Submitted: 11 February, 2025 Accepted: 02 July, 2025 Published: 30 October, 2025 DOI:10.22514/jomh.2025.125

ORIGINAL RESEARCH

JOMH Journal of Men's Health

Exosomal *TFF3* promotes colorectal cancer progression via the *LINC00941* pathway

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Abstract

Background: Colorectal cancer (CRC) is among the most prevalent malignancies globally, characterized by high mortality rate, and a significant reduction in patients' quality of life. Tumor-derived exosomes act as key mediators of interactions between the cells, and transmit oncogenic signals to nearby or distant cells and promote cancer progression. Trefoil factor 3 (*TFF3*) has vital function in metastasis, tumor growth, and oncogenic transformation in multiple carcinomas. Nevertheless, regulatory function of exosomal *TFF3* and molecular mechanistic processes behind its function in CRC progression are not well understood. **Methods**: The exosomes were characterized using transmission electron microscopy. Protein expression levels were determined by western blotting. Exosome uptake was assessed using PKH-26 fluorescence in immunofluorescence assays. The colony formation and Cell Counting Kit-8 (CCK-8) assays were used to measure cell proliferation, while Transwell and wound healing assays to evaluate cell invasion and migration abilities. *LINC00941* expression was quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Tumor growth was assessed *in vivo* assay, and Ki67 expression was examined through immunohistochemistry (IHC) assay. **Results**: This work revealed *TFF3* being overexpressed in CRC-derived exosomes. Exosomal *TFF3* substantially increased CRC cell invasion, migration, and proliferation. Mechanistically, exosomal *TFF3* upregulated *LINC00941* expression, thereby accelerating CRC progression. The exosomal *TFF3* enhanced tumor growth as verified through *in vivo* experimentation. **Conclusions**: The research herein demonstrates that CRC metastasis and cell proliferation are promoted by exosomal *TFF3* via the upregulation of *LINC00941*. These outcomes offer newer understanding of exosomal *TFF3* function in CRC pathogenesis and may inform future therapeutic strategies.

Keywords

Exosomal; *TFF3*; Colorectal cancer; *LINC00941*

1. Introduction

Colorectal cancer (CRC) is among the most prevalent malignancies, characterised by higher mortality and morbidity rates worldwide [1, 2]. Current therapeutics for CRC encompasses immunotherapy, radiotherapy, targeted therapy, chemotherapy, and surgery [3]. Among these, immunotherapy has evolved as reliable treatment, showing significant potential in the treatment of various malignancies [4, 5]. Cells secrete exosomes as small extracellular vesicles, which have vital functions in mediating intercellular communication and act as key modulators of cancer immunotherapy [6]. Therefore, identifying exosome-associated molecular targets holds great promise for improving therapeutic strategies against CRC.

Trefoil factor (TFF) is a tricyclic structured small, secreted peptide, and contains a highly conserved cysteine disulfide motif (can maintain the stability of proteins) [7]. *TFF3* has vital function in regulating various cancers. For instance, *TFF3* has been identified as a valuable target for modulating endocrine response in breast cancer [8]. In gastric cancer, *TFF3* promotes tumor progression by activating vascular endothelial growth factor (VEGF) signaling [9]. Cervical cancer also exhibited higher expression of *TFF3*, contributing to its malignant progression [10]. Furthermore, *TFF3* contributes to cell migration and angiogenesis in pituitary tumors by regulating VEGFA [11]. Interestingly, *TFF3* has also been reported to activate the signal transducer and activator of transcription 3 (STAT3)/Prostaglandin E Receptor EP4 (EP4) signaling pathway, thereby promoting colorectal cancer development [12]. Consistently, our previous work showed *TFF3* being overexpressed in CRC and facilitating epithelial-mesenchymal transition (EMT) in colon cancer cells [13]. But the detailed regulatory mechanisms by which *TFF3* influences CRC progression remain to be elucidated.

Exosomes are extracellular vesicles mediators derived from tumor cells that facilitate signal transduction between adjacent cells or distant cells, thereby contributing to the progression of cancers [14]. Increasing attention has been directed toward exosome-associated molecular targets due to their regulatory roles in various cancers [15]. However, the specific impact of exosomal *TFF3* on CRC progression is not well defined.

The research herein aims to explore regulatory function of exosomal *TFF3* in CRC progression. We demonstrate for the first time that exosomal *TFF3* enhances CRC cells migration and proliferation by upregulating *LINC00941*. The outcomes reveal that exosomal *TFF3* can act as reliable CRC therapeutic target.

2. Materials and methods

2.1 Tissue samples

During January 2024–November 2024, colorectal cancer (CRC) tissues (n = 20) and nearby healthy tissues (n = 20) were gathered from diseased individuals at the Tumor Hospital of Xinjiang Medical University. The signed informed consent was taken from all patients who had not received any prior therapy. The Ethics Committee of Tumor Hospital of Xinjiang Medical University authorized this study (No. G-2024010). All tissue samples were immediately stored in Liq. N_2 for further experimentation.

