Fusobacterium nucleatum promotes colorectal cancer metastasis by modulating KRT7-AS/KRT7

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ABSTRACT

The enrichment of Fusobacterium nucleatum (Fn) has been identified in CRC patients and associated with worse outcomes. However, whether Fn was involved in the metastasis of CRC was not well determined. Here, we found that the abundance of Fn was significantly increased in CRC patients with lymph nodes metastasis. To further clarify the role of Fn in CRC metastasis, we performed transwell and wound healing assays after incubating CRC cell lines with or without Fn and injected Fn-treated or untreated CRC cells into nude mice via tail vein. The results indicated that Fn infection promoted CRC cells migration in vitro, as well as lung metastasis in vivo. Interestingly, colonization of Fn was detected in metastatic lung lesions of nude mice by fluorescence in situ hybridization. Mechanistically, RNA sequencing and validation study revealed that Fn significantly upregulated the expression of long non-coding RNA Keratin7-antisense (KRT7-AS) and Keratin7 (KRT7) in CRC cells. Importantly, Fn-induced CRC lung metastasis was attenuated by the depletion of KRT7-AS. In addition, KRT7-AS facilitated CRC cells migration by upregulating KRT7. Subsequently, we found that NF-κB signaling pathway was involved in the upregulation of KRT7-AS upon Fn infection. In conclusion, Fn infection upregulated KRT7-AS/KRT7 by activating NF-κB pathway, which promoted CRC cell migration in vitro and metastasis in vivo.

Introduction

Colorectal cancer (CRC) is the fourth most common cancer and the second leading cause of cancer-related death worldwide. Approximately 25% of patients with CRC show synchronous metastases while another 25% develop metastases throughout the course of their disease. The prognosis of CRC patients with distant metastases is dismal with a 5-year survival rate of less than 10%. Unfortunately, the underlying mechanisms for the metastasis of CRC have not been fully clarified.

Recent evidence suggested that host–microflora interactions might play a key role in the initiation and progression of CRC. Study indicated that gavage of stool samples from patients with CRC promoted intestinal carcinogenesis in germ-free and conventional mice, which confirmed the direct pro-tumorigenic effect of gut microbiota. Identification of specific carcinogenic microbiota remains an important area for CRC research. Previous studies have documented that several microbiota were involved in carcinogenesis of CRC, including Peptostreptococcusanaerobius, enterotoxigenic Bacteroidesfragilis, Escherichia coli, and Fusobacterium nucleatum(Fn). However, previous studies mainly focused on the proliferative effects of microbiota, while their roles in the metastasis of CRC were not well explored.

Fn is a gram-negative anaerobic bacterium prevalent in the oral cavity which has gained significant attention for its potential role in CRC. The enrichment of Fn in CRC tissues compared to the adjacent normal tissues has been confirmed by several studies. Epithelial barrier defects...
that occurred in sites of dysplasia might allow Fn to thrive in the local tumor environment.\textsuperscript{15} Fn possesses key pathogenic features that may allow it to predominate. Fap2, a surface protein of Fn, could bind to tumor-expressed Gal-GalNAc, which resulted in the enrichment of Fn in CRC.\textsuperscript{16} FadA, a unique virulence factor of Fn, could bind to E-cadherin and activate β-catenin, which stimulated tumor cell growth in CRC. Additionally, introduction of Fn to Apc\textsuperscript{Min/+} mice accelerated tumorigenesis.\textsuperscript{17} Furthermore, the presence of Fn might be a poor prognostic biomarker in CRC. The level of Fn DNA presented in CRC tissues was correlated with higher CRC-specific mortality.\textsuperscript{18} Meanwhile, Fn was associated with CRC recurrence and resistance to chemotherapy by activating the autophagy pathway.\textsuperscript{19}

Recently, some clinical evidence suggested that the enrichment of Fn might be related to CRC metastasis.\textsuperscript{11,13,20–22} However, the role of Fn in CRC metastasis and its underlying mechanism remains unclear. This study first clarified the relationship between Fn infection and CRC metastasis. We found Fn infection upregulated KRT7-AS/KRT7 by activating NF-κB pathway, which promoted CRC cell migration \textit{in vitro} and metastasis \textit{in vivo}.

