50μg/ml), and treated with RNase A (100μg/ml). After staining, cells were assessed
with a flow cytometer (BD Accuri C6; BD Biosciences), with the frequencies of cells in the G$_0$/G$_1$, S, and G$_2$/M phases being compared. The resultant data were processed with the ModFit LT5 software.

### 2.12 Colony formation assays

Following transfection with siRNAs or constructs designed to knock down or overexpress METTL3 or MAP2K4 for 24 h, HCT-116 cells were added to 6-well plates (1 x 10$^5$/well) and incubated for 2 weeks. Colonies were then fixed with methanol, stained for 30 min with 0.1% crystal violet, imaged, and counted.

### 2.13 N6-methyladenosine RNA immunoprecipitation assay

In m$^6$A RNA binding assays, after being extracted from HCT-116 cells, RNAs were subjected to chemical fragmentation prior to incubation with protein A/G magnetic beads conjugated with anti-m$^6$A (ab208577, 1:150; Abcam) or control rabbit anti-IgG overnight at 4°C. Immunoprecipitated RNAs were then collected and analyzed by qPCR, with input being used for normalization.

### 2.14 Dual luciferase reporter assay

TargetScan 7.2 was utilized to predict potential sites of overlapping binding between miR-4717 and MAP2K4. Wild-type or MT versions of the identified binding site (Table S3) within the 3'-UTR of MAP2K4 were then inserted into the pMIR-REPORT luciferase reporter plasmid. HEK293 cells were cotransfected with these plasmids and either a miR-4717 inhibitor or mimic using Lipofectamine 3000 in a 24-well plate. A dual luciferase reporter assay system (Promega) was then used based on provided directions to measure luciferase activity after 48 h, with Renilla being used for normalization.

### 2.15 Mutagenesis assay

Point mutations were obtained using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (210.516; Agilent Technologies). For this analysis, mutagenic primers were designed in the Web-based QuikChange Primer Design Program based on the principle of requirements (agilent.com.cn/store/primerDesignProgram.jsp) and compiled in Table S4. We isolated the plasmid DNA template from the dam+ Escherichia coli strain by using a TIANprep Mini Plasmid Kit (DP103; Tiangen), prepared the mutant strand synthesis reactions for thermal cycling, digested the amplification products by DpnI, and transferred the digested products to the ultracompentent cells for transformation reaction. Finally, all transformed products were coated on a plate containing appropriate antibiotics and cultured overnight. The single colony samples were sent for sequencing.

### 2.16 Fusobacterium nucleatum in situ hybridization

Five-micrometer-thick sections were prepared and hybridized following the manufacturer’s instructions (BersinBio Biotechnology). The sequence of the F. nucleatum-targeted probe (Fus611; Dig-labeled) was 5’- CGCAATACAGAGTTGAGCCCTGC-3’. Slides were imaged with the Zeiss 800 confocal microscope in sequential scanning mode. Images were further analyzed with Zen 2012 software (Zeiss). Five random 400× magnification fields per sample were evaluated by an observer who was blinded to the experimental protocol, and the average number of bacteria per field was calculated. We defined a negative or positive of F. nucleatum as an average of <10 or >10 visualized Fus611 probes per field, respectively.

### 2.17 Statistical analyses

Analyses were completed in triplicate, and data are given as mean ± SD. Data were compared by two-tailed Student’s t-tests or one-way ANOVAs as appropriate, with $p < 0.05$ as the significance threshold (’$p < 0.05$; ’’$p < 0.01$; ’’’$p < 0.001$). Associations between F. nucleatum abundance and miR-4717 or MAP2K4 expression were evaluated using linear regression analysis. The diagnostic utility of miR-4717 or MAP2K4 was examined using ROC curves, with a specific focus on specificity, sensitivity, and AUC values with 95% CIs when utilized as a binary classifier system. GraphPad Prism 8 (GraphPad Software, Inc.) and IBM SPSS Statistics 20.0 (IBM, Inc.) were used for all statistical analyses.