2.2 Cell line and treatment

CRC cell lines (LoVo, SW480 and HCT116) had been used for this study [16, 17], and provided by the American Tissue Culture Collection (ATCC, USA). Dulbecco's Modified Eagle Medium (DMEM; 11965084, Gibco, Rockville, MD, USA) having 10% fetal bovine serum (FBS) was used for cell culturing in humidified incubator with 5% CO_2 at 37 °C. For exosome treatment, exosomes from LoVo cells were co-cultured with HCT116 and SW480 cells. All cell experiments were performed independently in triplicate.

2.3 Exosomes' isolation and detection

LoVo cells were used to isolate exosomes for culturing in media having exosome-depleted FBS. Briefly, cells and debris were removed by centrifuging cell culture supernatants at 12,000×g for 30 minutes. To pellet exosomes, resulting supernatants were further centrifuged for 2 hours at 120,000×g, followed by washing twice with phosphate buffered saline (PBS). For transmission electron microscope (TEM), the exosomes' suspension was applied to 200-mesh carbon-coated grids at room temperature for 3 minutes. After drying for one minute, 3% phosphotungstic acid was used to stain grids. Exosomes were visualized using JEM-2000EX TEM (JEOL, Tokyo, Japan) and imaged for analysis.

2.4 Cell transfection

GenePharma (Shanghai, China) provided the pcDNA3.1 vectors targeting *TFF3* (TFF3) with negative control (Vector) and shRNAs targeting *TFF3* (sh-TFF3) with negative control (sh-NC). LoVo cells underwent transfections employing Lipofectamine 2000 (11668-027, Invitrogen, Carlsbad, CA, USA) as per the company's instructions. Following transfection, the culture supernatants of diversely administered LoVo cells went through isolation of exosomes for subsequent experiments.

2.5 Western blot

Radio immunoprecipitation assay buffer (RIPA) lysis buffer was used to extract total proteins from tumor tissues or CRC cells. Protein samples were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and moved to polyvinylidene fluoride (PVDF) membrane (FFP26, Beyotime, Shanghai, China). Membranes after blocking were treated with primary antibodies at 4 °C overnight. Afterwards, incubation was made with secondary antibodies (1/2000; ab7090) for 2 h at room temperature. A chemiluminescence detection kit (89880, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to detect protein expressions.

The primary antibodies: β-actin (1 μg/mL; ab8226; Abcam, Shanghai, China), TFF3 (1/500; ab272927), tumor susceptibility gene (Tsg)101 (1/1000; ab125011), and cluster of differentiation (CD)63 (1/1000; ab134045).

2.6 Immunofluorescence (IF) assay

Red fluorescent membrane dye PKH67 (MIDI67, Sigma-Aldrich, St.Louis, MO, USA) was used to label exosomes by following provider's manual. After thorough washing, labeled exosomes were isolated by centrifuging at 12,000×g for 30 minutes and further purified using ExoQuick™. DMEM was then used to resuspend labeled exosomes, followed by their addition to HCT116 and SW480 cells for co-culture. After incubation, 10% formaldehyde was applied to fix the cells and 4′,6-Diamidino-2-phenylindole (DAPI) for counterstaining. Fluorescent signals were visualized and imaged using fluorescence microscope (80i, Nikon, Tokyo, Japan).

2.7 CCK-8 assay

SW480 and HCT116 cells (1000 cells/well) seeded into the 96-wells plate and treated with exosomes. Cells viabilities were evaluated by Cell Counting Kit-8 (CCK-8; CK04, Dojindo Laboratories, Kumamoto, Japan). 10 μL CCK-8 solution was added to each well after the treatment, followed by 2 h incubation at 37 °C. A spectrophotometer (ND-ONE-W, Thermo Fisher Scientific, Waltham, MA, USA) was used to measure absorbance at 450 nm.

2.8 Colony formation assay

HCT116 and SW480 cells were cultured in 6-well plates for 14 days to form colonies. 4% paraformaldehyde was used to fix the developed colonies, followed by staining via 0.1% crystal violet. After washing, visible colonies had been counted and analyzed.

2.9 Wound healing assay

HCT116 and SW480 cells were cultured in 6-wells plates till they attained 80–90% confluence. A linear scratch was created by sterile pipette tip (10 μL) across cell monolayer. The wound area was explored regarding cell migration and imaged at 0 and 24 h through light microscope.

2.10 Transwell assay for cell invasion

Matrigel (356234, BD Biosciences, Franklin Lakes, NJ, USA) was used to pre-coat transwell chambers having 8 μm pore membranes (pore size, 8 μM; 3470, Corning, NY, USA). The upper chambers were added with HCT116 and SW480 cells suspended in 200 μL serum-free medium, while lower chambers with 600 μL medium having 20% FBS as chemoattractant. After incubating for 48 h, 4% paraformaldehyde was used to fix invasive cells on lower membrane surface, followed by staining via 0.1% crystal violet. Number of invaded cells was microscopically counted (E200, Nikon, Tokyo, Japan).