\section*{Results}

\textbf{Fn promoted CRC cells migration and metastasis}

To identify the clinical significance of Fn, we performed qRT-PCR to quantify the abundance of Fn in fecal samples from CRC patients and healthy controls. The results indicated an enrichment of \textit{Fusobacterium spp.} in the feces of CRC patients compared with healthy controls. Meanwhile, CRC patients with positive lymph nodes metastasis showed more abundance of Fn in feces compared to CRC patients without lymph nodes metastasis (Figure 1a). To evaluate the effect of Fn infection on CRC cell migration, we performed transwell and wound healing assays after incubating HCT-116 or LoVo cells with Fn for 12 h. The results indicated that Fn infection significantly enhanced HCT-116 and LoVo cells migration compared with \textit{E. coli} DH5a or PBS control treatment (Figure 1b,c). However, heat-killed Fn was unable to promote HCT-116 and LoVo cells migration (Supplementary Figure 1a,b). To confirm the results \textit{in vitro}, we injected Fn-treated or PBS-treated HCT-116 cells into nude mice via tail vein. In accordance with the results \textit{in vitro}, Fn-treated cells induced more metastatic nodules in lung than PBS-treated cells (Figure 1d). Notably, after intravenous injection of Fn-treated cells into nude mice, Fn was detected in the metastatic lung lesions by FISH assay (Figure 1e).

\textbf{KRT7-AS/KRT7 expression is upregulated in Fn-treated CRC cells}

Long-non-coding RNAs (\textit{IncRNAs}) were reported to play essential roles in the metastasis of CRC.\textsuperscript{23,24} However, the interplay between Fn infection and \textit{IncRNA} has never been identified. To explore whether \textit{IncRNAs} were involved in Fn infection-induced CRC metastasis, we performed RNA sequencing to evaluate the expression profiles of \textit{IncRNAs} after incubating LoVo cells with Fn or PBS for 24 h. The RNA sequencing results showed that Fn treatment significantly upregulated 43 \textit{IncRNAs}, while downregulated 63 \textit{IncRNAs} (FDR<0.05, |log₂ (fold change)|>1) (Supplementary Table 1). Among these differentially expressed \textit{IncRNA}, we were particularly interested in the \textit{IncRNA Homo sapiens keratin 7 antisense RNA (KRT7-AS)}, which was one of the most differentially upregulated \textit{IncRNAs}(FDR <0.0001, fold change = 6.06) (Figure 2a, Supplementary Figure 2). KRT7-AS was reported to be a single antisense RNA that was transcribed from the negative strand of the keratin 7 (KRT7) (Figure 2a).\textsuperscript{25} Interestingly, RNA sequencing analysis also revealed that KRT7 mRNA was consistently upregulated in Fn-treated cells (FDR<0.0001, fold change = 7.5) (Figure 2c). To validate RNA sequencing results, we performed qRT-PCR assay after incubating HCT-116 or LoVo cells with live or heat-killed Fn, \textit{E.coli} or PBS control for 24 h. Consistently, both KRT7-AS and KRT7 were significantly upregulated in live Fn-treated cells, while not in \textit{E.coli} or heat-killed Fn-treated cells (Figure 2d). In addition, the expression of KRT7-AS and KRT7 were simultaneously increased with the increasing Fn.
Figure 1. Fn promoted CRC cells migration and metastasis. (a) The abundance of Fn in the feces of CRC patients (T, n = 49) versus healthy people (N, n = 30). The abundance of Fn is higher in the feces of CRC patients with lymph nodes metastasis (N1+ N2, n = 23) than those without metastasis (N0, n = 26). (b,c) HCT-116 and LoVo cells were incubated with PBS control, E. coli or Fn. The migration ability of cells was evaluated by transwell assay (b) or wound healing assay (c). In transwell assay, migrated cells were stained with DAPI and images were randomly taken under microscope. Every 6 fields were counted for each sample. (d) HCT-116 cells were incubated with Fn or PBS for 24 h and then injected into BALB/C nude mice via tail vein (n = 6 per group). The left panel shows macroscopic lungs of nude mice. The area of metastatic lesions was labeled by dashed circles. The middle panel indicates the hematoxylin-eosin (H&E) staining of lung metastasis (scale bar = 100 μm), two representative images per group. The right panel shows the statistical analysis of metastasis focuses. (e) Fn in lung metastasis tissues was detected by FISH. The colonization of Fn was detected in lung metastasis from mice injected with Fn-treated cells (1000 times magnification power). The white arrows indicate positive stain. All data are shown as mean ± SD, *p < .05, **p < .01, ***p < .001, ****p < .0001 (Mann–Whitney test was used in a, unpaired Student’s t test was used in b and d). Ctrl, control; E.coli, Escherichia coli; Fn, Fusobacterium nucleatum.
Figure 2. KRT7-AS/KRT7 expression is upregulated in Fn-treated CRC cells. (a) Heatmap of representative differentially expressed LncRNA between Fn-treated and PBS-treated LoVo cells (n = 3 per group, fold change > 2, logCPM > 2, FDR < 0.05). (b) The schematic representation of KRT7-AS (ensemble gene transcript ENST00000546688, on the negative DNA strand) and KRT7 mRNA (ReSeq gene NM_005556, on the positive strand). E7 indicates seventh exon of KRT7 gene. Black arrows indicates transcription direction. “+” indicates the positive strand. “−” indicates the negative strand. (c) Heatmap of representative differentially expressed mRNA between Fn-treated and PBS-treated cells (n = 3 each, fold change > 2, logCPM > 2, FDR < 0.05). (d) HCT-116 or LoVo were incubated with live or heat-killed Fn, E.coli or PBS for 24 h. The RNA expression level of KRT7-AS and KRT7 were analyzed by qRT-PCR. (d) HCT-116 cells were incubated with Fn in different multiplicity of infection (MOI) (10:1, 50:1, 100:1, 200:1) for 24 h and the expression level of KRT7-AS and KRT7 were analyzed by qRT-PCR. (f) HCT-116 or LoVo were incubated with live or heat-killed Fn, E.coli or PBS for 24 h. KRT7 protein was analyzed by Western blot. (g) HCT-116 were incubated with Fn or PBS for 24 h, immunofluorescence assay was conducted to show KRT7 protein in HCT-116 cells (scale bar = 50 μm). All data are shown as mean ± SD, **** p < .0001 (unpaired Student’s t test). Ctrl, control; Fn, Fusobacterium nucleatum.
multiplicity of infection (MOI) (Figure 2e). We confirmed that Fn infection increased the protein levels of KRT7 by both Western blot and immunofluorescence assays (Figure 2f,g). Collectively, these results showed that Fn infection upregulated both KRT7-AS and KRT7 in CRC cells.