### 3 RESULTS

#### 3.1 Fusobacterium nucleatum promotes in vitro and in vivo CRC cell proliferation through miR-4717

In a previously published microarray study, we found that miR-4717 was markedly upregulated in CRC tissues infected by F. nucleatum, and we further found that F. nucleatum was able to promote miR-4717 overexpression in CRC cells in vitro. However, the specific functional role played by miR-4717 in this oncogenic context remains to be established. To further explore its relevance in patients with CRC, we obtained 50 pairs of fresh-frozen CRC tumor samples and paired paracancerous tissues from individuals that were or were not positive for F. nucleatum infection. As shown in Table S1, the abundance of F. nucleatum was associated with depth of invasion ($p < 0.05$) and tumor stage (TNM, $p < 0.01$). A qPCR analysis revealed miR-4717 to be significantly upregulated in F. nucleatum-positive CRC tumors relative to matched paracancerous samples (Figure 1A), with a significant positive correlation between F. nucleatum abundance and miR-4717 expression level within these tumor tissues ($r = 0.5135$, $p = 0.0087$; Figure 1B).

A ROC curve analysis revealed that the AUC value for miR-4717 when used to differentiate between CRC tissues that were or were
not infected by *F. nucleatum* was 73.7% (95% CI, 59.3%–85.1%,
\( p = 0.004\); Figure 1C). Moreover, while *F. nucleatum* infection
promoted miR-4717 upregulation in HCT-116 cells, the same was
not observed following *B. fragilis* infection or by PBS treatment
(Figure 1D). Infection with *F. nucleatum* significantly increased the
expression of miR-4717 in all CRC cell lines (SW480, HT29, LoVo,
and HCT-116) and the normal colonic epithelial cell line (NCM460),
compared to cells without infection (Figure 1E; all \( p < 0.05\), and
the expression of miR-4717 was also F. nucleatum dose-dependent in HCT-116 and LoVo cells (Figure 1F).

To explore the potential ability of miR-4717 to regulate CRC onset or progression following F. nucleatum infection, we undertook CCK-8, EdU, and cell cycle progression assays using the LoVo and HCT-116 CRC cell lines in an effort to specifically assess the oncogenic role of this miRNA. In these assays, F. nucleatum promoted significantly enhanced tumor cell proliferation that was reversed by miR-4717 inhibitor treatment (Figure 1G–I). Consistently, we utilized a murine xenograft model in which animals were implanted with HCT-116 cells that had been subjected to F. nucleatum infection and/or miR-4717 antagonism treatment, revealing that miR-4717 inhibition was sufficient to reverse the ability of F. nucleatum to influence in vivo tumor growth (Figure 1J). Together, these data strongly indicate that F. nucleatum can readily promote the enhanced proliferation of CRC cells by inducing miR-4717 upregulation.

3.2 MicroRNA-4717-3p inhibits expression of MAP2K4 in Fusobacterium nucleatum-infected CRC cells

The TargetScan, MiRanda, miRDB, and gene annotation and targets of microarray verification tissues were next used together with microarray results to identify MAP2K4 as a promising tumor progression-related target for further analysis (Figure 2A). A putative miR-4717 binding site was identified within the MAP2K4 3′-UTR (Figure 2B), and luciferase reporter assays confirmed that the inhibition or overexpression of miR-4717 was sufficient to enhance or suppress the activity of a luciferase reporter construct harbor ing the WT but not MT version of this 3′-UTR sequence (Figure 2C). We further found miR-4717 mimic transfection reduced MAP2K4 mRNA and protein levels within HCT-116 cells, whereas miR-4717 inhibitors had the opposite effect (Figure 2D,E).

Given that MAP2K4 appears to be a promising and novel miR-4717 target gene, we next examined the potential relationship between the two in CRC patient samples and cell lines. MAP2K4 mRNA levels were significantly lower in F. nucleatum-positive CRC tumor samples relative to paracancerous tissues (Figure 2F). Moreover, MAP2K4 protein levels were significantly lower in Fn+CRC compared to Fn−CRC (Figure 2G). Fusobacterium nucleatum abundance and MAP2K4 mRNA levels were negatively correlated (r = −0.5249, p = 0.0071; Figure 2H). Consistently, F. nucleatum infection was associated with the significant downregulation of MAP2K4 at the mRNA and protein level in HCT-116 cells as compared to B. fragilis infection or PBS treatment (Figure 2I,J). Transfection of a miR-4717 mimic was sufficient to inhibit MAP2K4 expression, whereas miR-4717 inhibitor transfection reversed the downregulation of this gene in the context of F. nucleatum infection (Figure 2K). To further explore the effects of interactions between miR-4717 and MAP2K4 in F. nucleatum-infected CRC, we undertook siRNA-mediated knockdown and rescue assays. The overexpression of miR-4717 was sufficient to promote MAP2K4 protein downregulation, while inhibiting miR-4717 led to increases in the MAP2K4 protein levels in HCT-116 cells (Figure 2L–N). Together, these results highlight MAP2K4 as a direct miR-4717 target that is closely linked to F. nucleatum-associated CRC.