2.11 RT-qPCR

TRIzol reagent (15596018, Invitrogen, Carlsbad, CA, USA) was employed to extract total RNA by following company's manual. PrimeScript™ RT Master Mix kit (RR036A Takara, Dalian, China) was applied to perform reverse transcription of RNA into cDNA. qPCR was carried out by employing SYBR Premix Ex Taq™ kit. The $2^{-\Delta\Delta Ct}$ methodology was adopted to calculate relative *LINC00941* expression level. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control.

LINC00941:
Forward, 5′-CTTCTCTGAACTGCGGCTCA-3′;
Reverse, 5′-GGCCTCCTTGCTGACTGATT-3′.
GAPDH:
Forward, 5′-CTGGGCTACACTGAGCACC-3′;
Reverse, 5′-AAGTGGTCGTTGAGGGCAATG-3′.

2.12 *In vivo* assay

The Ethics Committee of Tumor Hospital of Xin Jiang Medical University approved animal experimentation which was executed in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines (No. IACUC-20220306-27). The Vital River company (Beijing, China) provided the male BALB/c nude mice (n = 16). In alignment with the 4R principles of animal research—reduction, refinement, replacement, and responsibility—10 mice were ultimately used for this study. Mice had been randomly grouped into two and administered by either sh-TFF3-Exo or sh-NC-Exo. After 35 days, 2% pentobarbital sodium was used to euthanize mice for dislocating cervix. Tumors had been harvested and processed for subsequent analysis (Approval No. IACUC-20220306-27).

2.13 IHC assay

4 μm thick sections of tumor tissue embedded in paraffin had been dewaxed, rehydrated, and subjected to antigen retrieval. After blocking, the sections underwent overnight incubation at 4 °C with 1 μg/mL primary Ki67 antibody (ab15580, Abcam, Shanghai, China). Next day, the sections underwent incubation at room temperature with secondary antibody (1:1000; ab7090). Staining was performed by diaminobenzidine (DAB) and counterstaining by hematoxylin. Ki-67 expression was microscopically observed and evaluated (E200, Nikon, Tokyo, Japan).

2.14 Statistical analyses

The data were shown as mean ± standard deviation (SD). GraphPad Prism Software 9 (GraphPad Software, San Diego, CA, USA) was used to perform statistical analysis. Two groups were compared and analyzed by *t*-test, while multiple groups by one-way analysis of variance (ANOVA). *p*-value of less than 0.05 had been of statistical significance.

3. Results

3.1 TFF3 is highly expressed in CRC tissue

Analysis using gene expression profiling interactive analysis (GEPIA) online database revealed that *TFF3* expression was substantially higher in colon adenocarcinoma (COAD) tissues in comparison to normal (Fig. 1A). Consistently, western blot analysis further confirmed that TFF3 protein levels were upregulated in CRC tissues in comparison to the matched normal (Fig. 1B). These outcomes indicate that *TFF3* is overexpressed in CRC.

3.2 TFF3 is upregulated in CRC-derived exosomes

TEM confirmed the isolated exosomes by revealing typical vesicle morphology (Fig. 2A). Western blotting depicted higher expressions of exosomal markers Tsg101 and CD63 in isolated exosomes (Fig. 2B). Notably, CRC-derived exosomes had substantially enhanced TFF3 protein expressions (Fig. 2C). These results suggest enriched *TFF3* in CRC cells derived exosomes.

3.3 Exosomal TFF3 promotes CRC cell growth

IF analysis showed that the PKH-26 fluorescence intensity decreased following *TFF3* knockdown and increased with *TFF3* overexpression, indicating altered exosome uptake (Fig. 3A). Western blot analysis confirmed that *TFF3* protein levels were reduced upon silencing and elevated upon overexpression (Fig. 3B). CCK-8 assays exhibited substantially reduced cell viability with *TFF3* knockdown, while it increased with *TFF3* overexpression (Fig. 3C). Similarly, colony formation assays demonstrated reduced cell proliferation in the *TFF3*-silenced group and enhanced proliferation in the overexpression group (Fig. 3D). In conclusion, these outcomes reflected that exosomal *TFF3* triggered CRC cell growth.