**KRT7-AS promoted metastasis of CRC cells by regulating KRT7**

To elucidate the interaction between KRT7-AS and KRT7, we then performed loss and gain of function assays in CRC cells. We found that knockdown of KRT7-AS in HCT-116 significantly suppressed mRNA and protein levels of KRT7 (Figure 3a,b). Meanwhile, overexpression of KRT7-AS in HCT-116 or LoVo cells upregulated the expression of KRT7 (Figure 3a,b, Supplementary Figure 3a). In contrast, knockdown or overexpression of KRT7 had no effect on the expression of KRT7-AS (Figure 3a,b, Supplementary Figure 3a,b).

Then, we performed wound healing and transwell assays to evaluate the role of KRT7-AS and KRT7 in CRC cell migration. Knockdown of KRT7-AS or KRT7 in HCT-116 significantly inhibited cell migration, while overexpression of KRT7-AS or KRT7 in HCT-116 or LoVo promoted cell migration (Figure 3c,d, Supplementary Figure 3c). To confirm the function of KRT7-AS in vitro, we stably transfected HCT-116 cells with KRT7-AS shRNA or control lentivirus and then injected these infected cells into nude mice. Result indicated knockdown of KRT7-AS significantly inhibited lung metastasis of nude mice (Figure 3e, Supplementary Figure 3d). In addition, overexpression of KRT7 restored the suppressed migration properties of HCT-116 cells induced by knockdown of KRT7-AS (Figure 3f). These results indicated that KRT7-AS promoted metastasis of CRC cells by upregulating the expression of KRT7-AS/KRT7.

**Fn promoted metastasis of CRC by modulating KRT7-AS/KRT7**

To determine whether the effect of Fn on the metastasis of CRC cells was mediated by KRT7-AS/KRT7, we treated HCT-116 cells with siRNA targeting KRT7-AS alone or together with Fn. We observed that knockdown of KRT7-AS attenuated Fn-induced upregulation of KRT7 (Figure 4a,b). The pro-migratory effect induced by Fn infection was abolished by knockdown of KRT7-AS or KRT7 in HCT116 cells (Figure 4c). To confirm these results in vitro, HCT-116 cells which were stably transfected with KRT7-AS shRNA or control lentivirus were incubated with Fn or PBS for 24 h and then injected into nude mice. Consistent with above result, Fn treatment significantly induced more lung metastatic nodules. Importantly, depletion of KRT7-AS attenuated Fn-induced CRC lung metastasis (Figure 4d). These results suggested that Fn promoted metastasis of CRC cells by upregulating the expression of KRT7-AS/KRT7.