3.3 Methyltransferase-like 3-mediated m^6^A methylation promotes miR-4717 upregulation in CRC cells

To more fully clarify the mechanisms whereby F. nucleatum infection can affect the expression of miR-4717, we next assessed pri-miR-4717 levels in HCT-116 cells. The pri-miR-4717 expression was decreased when comparing control cells to those infected with F. nucleatum (Figure 3A), suggesting that such infection must stabilize this transcript rather than promote its upregulation. The m^6^A methylation of primary miRNAs plays an important role in their maturation. To test whether pri-miR-4717 processing is subject to m^6^A methylation-related regulatory activity, the SRAMP database (http://www.cuilab.cn/sramp) was queried to identify putative sites for m^6^A modification within the pri-miR-4717 sequence, leading to the high confidence position of three predicted RRACH m^6^A sequence motifs (Figure 3B). A methylated RNA immunoprecipitation assay was then used to assess m^6^A modification status, revealing that m^6^A modification was enriched with the pri-miR-4717 sequence (Figure 3C). To determine whether these sites participated in the m^6^A modification of pri-miRNA-4717, we constructed and transfected HEK293T cells with equal amounts of WT and MT pri-miRNA-4717 vectors. The expression of pri-miR-4717 and mature miR-4717 were detected between WT and MT cells (Figure 3D,E). There were no difference between WT and MT groups in the expression of pri-miRNA-4717, while mature miR-4717 expression was decreased in the MT group. We also found that the m^6^A level in MT pri-miRNA-4717 was decreased in the WT group (Figure 3F). These findings indicated that the sites we predicted were closely related to the m^6^A modification of pri-miRNA-4717. Following F. nucleatum infection, METTL3 expression levels were significantly increased as compared to noninfected cells (Figure 3G), with the same being true at the protein level in HCT-116 and LoVo cells (Figure 3H). Mature miR-4717 downregulation was evident following the knockdown of METTL3 in CRC cells, whereas the opposite was true when METTL3 was overexpressed (Figure 3I). In addition, the m^6^A modification of pri-miR-4717 was significantly decreased in the siMETTL3 group (Figure 3J). Reduction of m^6^A levels is associated with the arrest of pri-miRNA processing and reduction in corresponding mature miRNA levels, and as such, METTL3/m^6^A-dependent miRNAs show positive correlations with METTL3.

3.4 Methyltransferase-like 3 mediates in vitro and in vivo Fusobacterium nucleatum-induced proliferation of CRC cells

To confirm the pivotal role of METTL3 in F. nucleatum-induced CRC, we undertook CCK-8, EdU, and cell cycle progression assays using the HCT-116 CRC cell line. Fusobacterium nucleatum was able to
readily promote the in vitro cell proliferation of HCT-116 cells in a manner that was inhibited by si-METTL3 pretreatment (Figure 4A-C). To confirm that METTL3 was able to promote in vivo tumor growth, we next evaluated murine xenograft tumor models that had been implanted with HCT-116 cells in which METTL3 had been knocked down and/or cells had been infected with *F. nucleatum* for...
Knocking down METTL3 was sufficient to inhibit *F. nucleatum* infection-induced tumor growth (Figure 4D, left panel). Consistently, *F. nucleatum* infection enhanced Ki-67 expression (Figure 4D, middle panel) and tumor weight in these tumors (Figure 4D, right panel), whereas METTL3 knockdown reversed these changes.

To identify the mechanisms whereby miR-4717 could function as a downstream METTL3 target to shape CRC progression, we utilized a miR-4717 inhibitor to knock down this miRNA in LoVo and HCT-116 cells overexpressing METTL3 (Figure 5A), revealing that while METTL3 upregulation was associated with enhanced CRC cell proliferation, miR-4717 inhibition was sufficient to reduce such proliferative activity in both cell lines (Figures 5B,E, left panels). Finally, we utilized miR-4717 mimic constructs to overexpress this miRNA in CRC cells in which METTL3 had been knocked down (Figure 5C).
revealing that while METTL3 knockdown was associated with reduced proliferation in both cell lines, miR-4717 overexpression reversed this effect (Figures 5D, E, right panels). We then measured miR-4717 and METTL3 expression in CRC tissue samples infected by *F. nucleatum*, revealing a positive correlation between the two ($r = 0.5610, p = 0.0035$; Figure 5F). These data thus provide strong evidence that METTL3 can promote the proliferation of CRC cells in the context of *F. nucleatum* infection through mechanisms dependent on miR-4717.

