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LINC00704 boosts the immunologic escape of colorectal cancer cells by upregulating TLR4 by binding with miR- 203a- 3p

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Abstract

Background Colorectal cancer (CRC) is a common malignant tumor and is the second most common cause of cancer-related deaths worldwide. Immune escape suppresses anti-tumor immunity and facilitates tumor cells to proliferate. MiR- 203a- 3p regulates cancer progression and LINC00704 may bind with miR- 203a- 3p to inhibit its effects.

Methods In this study, the levels of miR- 203a- 3p and LINC00704 were tested in tumor tissue and non-cancer tissues in vivo. In further in vitro experiments, transfection, cell vitality, apoptosis, and proliferation ability were detected. The expression level of TLR4 was also examined. Finally, a luciferase assay was conducted to detect whether LINC00704 could bind with miR- 203a- 3p.

Results A rise in LINC00704 mRNA was observed in CRC tissues while miR- 203a- 3p was reduced. LINC00704 boosts the proliferation of cells and inhibits cell apoptosis. LINC00704 regulates Toll- 1ike receptor- 4 (TLR4) expression through miR- 203a- 3p, thereby modulating cell viability. CRC cell immune escape was facilitated by LINC00704 via miR- 203a- 3p.

Conclusion LINC00704 promotes CRC cell immunologic escape by upgrading TLR4 by binding with miR- 203a- 3p.

Keywords LINC00704, Immune escape, Colorectal cancer, TLR4, MiR- 203a- 3p

Introduction

Colorectal cancer (CRC) is a prevalent malignant tumor and the second leading cause of cancer-related mortality worldwide [1]. The incidence and mortality rates are rising, particularly in transitioning countries and younger populations [2–4]. Therefore, elucidating the pathogenesis of CRC is crucial for improving treatment outcomes.

Immune escape, the suppression of anti-tumor immunity, facilitates tumor proliferation [5–7]. The role of Toll-like receptors (TLRs) expressed on tumor cells in immune escape has gained significant attention. TLR4, in particular, promotes tumor cells to secrete immunosuppressive factors, thereby inhibiting immune responses and enhancing tumor survival [8]. Our previous studies demonstrated that TLR4 signaling contributes to the immune escape of colon cancer cells [9, 10]. Thus, targeting tumor immune escape may represent a promising therapeutic strategy for CRC.

MicroRNAs (miRNAs), small non-coding RNAs, regulate key biological processes such as immune responses, apoptosis, and proliferation [11, 12]. MiR- 203a- 3p plays a crucial role in cancer progression by suppressing CRC cell proliferation, migration, and invasion [13]. However, its precise regulatory mechanisms remain unclear.

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Long non-coding RNAs (lncRNAs) have recently attracted increasing attention [14, 15]. As competitive endogenous RNAs (ceRNAs), lncRNAs can prevent miRNAs from binding to their target mRNAs [16]. Evidence suggests that lncRNAs are involved in CRC modulation [17, 18], yet the underlying mechanisms remain poorly understood. LINC00704, a novel lncRNA first identified in breast cancer cell lines [19], has been implicated in the malignant progression of various cancers. Notably, LINC00704 exhibits low expression in normal tissues but is highly expressed in tumors, suggesting a potential role in tumor development [19, 20]. However, its function in CRC remains unexplored, highlighting its potential as a therapeutic target.

Our previous study predicted that LINC00704 interacts with miR- 203a- 3p. This study investigates whether LINC00704 promotes TLR4 expression by competitively binding miR- 203a- 3p and examines its role in CRC progression. By elucidating the mechanisms underlying CRC metastasis and recurrence, our findings may provide a theoretical foundation for improving CRC prognosis.

Materials and methods

Human samples

Tumor tissues and non-carcinoma adjacent tissues of cancer were collected from patients with CRC. The expression levels of miR- 203a- 3p and LINC00704 from different tissues were detected by reverse transcription-polymerase chain reaction (RT-PCR). The Ethics Committees of Wuhan University authorized this research (Approval No. 2023024 k).