**Fn regulated KRT7-AS through NF-κB signaling pathway**

The mechanism for the upregulation of KRT7-AS upon Fn infection remained unclear. Therefore, we aimed to find certain pathway regulated by Fn and identify the upstream regulators of KRT7-AS. KEGG analysis based on RNA sequencing results demonstrated that NF-κB pathway was activated by Fn infection (Figure 5a). We validated this result by performing Western blot after incubating HCT-116 or LoVo cells with live or heat-killed Fn, E.coli or PBS control for 24 h. Consistently, live Fn treatment dramatically upregulated phospho-p65 (activated NF-κB subunit), and downregulated 1κB-α (NF-κB inhibitor) compared with heat-killed Fn, E.coli or PBS control (Figure 5b). Similarly, we observed increased nuclear localization of NF-κB p65 in Fn-treated CRC cells (Figure 5c).

We next used two software packages (TESS and FIMO)26,27 to predict transcriptional factor binding to the promoter region of KRT7-AS. Interestingly, the consensus binding sequence (−2470-2461 bp 5′-GGGAACCCCA-3′) for NF-κB p65 in the promoter region of KRT7-AS was predicted. Knockdown of NF-κB p65 in HCT-116 or LoVo significantly decreased the expression of KRT7-AS as well as KRT7 (Figure 5d,e, Supplementary Figure 3e). Administration with an NF-κB inhibitor, BAY-117082, at different concentrations to HCT-116 cells also significantly decreased expression of KRT7-AS and KRT7 (Figure 5f, Supplementary Figure 3f). In addition,
Figure 3. KRT7-AS promoted metastasis of CRC cells by regulating KRT7. (a) RNA level of KRT7-AS and KRT7 was detected by qRT-PCR after knockdown of KRT7-AS (upper left panel) or overexpression of KRT7-AS (upper right panel), respectively, in HCT-116 cells. Expression level of KRT7-AS and KRT7 were detected by qRT-PCR after knockdown of KRT7 (lower left panel) or overexpression of KRT7 (lower right panel), respectively, in HCT-116 cells. (b) KRT7 protein level was detected by Western blot after knockdown or overexpression of KRT7-AS or KRT7. (c, d) HCT-116 cells were transfected with siRNA targeting KRT7-AS (KRT7) or KRT7-AS (KRT7) plasmids. Wound healing assays (c) and transwell assays (d) were performed. For transwell assay, migrated cells were stained with DAPI and images were randomly taken under microscope. Every 6 fields were counted for each sample. (e) HCT-116 cells stably transfected with KRT7-AS shRNA lentivirus were established and injected into nude mice via tail vein. The number of metastatic nodules in each slide was counted after H&E staining. (n = 8 mice per group). GFP signal shows metastatic cells infected by lentivirus in lungs. (f) HCT-116 cells were transfected with shKRT7-AS lentivirus alone or combined with KRT7 overexpression plasmid, and transwell assay was performed. Migrated cells were stained with DAPI and images were randomly taken under microscope. Every 6 fields were counted for each sample. All data are shown as mean ± SD, *p < .05, **p < .01, ***p < .001, ****p < .0001 (unpaired Student’s t test). NC, negative control; Fn, Fusobacterium nucleatum; vec, vector; si, siRNA.
the upregulation of KRT7-AS by Fn infection was attenuated when HCT-116 cells were pretreated with NF-κB inhibitor, BAY-117082 (Figure 5f). Next, we constructed luciferase reporter plasmid containing promoter region of KRT7-AS to determine the interaction between p65 and KRT7-AS promoter. The luciferase reporter assay showed that knockdown of p65 significantly decreased the transcriptional activity of KRT7-AS (Figure 5g). These results demonstrated that Fn infection increased KRT7-AS expression through activating NF-κB signaling pathway.