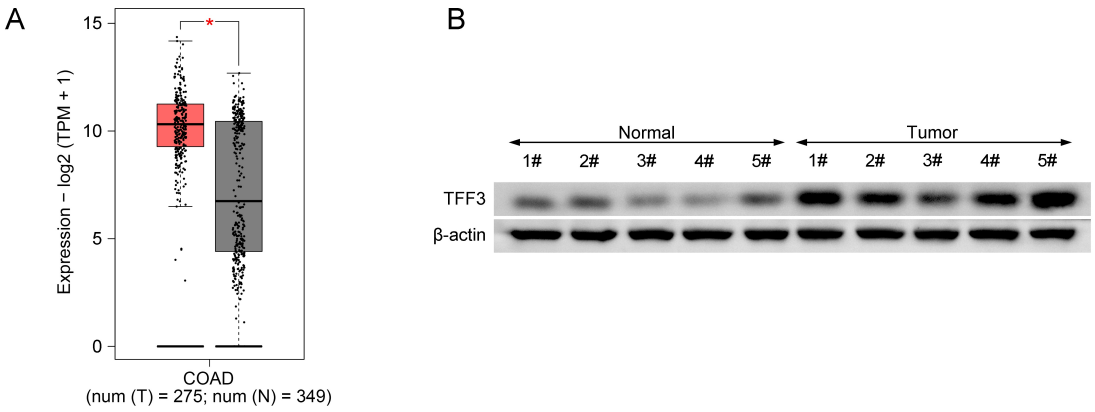

FIGURE 1. Overexpressed *TFF3* in CRC tissue. (A) GEPIA online database was used to analyze *TFF3* expression in normal and colon adenocarcinoma (COAD) tissues. $*p < 0.05$. (B) Western blotting was employed to examine TFF3 protein levels in normal (n = 20) and CRC tissues (n = 20). TFF: Trefoil factor; TPM: Transcripts per million.

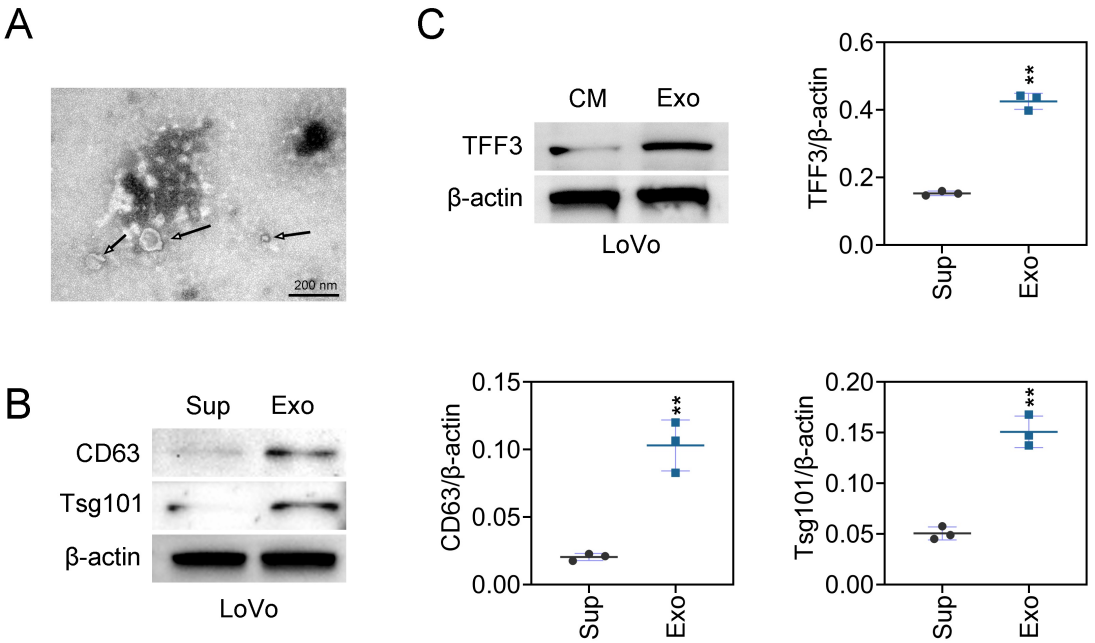

FIGURE 2. *TFF3* is upregulated in CRC-derived exosomes. (A) Isolated exosomes' morphology was confirmed by TEM. Arrows indicated exosomes. Scale bar = 200 nm. (B) Protein expressions of Tsg101 and CD63 exosomal markers were detected by western blotting (n = 3). (C) TFF3 protein expression in CRC-derived exosomes was evaluated through western blotting (n = 3). $**p < 0.01$. TFF: Trefoil factor; CD: Cluster of differentiation; Sup: Supernatant; Exo: Exosomes; CM: Conditioned medium; Tsg: Tumor susceptibility gene.

3.4 Exosomal *TFF3* enhances cells invasion and migration in CRC

Assays from wound healing depicted that CRC cells' migration rate was substantially decreased upon *TFF3* knockdown and enhanced following *TFF3* overexpression (Fig. 4A). Similarly, Transwell assays showed that cell invasion was suppressed by *TFF3* silencing and promoted by *TFF3* overexpression (Fig. 4B). These results demonstrate that exosomal *TFF3* enhances CRC cells' invasion and migration potential.

3.5 Exosomal *TFF3* upregulates *LINC00941* to promote CRC progression

RT-qPCR analyses showed substantially downregulated *LINC00941* expression following *TFF3* knockdown and upregulated upon *TFF3* overexpression (Fig. 5A). In addition, cells viabilities were decreased following *TFF3* knockdown, however the change was rescued by *LINC00941* overexpression (Fig. 5B). Similarly, Transwell and wound healing assays revealed that *TFF3* knockdown suppressed cells invasion and migration, while *LINC00941* overexpression reversed these effects (Fig. 5C,D). These findings suggest that exosomal *TFF3* promotes CRC progression by upregulating *LINC00941*.