Cell culture and treatment

The SW480 cell line was purchased from the American Type Culture Collection (CCL- 228) and used to simulate the growth of CRC cells. Cells were cultured in complete Leibovitz- 15 (L15) medium (11415064, Gibco) with 10% fetal bovine serum (FBS10099 -141, Gibco), and antibiotics (15140–122, Gibco) at 37 °C with 5% CO_2. The small interfering RNA (siRNA) and overexpression vector of LINC00704 were synthesized for SW480 transfection. For overexpression vector transfection, Opti-MEM (11058021, Gibco, USA) was used with Lipofectamine ™ 3000 (L3000015, Invitrogen, USA) dilution and vectors were diluted using Opti-MEM with with P3000™. Lipofectamine ™ 3000 was added to the diluted vectors (1:1) [21]. For siRNA transfection, Lipofectamine™ RNAiMAX (13778150, Invitrogen, USA) was used according to the procedure. The siRNAs and Lipofectamine™ RNAiMAX were diluted in Opti-MEM. Subsequently, Lipofectamine™ RNAiMAX was mixed with siRNAs and incubated for 15 min. Finally, the complexes were added to the plate.

Cell proliferation detection

EdU (5-ethyl- 2-deoxyridine) is a thymidine deoxyribonucleoside analog that can replace thymidine deoxyribonucleoside during DNA synthesis and be incorporated into newly synthesized DNA. In this study, the EdU Kit (MA0424, meilunbio, Guangzhou) was used to detect cell proliferation. Briefly, cells were cultured overnight and transfected. The 2× EdU working solution was prepared and preheated at 37 ℃ and equal amounts were added to each well to dilute the final concentration of EdU to 1×. Following EdU labeling, the cells were fixed and washed. Click-iT Additive Solution was prepared and added to each well for incubation for 30 min. Thereafter, DAPI (4',6-diamidino2-phenylindole) staining solution was used to stain the nuclei. Images were captured using microscopy (Nikon, ECLIPSE Ci) and proliferating cells were labeled with bright green fluorescence with blue nuclei.

Flow cytometry

Following the corresponding treatments, cells were digested using pancreatic enzymes (25200–072, Gibco, USA) and re-suspended with PBS/BSA twice, then diluted at 1×10^6 cells/ml in PBS/BSA buffer. Then 100 µL cell suspension was added in a flow cytometry tube and Annexin V/FITC (FXP018, 4 A Biotech, Suzhou) was added for incubation. Afterward, 10 µL iodized propidium solution (PI, 20 µg/mL) and 400 µL PBS were added and a cytometer (BeamCyte- 1026, BEAMDIAG, Suzhou) was used for detection.

RT-PCR

After different treatments, cells were collected for total mRNA extraction. The RNA extraction kit (RNAfast200, 220011, Shanghai) was used for total RNA extraction according to the manufacturer's instructions. cDNA was obtained using the reagent kit (RR047 A, TaKaRa). In addition, the cDNA was detected by RT-qPCR using the LightCycler® 480 SYBR® Green I Master with the Light

Table 1 Primer sequences

Primer	Sequences
miR- 203a- 3p	Forward: 5'- CTCAACTGGTGTCGTGGA – 3' Reverse: 5'- TCGGCAGGGTGAAATGTTTAGGAC- 3'
U6	Forward: 5'-CGGCAGCACATATACTAAAATTGGA- 3' Reverse: 5'-ATTTGCGTGTCATCCTTGCG- 3'
LINC00704	Forward: 5'- TAGATCCAGCTTGCTCTCAC – 3' Reverse: 5'- GCCTGTTTACATCAGCGTTT- 3'
TLR4	Forward: 5'- ATCTTTGGCTGAATTAGCTG- 3' Reverse: 5'- TCACTACCTCTATCGCTGT- 3'
GAPDH	Forward: 5'- ACATCATCCCTGCATCCACT – 3' Reverse: 5'- GCGGCATGTCAGATCCACAAC – 3'