The expression of KRT7-AS/KRT7 in CRC patients
To identify the clinical significance of KRT7-AS and KRT7, we performed qRT-PCR to quantify the expression of KRT7-AS and KRT7 using fresh frozen tissues from CRC patients. The expression of KRT7-AS and KRT7 was significantly increased in CRC tissues compared with adjacent normal tissues (n = 83) (Figure 6a). Consistently published data from The Cancer Genome Atlas (TCGA) also demonstrated that KRT7-AS and KRT7 expression were significantly elevated in colon cancer tissues.
Figure 5. Fn regulated KRT7-AS through NF-κB signaling pathway. (a) The KEGG analysis based on RNA sequencing result shows activation of NF-κB induced by Fn infection. (b) HCT-116 or LoVo cells were incubated with live or heat-killed Fn, E.coli or PBS for 24 h. The protein level of phospho-p65, p65, and IκB-α was measured by Western blot. (c) HCT-116 or LoVo were incubated with Fn or PBS control for 2 h. Immunofluorescence assay was performed to detect the p65 in the nucleus. DAPI was used to stain cell nucleus (scale bar = 50 μm). (d) Western blot shows the efficiency of two siRNA targeting p65. (e) HCT116 or LoVo was transfected with siRNA targeting p65. After 48 h, the qRT-PCR was performed to analyze the expression of KRT7-AS. (f) HCT-116 cells were treated with BAY-117082 (Selleck, USA) in different concentrations (5 μM, 10 μM, 20 μM) for 2 h (upper panel). HCT-116 cells were treated with 20 μM BAY-117082 for 2 h followed by Fn incubation for 4 h (lower panel). The qRT-PCR was performed to analyze the expression of KRT7-AS. (g) HEK293T and HCT-116 cells were transfected with luciferase reporter plasmid along with p65 siRNA or negative control. After 48 h, the luciferase activity was measured. The transfection efficiency data were normalized by dividing the Firefly luciferase activities with that of Renilla luciferase. All data are shown as mean ± SD, *p < .05, **p < .01, ***p < .001, ****p < .0001 (unpaired Student’s t-test). Ctrl, control; NC, negative control. Fn, Fusobacterium nucleatum; si, siRNA.
Figure 6. The expression of KRT7-AS/KRT7 in CRC patients. (a) The qRT-PCR analysis of the expression of KRT7-AS and KRT7 in CRC tissues and their adjacent normal tissues (n = 83 pairs). (b) Correlation analysis of the expression of KRT7-AS with KRT7 in our tested CRC tissues (n = 83, spearman r = 0.409, p < .0001). (c) Data in TCGA database showed the expression levels of KRT7-AS and KRT7 in colon cancer compared with those of normal controls (n = 456 vs. 41). (d) Correlation analysis of the expression of KRT7-AS with KRT7 in TCGA database (n = 456, spearman r = 0.71, p < .0001). (e, f) The expression of KRT7-AS and KRT7 among CRC patients with different N stage (N0, N1, N2) in our tested CRC tissue (e) and TCGA database (f). All data are shown as mean ± SD, *p < .05, **p < .01, ***p < .001, ****p < .0001 (Wilcoxon matched-pairs signed-rank test was used in a, Mann–Whitney test was used in c, spearman correlation analysis was used in b and d, one-way ANOVA was used in e and f). Fn, Fusobacterium nucleatum.
compared with normal tissues (Figure 6c). Furthermore, the KRT7-AS expression was positively associated with KRT7 expression both in our tested CRC tissues and TCGA database (Figure 6b, d). Though the expression of KRT7-AS and KRT7 were not increased in N1 patients compared with N0 patients, both our tested CRC tissues and TCGA database consistently showed that the expression of KRT7-AS and KRT7 were significantly increased in CRC tissue of N2 patients. These results indicated that both KRT7-AS and KRT7 were positively associated with more advanced N stage in CRC patients (Figure 6e,f).

Discussion

In the present study, we observed that the abundance of Fn was significantly increased in CRC patients with lymph nodes metastasis than those without lymph nodes metastasis. Furthermore, we reported that Fn infection enhanced CRC cells migration in vitro and increased lung metastasis in vivo. Several previous studies also indicated that the abundance of Fn was associated with TNM stage and survival outcomes in CRC patients. Interestingly, Bullman et al. recently found identical Fusobacterium strains in human primary CRC tissues and paired metastatic tumors in liver, suggesting these bacteria may migrate with colorectal cancer cells to distant sites. Inconsistent with this assumption, we also found the existence of Fn in metastatic lung lesions from mice which were intravenously injected with Fn-treated cells. These findings indicated that Fn not only plays a pro-tumorigenic role in CRC but also promotes distant metastasis.

The mechanisms for pro-metastatic effect upon Fn infection are not fully understood. Long non-coding RNAs (lncRNAs) were reported to play essential roles in the metastasis of CRC. The crosstalk between Fn and lncRNA has never been clarified. We hypothesized that lncRNA might be involved in the progression of CRC upon Fn infection. Interestingly, we discovered a great number of differentially expressed lncRNAs induced by Fn infection, among which KRT7-AS is one of the most differentially upregulated lncRNA. Prior study has demonstrated that overexpression of KRT7-AS in gastric cancer cells promoted cell growth, S-phase entry, and migration. However, its role in CRC was unknown. We found that KRT7-AS was associated with advanced N stage and Fn infection promoted CRC metastasis in a KRT7-AS dependent manner.