3.5 | *Fusobacterium nucleatum* induces proliferation of CRC cells through the METTL3/miR-4717/MAP2K4 pathway

To more fully explore the relationship between *F. nucleatum* infection and METTL3 expression, we explored the levels of this methyltransferase in our paired tumor and paracancerous tissue samples from CRC patients that were or were not positive for *F. nucleatum* infection. The expression of METTL3 was higher in *F. nucleatum*-positive CRC tissues (Figure 6A), and these levels were negatively correlated with MAP2K4 expression in CRC tissues ($r = -0.6752, p = 0.0002$; Figure 6B). Moreover, a high abundance of *F. nucleatum* in tissues was often accompanied by high levels of METTL3 expression and low levels of MAP2K4 expression (Figure 6C). In addition, MAP2K4 protein levels were significantly elevated following the knockdown of METTL3, whereas its overexpression had the opposite effect (Figure 6D).

To confirm the regulatory importance of the METTL3/miR-4717/MAP2K4 axis, we knocked down or overexpressed this methyltransferase in HCT-116 cells that had been transfected with miR-4717/miR-4717/MAP2K4 axis, we knocked down or overexpressed this methyltransferase in HCT-116 cells that had been transfected with miR-4717/miR-4717/MAP2K4 axis, we knocked down or overexpressed this methyltransferase in HCT-116 cells that had been transfected with miR-4717 mimic or inhibitor constructs, followed by *F. nucleatum* infection. In these experiments, si-METTL3-mediated increases in MAP2K4 expression were reversed by miR-4717 mimic transfection or *F. nucleatum* infection (Figure 6E). Conversely, MAP2K4 downregulation following METTL3 overexpression was reversed.

**Figure 4** Methyltransferase-like 3 (METTL3) induces the proliferation of *Fusobacterium nucleatum* (*Fn*)-associated colorectal cancer in vitro and in vivo. (A–C) Ability of HCT-116 cells to proliferate following *F. nucleatum* and/or si-METTL3 treatment was assessed by (A) EdU, (B) cell cycle analyses, and (C) CCK-8. (D) Xenograft tumor models were generated, with representative xenograft tumors (D, left panel) being shown. Ki-67 staining of these xenografts (D, middle panel) were also carried out, and average tumor weights for xenograft tumors (D, right panel) were calculated. Data are means± SD from triplicate experiments. *p < 0.05. Scale bar, 125 μm.
following miR-4717 inhibitor transfection in these cells (Figure 6F). Methyltransferase-like 3 was able to enhance cellular proliferation, while MAP2K4 suppressed such proliferation in HCT-116 cells upon METTL3 overexpression (Figure 6G,H). Knocking down METTL3 reduced such proliferation, while MAP2K4 knockdown conversely enhanced such proliferation in HCT-116 cells following METTL3 knockdown. Overall, these results provide strong evidence that F. nucleatum can regulate CRC cell proliferation through the METTL3/miR-4717/MAP2K4 axis.

**DISCUSSION**

Herein, we determined that the METTL3-mediated maturation of pri-miR-4717 could play a key role in the onset and progression of F. nucleatum-related CRC. Specifically, we found that pri-miR-4717 showed increases in m6A RNA modification levels, with METTL3 being the primary m6A methyltransferase that was differentially active in the context of F. nucleatum infection. Functionally, METTL3 was able to promote the maturation of miR-4717 through the m6A
modification of pri-miR-4717, ultimately contributing to enhanced CRC cell proliferation in the context of *F. nucleatum* infection by driving enhanced pri-miR-4717 maturation to ultimately inhibit the miR-4717 target gene MAP2K4 (Figure 7).