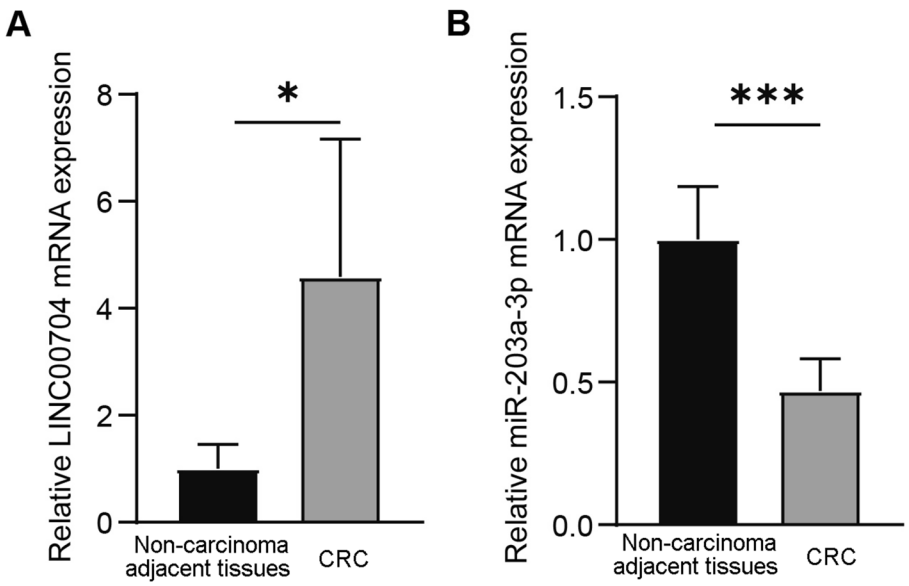

Fig. 1 LINC00704 expression was increased in CRC tissues while miR- 203a- 3p expression was decreased. **A**: RT-PCR was performed to detect the mRNA level of LINC00704 in indicated tissues. GAPDH was used as the loading control. **B**: RT-PCR was performed to detect the expression level of miR- 203a- 3p in tumor tissues and non-carcinoma adjacent tissues. U6 was used as the loading control. n = 5 per group. *$P < 0.05$, vs. non-carcinoma adjacent tissues, ***$P < 0.001$, vs. non-carcinoma adjacent tissues

Cycler480 instrument. The primer sequences are listed in Table 1.

Western blot

After the treatment described above, total protein was obtained from cells using Cell Lysis (MDL91201, MDL) containing the protease inhibitor cocktail (MD912893, MDL). Western blot was performed following the standard protocol [22]. Briefly, proteins were separated by 10% SDS-PAGE gel (BIO-Rad) and then transferred on 0.22 μm PVDF membranes (ISEQ00010, Millipore). Then, the blots were blocked using 5% BSA buffer and incubated with the indicated primary antibodies. The next day, the blots were incubated with HRP-conjugated secondary antibodies. The primary antibodies were TLR4 (bs-20594R, bioss) and β-actin (AF7018, Affity).

Cell counting Kit- 8 (CCK8) assay

After transfection, a CCK8 assay (F25, SciBioCold, Beijing) was used for cell proliferation detection in vitro. SW480 cells were seeded into a 96-well plate and CCK- 8 solution was added for incubation at 37 ℃ for 1 to 4 h. The optical density value was detected at a wavelength of 450 nm.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of interleukin 10 (SEA056Hu, Uscn Life Science Inc, Wuhan), IFN γ (Interferon γ, MDL), and transforming growth factor β1 (TGFβ1, MDL) of SW480-supernatant were measured using ELISA kits according to instructions.

Luciferase assay

After transfection, cells were collected and lysed on ice for 5 min, and 20 μL cell lysate was added to a black enzyme-linked plate. Then, the firefly luciferase reaction solution was added to the plate and shaken to mix well. The activity of firefly luciferase was detected within 30 min. Subsequently, Ranilla luciferase reaction solution was added to the wells and the activity was detected within 30 min.

(See figure on next page.)

Fig. 2 LINC00704 promotes cell proliferation and inhibits cell apoptosis. **A**, SW480 cells were transfected with overexpression negative control vector (oe-NC), LINC00704 vector (oe-LINC00704), negative control small interfering RNA (si-NC), and 3 different LINC00704 siRNAs (si-LINC00704). The control group was not transfected with any vector or siRNA. RT-PCR was performed (n = 3). ***$P < 0.001$, vs. Control. **B**, EdU images of indicated groups taken from microscopy (200 ×). **C**, Data analysis of Fig. 2B (n = 3). **$P < 0.01$, vs. Control, ***$P < 0.001$, vs. Control. D, Flow cytometry was performed to detect the level of apoptosis in indicated cells. **E**, Data analysis of Fig. 2D (n = 3). **$P < 0.01$, vs. oe-NC, ***$P < 0.001$, vs. si-NC