Previous study indicated that KRT7-AS upregulated protein and mRNA levels of KRT7 by stabilizing KRT7 mRNA in gastric cancer. In our study, we also found the similar interaction between KRT7-AS and KRT7 in CRC cells. KRT7 belongs to type II cytokeratin, which is the component of cytoskeleton and epithelial intermediate filaments. In addition to its role in maintaining the structural integrity of the cells, intermediate filaments, such as KRT7, have interesting properties that may promote motile activities. KRT7 was expressed in approximately 10% of all colorectal tumors and associated with high extent of tumor budding and worse outcomes. Reyhan et al. demonstrated that KRT7 expression was more common in CRC with lymph nodes metastasis compared to those without lymph nodes metastasis. Though KRT7 overexpression has been observed in CRC, the biological significance is unknown. We found that KRT7 was a downstream target of KRT7-AS and could regulate the migration of CRC cells. In this study, we attempted to clarify the mechanism for the upregulation of KRT7-AS by Fn infection. We found that Fn infection activated NF-κB pathway. The activated NF-κB P-p65 might upregulated KRT7-AS by enhancing the transcriptional activity of KRT7-AS.

In conclusion, our study confirmed an association between Fn and CRC metastasis and uncovered a novel mechanism by which Fn promoted CRC metastasis. This raises important questions on whether the depletion of Fn can prevent the early-stage CRC patients from an exacerbation and whether treatment regimens should be tailored based on the presence or absence of Fn in the metastatic spread of CRC patients.

Materials and methods
Bacterial strain and cell culture

Fn was purchased from American type culture collection (ATCC, Fusobacterium nucleatum subsp. nucleatum ATCC 25586). Fn was grown in Columbia blood agar.
(bioMerieux Biotech Co., Shanghai, China) in an anaerobic jar (MITSUBISHI Gas Chemical Co., Japan) at 37°C. Heat-killed Fn was prepared by heating the bacteria at 95°C for 1 h. The E. coli strain DH5α (Takara, Japan) was cultured in Luria-Bertani medium. CRC cell lines (HCT-116, LoVo) were purchased from ATCC. HCT-116 were cultured in Maccoy 5A (Genom, China) supplemented with 10% fetal bovine serum (FBS), while LoVo was cultured in F-12K (Genom, China) supplemented with 10% FBS. All CRC cell lines were cultured at 37°C in humidified 5% CO₂ atmosphere.

**Patients and public involvement**

Fecal samples were collected from 49 previously untreated CRC patients and 30 healthy people from Sir Run Run Shaw Hospital, Zhejiang University School of Medicine. Fresh cancer and adjacent normal tissues were obtained from 83 CRC patients. In addition, published data of 456 colon carcinomas (with 41 normal tissues) from The Cancer Genome Atlas (TCGA) were analyzed. Written informed consents were obtained from all participants. The study was approved by the Institutional Review Board of Sir Run Run Shaw hospital.

**RNA extraction and quantitative real-time PCR**

Total RNAs were extracted from CRC cell lines or fresh frozen tissues using Trizol reagent (Ambion, USA). 1 μg of total RNAs were reverse transcribed to cDNA using PrimeScript RT Reagent Kit (Takara, Japan). Quantitive real-time PCR was performed in ROCHE LightCycler®480 System (Rotor gene 6000 Software, Sydney, Australia). Each reaction was assayed in triplicate in 10 μl reactions containing SYBR Premix Ex Taq (Takara, Japan), primers and template DNA. The Ct values obtained from samples were compared using 2−ΔΔCt method. GAPDH or U6 served as internal reference genes. The following primer sets were used:

KRT7-AS-F, 5′-TCCAACGCCTATGTTCCAGTTCGCTTC
KRT7-AS-R, 5′-ACATTGTGCCACGGACATCTTG-3′;
KRT7-F, 5′-CGGCATCATCGGTAGGTCAAA-3′;
KRT7-R, 5′-GCCTGGAGGTCTCACAATTG-3′;
U6-F, 5′-CTCGCTTC-GGCAGCACAA-3′;
U6-R, 5′-AACGCTTCCAGAATTTGCGT-3′;
GAPDH-F, 5′-GCACCGTCAAGGCTGAGAAC-3′;
GAPDH-R, 5′-TGTTGAAGACGCAGTGTTGA-3′.