3.6 Exosomal *TFF3* promotes tumor growth *in vivo*

Finally, *in vivo* experiments demonstrated that tumors weight, volume, and size had been significantly reduced following

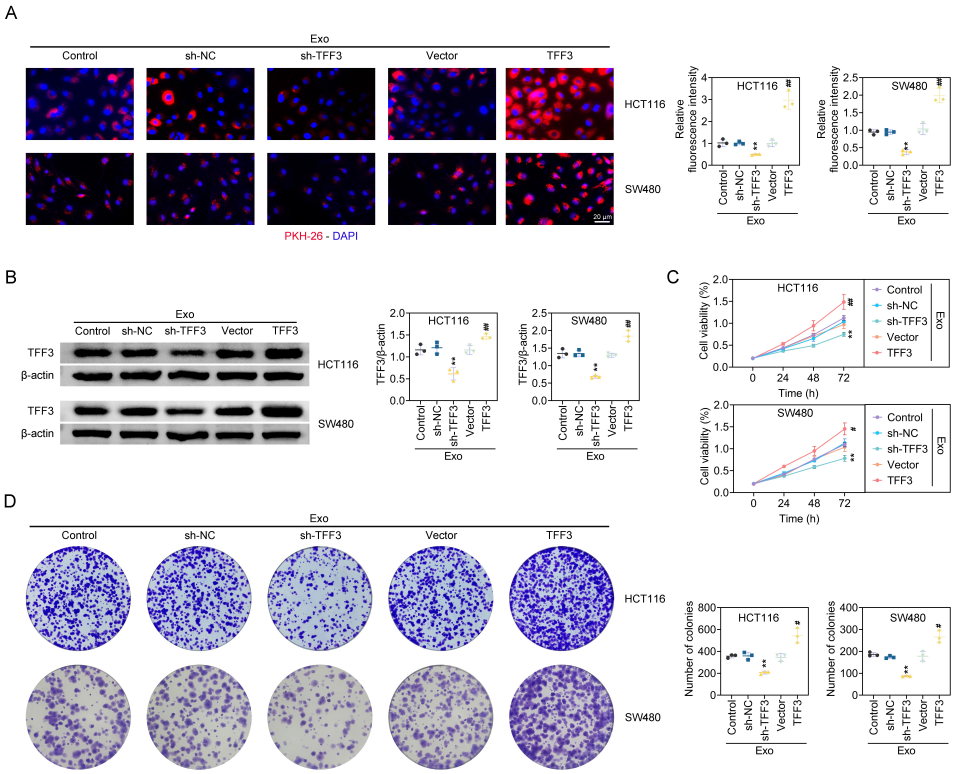

FIGURE 3. Exosomal *TFF3* promotes CRC cell growth. Groups had been segregated to Exo, sh-NC-Exo, sh-TFF3-Exo, Vector-Exo and TFF3-Exo group. (A) The fluorescence intensity of PKH-26 was examined through IF assay and analyzed through Image J software. Bar = 20 μm. (n = 3). (B) Western blotting assessed TFF3 protein expression. (n = 3). (C) CCK-8 assays evaluated cells viabilities. (n = 3). (D) Colony formation assay explored cells proliferation. N = 3. $^{**}p < 0.01$ *vs.* sh-NC-Exo group; $\#p < 0.05$, $\#\#p < 0.01$ *vs.* Vector-Exo group. TFF: Trefoil factor; Exo: Exosomes; sh-NC: shRNA-negative control; DAPI: 4′,6-Diamidino-2-PhenylIndole.