Recently, miRNAs have emerged as valuable diagnostic biomarkers for CRC.\(^{15-17}\) For example, miR-143 and miR-145 were consistently downregulated in CRC tumor tissues of different stages relative to normal colonic mucosal tissues.\(^{18}\) In contrast, other miRNAs (miR-21, miR-29a, miR-92a, and miR-135b) are reportedly upregulated in CRC patient tumor tissues.\(^{19-21}\) A growing body of evidence further suggests that *F. nucleatum* is linked to CRC onset, progression, and metastasis, with this bacterial infection potentially altering miRNA expression in the context of CRC progression.\(^{7,22-24}\) As such, *F. nucleatum*-related changes in miRNA expression could be of key relevance to the diagnosis and/or treatment of CRC. For example, prior evidence indicates that *F. nucleatum* can enhance...
CRC cell proliferation by promoting miR-21 upregulation, with *F. nucleatum*-infected patients experiencing higher-risk diseases and poorer clinical outcomes. Levels of miR-1246/92b-3p/27a-3p within circulating exosomes have been tied to both *F. nucleatum* abundance and tumor staging in CRC patients. Using a miRNA array platform and RT-PCR analyses, we previously identified miR-4717 as being upregulated in Fn+ CRC. Bioinformatics analyses have further revealed CREBBP to be the most significantly differentially expressed gene in *F. nucleatum*-related CRC. However, there was no significant relationship between CREBBP expression and *F. nucleatum* abundance in clinical samples (\( p > 0.05 \), data not shown). Herein, ROC curve analyses were used to assess miR-4717 diagnostic performance (AUC = 73.7%, \( p = 0.004 \)), revealing *F. nucleatum* abundance to be positively correlated with miR-4717 levels (\( r = 0.5135 \), \( p = 0.0087 \)). As such, miR-4717 could serve as a valuable diagnostic biomarker in *F. nucleatum*-infected patients.

Mitogen-activated protein kinase kinase 4 is a known tumor suppressor that phosphorylates JNKs and p38 family kinases, leading to their activation and regulating key downstream tumor-suppressive signaling cascades, including those mediated by the transcription factor c-Jun. Mitogen-activated protein kinase kinase 4 might also promote tumor senescence, which can contribute to the inhibition of oncogenic growth. However, the specific tumor suppressor role of MAP2K4 remains a matter of some controversy, with some articles having reported it to play an oncogenic role in breast, gallbladder, and pancreatic cancers. Indeed, there is evidence that MAP2K4 can promote or inhibit tumor progression in a manner dependent on the local genetic context, although it appears to suppress oncogenic processes in most cancers. Herein, we observed MAP2K4 downregulation in Fn+ CRC tissues, with *F. nucleatum* infection similarly promoting MAP2K4 downregulation in CRC cells. Consistently, CRC tissues showed a negative correlation between *F. nucleatum* abundance and MAP2K4 mRNA levels (\( r = -0.5249 \), \( p = 0.0071 \)). Both MAP2K4 mRNA and protein levels were influenced by miR-4717, with this kinase influencing HCT-116 cell proliferation in the context of *F. nucleatum* infection. Overall, our results suggest that *F. nucleatum* can suppress MAP2K4 expression in a miR-4717-dependent fashion.

![Schematic overview of *Fusobacterium nucleatum*-induced m6A modification-dependent microRNA-4717-3p (miR-4717) overexpression as a driver of colorectal cancer cell proliferation. MAP2K4, mitogen-activated protein kinase kinase 4; METTL3, methyltransferase-like 3; pri-miRNA-4717, primary miR-4717.](image-url)
F. nucleatum exposure, potentially contributing to F. nucleatum-related CRC progression.

AUTHOR CONTRIBUTIONS
Dongzhu Zeng, Xuhu Mao, and Bin Tang designed the research and supervised the project. Qiaolin Xu, Xiaoxue Lu, and Jing Li executed all experiments. Yuyang Feng, Yilan Mao, and Tao Zhang were responsible for clinical sample collection. Lv Hu and Jie Tang performed statistical analysis of data. Qian Li, Ling Deng, Xiaoyi He, Yuanzhi Lan, Huaxing Luo, Linghai Zeng, Yan Zhang, and Yuanyuan Xiang provided support experimental technical support. Bin Tang and Xuhu Mao wrote the manuscript. All authors read and approved the final manuscript.

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DISCLOSURE
The authors have no conflict of interest.

ETHICS STATEMENT
Approval of the research protocol by an Institutional Review Board. The present study was approved by the Ethics Committee of the Third Affiliated Hospital to Chongqing Medical University (2018-17).

INFORMED CONSENT
All subjects having provided written informed consent prior to sample collection.

ANIMAL STUDIES
All animal experiments were performed in accordance with protocols approved by the Laboratory Animal Welfare and Ethics Committee of the of the Third Military Medical University (AMUWEC20191778).

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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