**Fn quantification**

Fn quantification was performed as described previously.¹⁷,¹⁹ DNA from fecal samples were extracted using QIAGEN stool kit (QIAGEN, Germany). Fn quantification was performed by real-time PCR. Relative abundance was calculated by 2−ΔΔCt method. Universal Eubacteria 16s was used as reference gene. The following primer sets were used:

Fusobacterium spp-F, 5′-CGGGTGAGTAACGGTAAAG-3′;
Fusobacterium spp-R, 5′-ACATTGTGCCAGCATCTTG-3′;
universal Eubacteria 16s-F, 5′-CGGCAGGAGGCGCAACC-3′;
universal Eubacteria 16s-R, 5′-CCATTGTAGCCAGCAGTGCAGCC-3′.

**Western blotting**

Total proteins were extracted from CRC cell lines or fresh frozen tissues using RIPA lysis buffer and quantified using BCA Protein Assay Kit (Beyotime, China). Protein was electrophoresed through 10% SDS polyacrylamide gels and then transferred to PVDF membranes. The membranes were blocked with 5% fat-free milk for 2 h and incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: KRT7 (1:1000 CST#4466), p65 (1:1000 CST#8242), P-p65 (1:1000 CST#3033) and IκB-α (1:1000 CST#9294). Membranes were then incubated with second antibodies labeled with HRP at room temperature on the following day and the signal was detected using an ECL kit (Fdbio science, China). GAPDH was used as a reference gene.

**Immunofluorescence (IF)**

CRC cells were seeded on chamber slides and then fixed with 4% paraformaldehyde for 10 min. The slides were incubated with 0.2% TritonX-100 (Beyotime, China) for 15 min and blocked in 3%
BSA for 0.5 h. After washing by PBS for three times, the cells were incubated with primary antibodies against KRT7 (1:100 CST#4465) or NF-κB (1:400, CST #8242) at 4°C overnight in wet chamber. On the following day, the slides were washed by PBST for three times and incubated with secondary antibodies at room temperature for 1 h. The cells were then stained with DAPI.

**Transwell migration assays**

CRC cells were incubated with Fn or E. coli DH5a at multiplicity of infection (MOI) of 100:1 for 12 h. Cells treated with PBS were set as negative control. The infected cells were resuspended in 200 μl medium without serum and were seeded into upper chambers of transwell chambers (Corning, USA) while lower chambers were filled with medium containing 10% FBS. After 20 h, the cells remaining in the upper chamber were removed while cells that had migrated across the transwell membrane were stained with DAPI. Images were randomly taken under fluorescence microscope. Every 6 fields were counted for each sample.

**Wound healing assays**

CRC cells were seeded in 6-well plates and left to grow until confluent. After wounding with yellow pipette tips, the cells of each well were incubated with Fn or E. coli DH5a at MOI of 100:1. Cell images were taken at 6, 24, 36 and 48 h, respectively.

**Small interfering RNAs (siRNAs), lentivirus shRNA and plasmid**

Two different siRNAs which exclusively targeted KRT7-AS RNA

\[ (5'-CUAGAUGUGUCCGAGUCAATT-3'); \]
\[ (5'-GAGGAUGAGAAUGAGAAUAT-3') \]

and siRNAs targeted against KRT7 mRNA non-overlapping region

\[ (5'-UGGAGGACUUCAGAAUAAT-3'); \]
\[ (5'-ACAAGCUUCAGACCAATT-3') \]

were used. In addition, two different siRNA were used to target NF-κB p65

\[ (5'-GCTGATGTGCAAGAAATT-3'); \]
\[ (5'-TTCTCCGAACGTGTCACGT-3') \]

were used as negative control (NC). All siRNAs were synthesized by GenePharma Co. China. Lentivirus shRNAs were constructed by Genechem Co. China. Briefly, the siRNA targeting KRT7-AS (5'-GAGGATGAATGAGATAAT-3') was synthesized and a non-targeting sequence 5'-TTCTCCGAACGTGTCACGT-3' was set as negative control (NC). The DNA fragments containing loop-structure for specific short-hairpin RNA were cloned into GV248 (hU6-MCS-Ubiquitin-EGFP-IRES-puromycin). KRT7-AS and KRT7 overexpression plasmids were constructed by Genechem Co. China. Genes were cloned into pCDNA 3.1(+).

**Cell transfection**

The siRNAs were transfected into cells using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, Massachusetts, USA) in opti-MEM (Genom, China) according to the manufacturer’s protocol. Plasmids were transfected into cells using FuGENE HD transfection reagent (Promega, USA) in opti-MEM according to the manufacturer’s protocol.