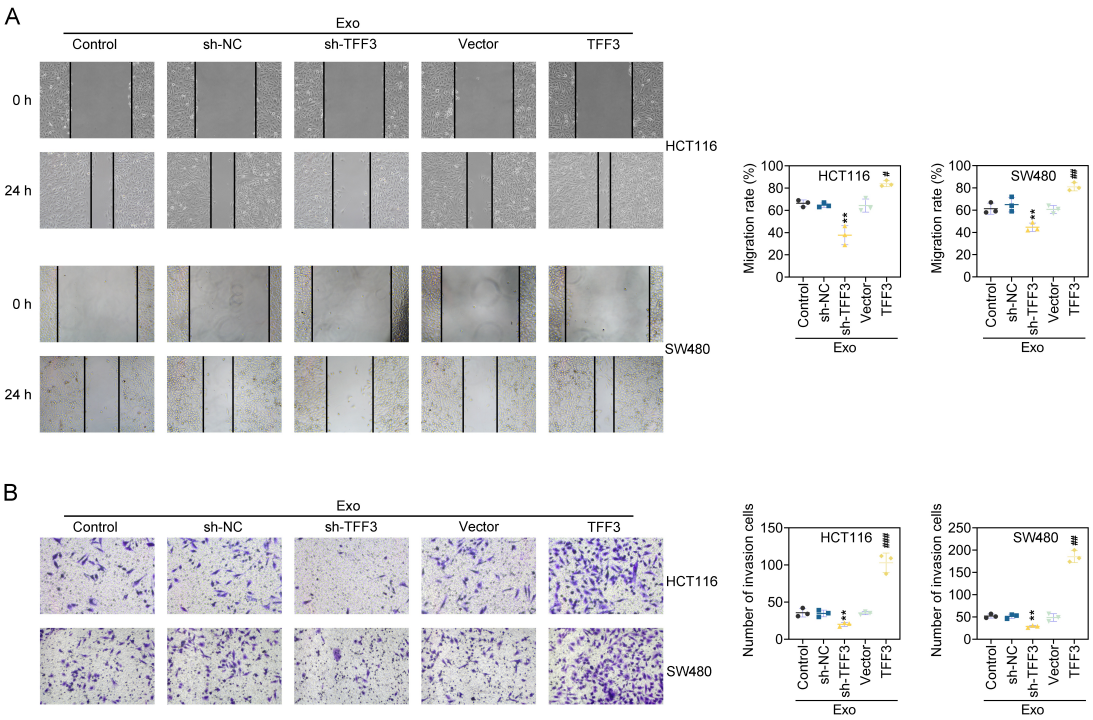

FIGURE 4. Exosomal *TFF3* enhances cells invasion and migration in CRCs. Groups had been segregated to Exo, sh-NC-Exo, sh-TFF3-Exo, Vector-Exo and TFF3-Exo groups. (A) The cell migration was evaluated by wound healing assay. (n = 3). (B) Transwell assay was used to assess cells invasion. (n = 3). $^{**}p < 0.01$ *vs.* sh-NC-Exo group; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ *vs.* Vector-Exo group. TFF: Trefoil factor; Exo: Exosomes; sh-NC: shRNA-negative control.

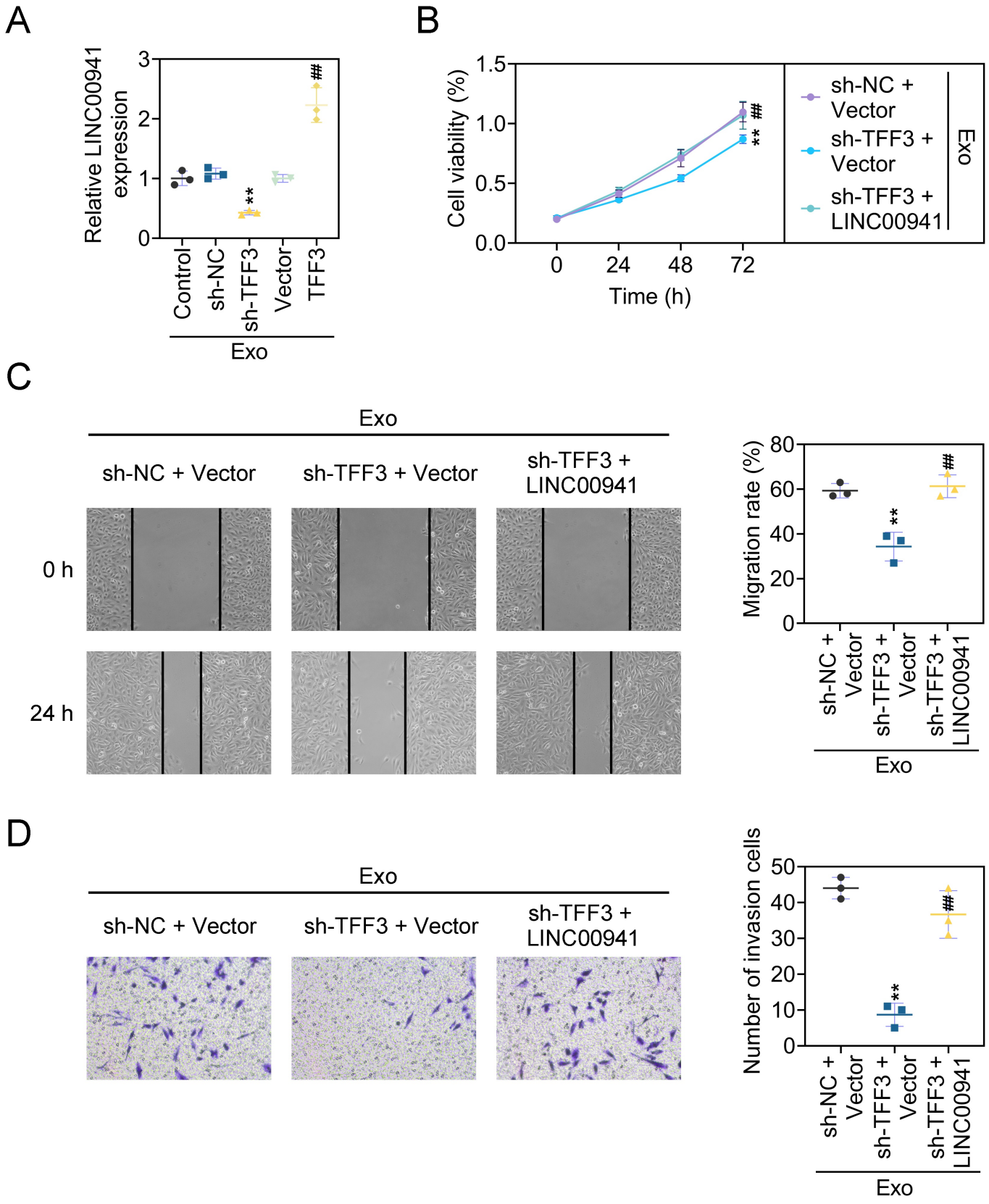

FIGURE 5. Exosomal *TFF3* upregulates *LINC00941* to promote CRC progression. (A) The *LINC00941* expression was examined through RT-qPCR. (n = 3). Groups had been segregated to Exo, sh-NC-Exo, sh-TFF3-Exo, Vector-Exo and TFF3-Exo group. $^{**}p < 0.01$ *vs.* the sh-NC-Exo group; $^{\#\#}p < 0.01$ *vs.* the Vector-Exo group. (B) Cells viabilities were evaluated by CCK-8 assays. (n = 3). (C) The cells migration by wound healing assay. (n = 3). (D) The cell invasion was examined through Transwell assay. Groups had been segregated to sh-NC + Vector-Exo, sh-TFF3 + Vector-Exo and sh-TFF3 + LINC00941-Exo group. N = 3. $^{**}p < 0.01$ *vs.* the sh-NC + Vector-Exo group; $^{\#\#}p < 0.01$ *vs.* the sh-TFF3 + Vector-Exo group. TFF: Trefoil factor; Exo: Exosomes; sh-NC: shRNA-negative control.

TFF3 knockdown (Fig. 6A). Western blot analysis confirmed a decrease in TFF3 protein expression in tumor tissues from the *TFF3*-silenced group (Fig. 6B). Consistently, RT-qPCR analysis showed that *LINC00941* expression was also downregulated after *TFF3* suppression (Fig. 6C). IHC staining revealed a marked reduction in Ki67 expression, indicating decreased cell proliferation in the *TFF3* knockdown group (Fig. 6D). These findings indicate that exosomal *TFF3* promotes tumor growth *in vivo*.

4. Discussion

Exosomes being the smaller extracellular vesicles have size range from 30 to 150 nm and carry proteins, RNAs, and lipids [18]. In recent years, exosomes derived from tumor have played vital functions in tumorigenesis and cancer progression [19]. Multiple studies have investigated exosomal-molecular targets in the regulation of CRC. For example, exosomal B7-H3 enhances metastasis and angiogenesis in CRC by modulating protein kinase B1 (AKT1)/mammalian target of rapamycin (mTOR)/vascular endothelial growth factor A (VEGFA) signaling pathway [20]. Additionally, tumor-derived exosomal miR-934 facilitates metastasis of liver via the induction of macrophage M2 polarizations in CRC [21], whereas exosomal ANGPTL1 suppresses MMP9-induced vascular leakiness to inhibit liver metastasis [22]. Exosomal lncRNA HOTAIR has also been reported to attenuate anti-tumor immunity, thereby promoting CRC progression [23].

TFF3 has crucial function in the progression of multiple cancers [8–11] and is upregulated in CRCs serum and tissues [12, 13]. Nevertheless, functions and mechanisms of exosomal *TFF3* in CRC progression are not clearly defined. In the present work, we confirmed the presence of exosomes secreted by LoVo cells and first time demonstrated that *TFF3* is highly expressed in exosomes derived from CRC. Moreover, it is observed that exosomal *TFF3* enhances CRC cells invasion, migration, and proliferation. *LINC00941*, also recognized as lncRNA-MUF, is novel and longer non-coding RNA (lncRNA) that is implicated in promoting metastasis and tumor growth across multiple carcinomas. For example, *LINC00941* enhances the progression of pancreatic cancer by positively regulating ANXA2 [24]. *LINC00941* activates MYC in colon cancer, thereby promoting cell proliferation and invasion [25]. Additionally, *LINC00941* communicates with miR-877-3p to control VEGFA, contributing to non-small cell lung cancer [26]. It also facilitates metastasis and tumor growth in gastric carcinoma [27]. Notably, *LINC00941* was found to activate transforming growth factor-beta (TGF-β)/mothers against decapentaplegic homolog (SMAD)2/3 pathway and inhibit degradation of SMAD4, thereby accelerating CRC metastasis [28]. However, the regulatory influences of exosomal *TFF3* on *LINC00941* expression in CRC development remain elusive. In the work herein, it was observed that *LINC00941* expressions were substantially upregulated in response to increased exosomal *TFF3* levels. Furthermore, exosomal *TFF3* promoted CRC progression by enhancing *LINC00941* expression. *In vivo* data further confirmed that exosomal *TFF3* aggravated tumor growth, supporting its role as a facilitator of CRC progression. These findings suggest that exosomes may serve as carriers of *TFF3*, delivering it to recipient cells and amplifying oncogenic signaling via *LINC00941*.