**RNA sequencing**

LoVo cells were incubated with Fn (MOI = 100:1) or PBS for 24 h. Total RNAs were extracted from LoVo treated by Fn or PBS using Trizol reagent, and ribosomal RNA was removed using the Ribo-Zero™kit (Epicenter, Madison, WI, USA). Subsequently, the purified RNAs were subjected to first strand and second strand cDNA synthesis followed by adaptor ligation and enrichment with a low-cycle according to instructions of NEBNext® Ultra™ RNA Library Prep Kit for Illumina (San Diego, CA, USA). The purified library products
were evaluated using the Agilent 2200 TapeStation and Qubit®2.0 (Life Technologies, USA). The libraries were paired-end sequenced (PE150, Sequencing reads were 150 bp) at Guangzhou Ribobio Co., Ltd. (China) using Illumina HiSeq 3000 platform. Raw fastq sequences were treated with Trimmomatic tools (v 0.36) to remove trailing sequences below a phred quality score of 20 and to achieve uniform sequence lengths for downstream clustering processes. Paired-end reads were aligned to the mouse reference genome mm10 and reads numbers mapped to each gene were counted by using Gfold (V1.1.2). The whole samples expression levels were presented as RPKM (expected number of Reads Per Kilobase of transcript sequence per Million base pairs sequenced).

The statistically significant differentially expressed genes were obtained by an adjusted p-value threshold of <0.05 and |log2(fold change)| > 1 using the edger (v 3.10.0) software. All the p-values were adjusted by FDR (False discovery rate) in multiple comparisons. To look for meaningful Fn-targeted lncRNA and mRNA, we filtered out lncRNA and mRNA with very low expression level (logCPM<2) among all the differentially expressed lncRNA and mRNA and obtained top 10 upregulated lncRNA and top 20 upregulated mRNA to do heatmap. All differentially expressed genes were used for KEGG ontology enrichment analyses. For KEGG enrichment analysis, a p value <0.05 was used as the threshold to determine the significant enrichment of the gene sets. All differentially expressed lncRNA and mRNA list as well as KEGG pathways were shown in Supplementary Tables 1–3.

**Lung metastasis in vivo**

BALB/c female nude mice aged 5 weeks were purchased from Shanghai SLAC Laboratory Animal Co. China. HCT-116 cells were incubated with Fn at MOI of 100:1 for 24 h. Cell resuspension solutions were injected via tail vein, $10^6$ cells in 100 μl per mouse. Two months later, the mice were sacrificed and their lungs were surgically excised, photographed, and measured. Subsequently, lung tissues were fixed in 10% formalin and embedded in paraffin for hematoxylin and eosin (H&E) staining to confirm histology. All nude mice were raised in a specific-pathogen-free condition. All animal procedures were performed in compliance with ethical standards and approved by the Animal Care Committee of Zhejiang University.

**Fluorescence In Situ Hybridization (FISH)**

Detection of Fn was performed by FISH on formalin-fixed paraffin-embedded (FFPE) section of lungs tissue from nude mice as described previously. The sequence of Fn targeted probe (5’-CGCAATACAGAGTTGAGCCCTGC-3’11) was synthesized by Guangzhou EXON Biological Technology Co. China.

**Luciferase reporter assay**

The DNA fragment of KRT7-AS promoter was amplified and cloned into the pGL3-Enhancer Vector (Taihe Biotechnology Co. China). The reporter plasmid was co-transfected with p65 siRNA or negative control siRNA into HEK293T or HCT-116 cells. Luciferase assays were performed 48 h after transfection by using Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activity was used as a reference. The transfection efficiency data were normalized by dividing the Firefly luciferase activities with that of Renilla luciferase.

**Statistical analysis**

Experimental results were evaluated using paired or unpaired Student’s t-test for normally distributed data and Mann–Whitney U test or Wilcoxon Sign-Rank test for non-parametric data. One-way ANOVA and Tukey’s multiple comparison were used for test among three groups. The Correlation analysis was performed using Spearman rank correlation test. Data were shown as mean ± standard deviation (SD). All p values were two-tailed and p values less than 0.05 were considered statistically significant. All statistical analyses were conducted using SPSS 22.0 or GraphPad Prism 7.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.
Authors’ contributions

Li.W., J.S. and W.Z. designed the experiment and supervised the study. T.S. and S.C. performed the experiments, analyzed the data and wrote the manuscript. Y.Z., J.H., Q.G., and L.W. performed the experiments. Li.W. and A.L. reviewed and revised the manuscript.

Funding

This work was supported by National Key R&D Program of China [2016YFC1303200] and the National Natural Science Foundation of China [81702308, 81972276].

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