5. Conclusions

This study is the first to demonstrate that exosomal *TFF3* increases CRCs cells proliferation and migration via the upregulation of *LINC00941* expressions. However, this study has several limitations. These include the absence of validation in clinical tissue samples, limited investigation into other ma-

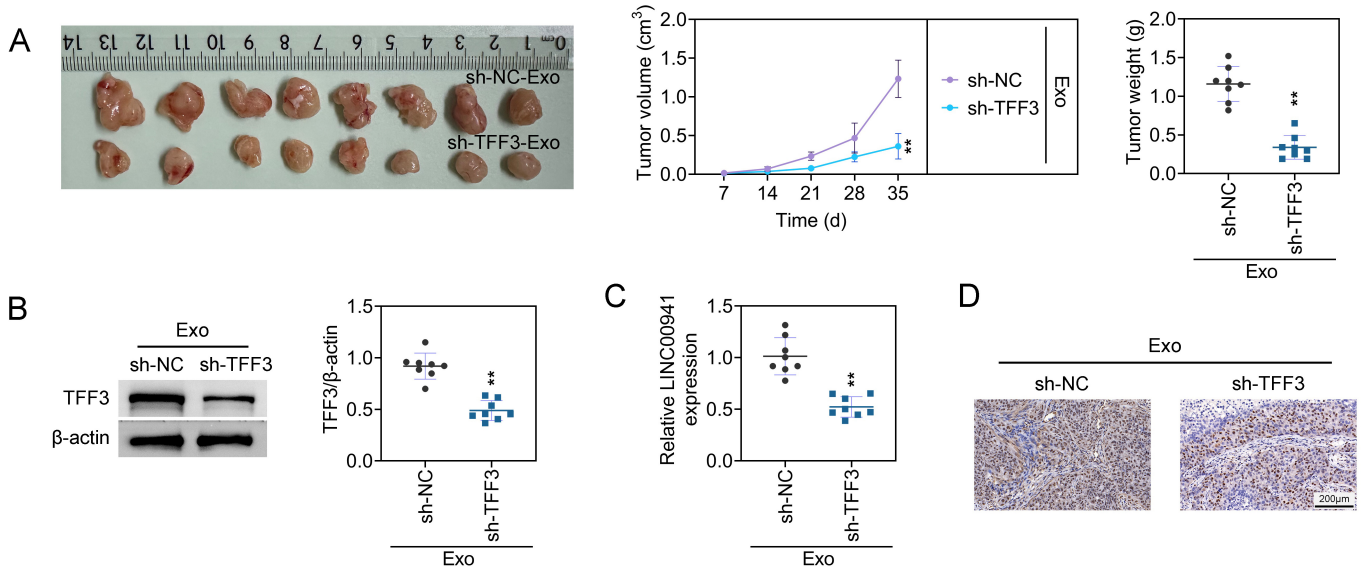

FIGURE 6. Exosomal *TFF3* promotes tumor growth *in vivo*. Groups had been segregated to sh-TFF3-Exo and sh-NC-Exo group. (A) Tumor growth in mice was evaluated. (n = 8). (B) TFF3 protein expressions were evaluated by western blotting. (n = 8). (C) *LINC00941* expression had been confirmed via RT-qPCR. (n = 8). (D) The Ki67 expression was determined through IHC assay. Scale Bar = 200 μm. (n = 8). $^{**}p < 0.01$. TFF: Trefoil factor; Exo: Exosomes; sh-NC: shRNA-negative control.

lignant phenotypes such as apoptosis or drug resistance, and insufficient exploration of additional signaling pathways that may be involved. Future studies should aim to validate these findings in clinical settings and further elucidate the broader oncogenic role of exosomal *TFF3* in CRC pathogenesis and therapeutic response. Further studies must emphasize on elucidating clinical importance of exosomal *TFF3* and its broader role in CRC pathogenesis and treatment.

AVAILABILITY OF DATA AND MATERIALS

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

AUTHOR CONTRIBUTIONS

AY—Performed Conceptualization, Methodology, and Writing-Original Draft. PS—Performed Formal analysis, Resources, and Investigation. YS—Performed Formal analysis, Visualization and Data Curation. JLC—Performed Project administration, Supervision, and Validation. SS and SA—Performed Validation, Supervision, and Writing-Review & Editing. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures performed in studies involving human participants were in accordance with the standards upheld by the Ethics Committee of Tumor Hospital of Xin Jiang Medical University and with those of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects (Approval No. Approval No. G-2024010). Written informed consent was obtained from a legally authorized representatives for anonymized patient information to be published in this article.

All animal experiments were approved by the Ethics Committee of Tumor Hospital of Xin Jiang Medical University for the use of animals and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines (Approval No. IACUC-20220306-27).

ACKNOWLEDGMENT

Not applicable.

FUNDING

This work was supported by the General Program of the Natural Science Foundation of Xinjiang Uyghur Autonomous Region (Grant No. 2019D01C253) and the "Tianshan Talents" High-level Medical and Health Personnel Training Project – Young and Middle-Aged Core Talents Cultivation Project (Grant No. TSYC202301B044).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Aikeremu Yusufu, Paerhati Shayimu, Yin Shu, Jiale Chen, Subiyinuer Sulayiman, Saibihutula Ababaike. Exosomal *TFF3* promotes colorectal cancer progression via the *LINC00941* pathway. Journal of Men's Health. 2025; 21(10): 35-43. doi: 10.22514/jomh.2025.125.