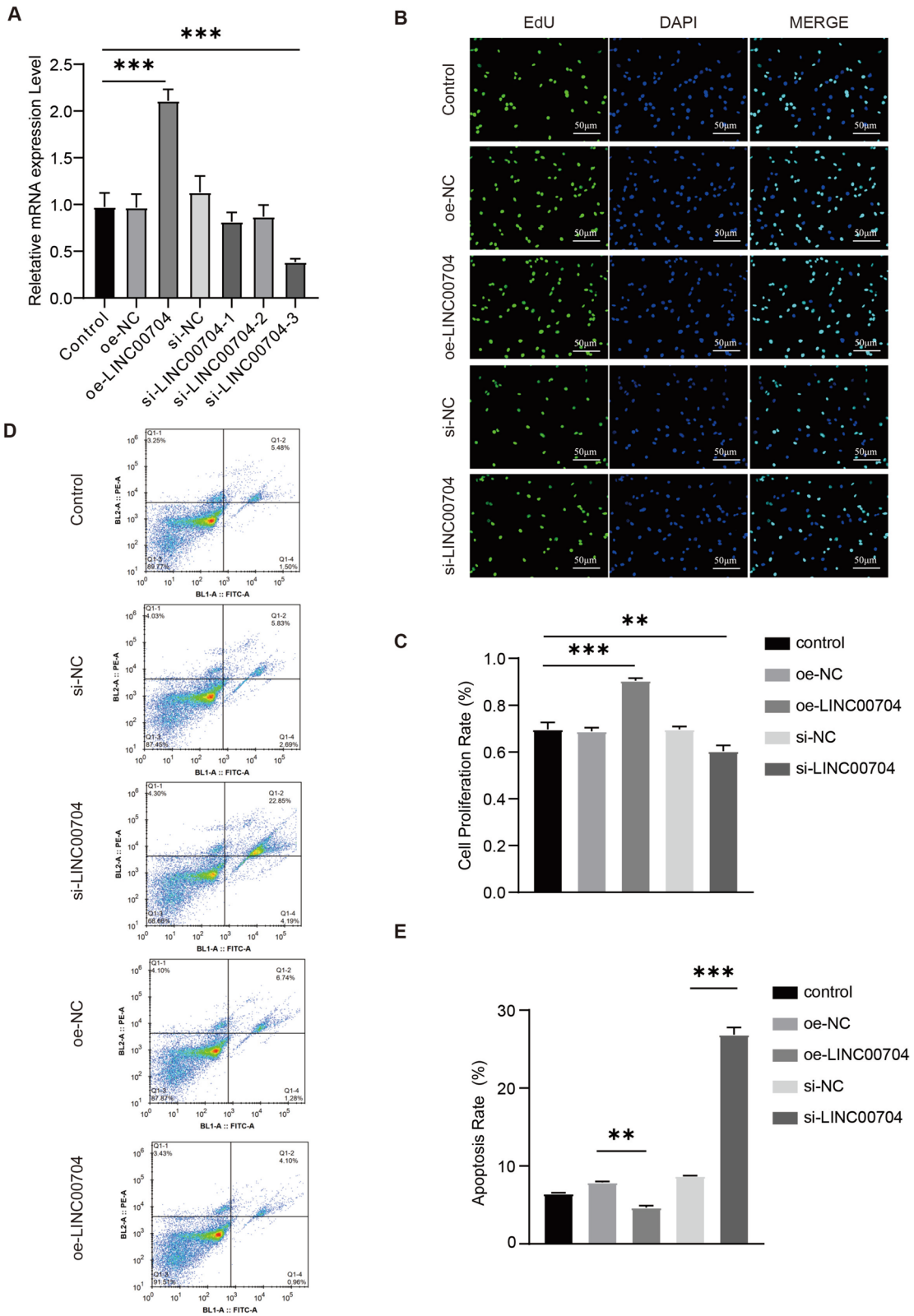

Fig. 2 (See legend on previous page.)

Statistical analysis

The GraphPad Prism 8 software was used for data analysis and values were expressed as the mean ± SD. The Shapiro–Wilk test was used to evaluate the normality after the variance test. The ordinary one-way ANOVA was used to compare means among three or more groups. In this study, $P < 0.05$ was considered statistically significant.

Results

LINC00704 level was increased in CRC tissues while miR-203a- 3p expression decreased

To explore the difference in LINC00704 and miR- 203a-3p expression levels between two types of tissues, two different tissues were collected from patients with CRC. Then, RT-PCR revealed that the mRNA level of LINC00704 was increased in CRC tissues compared with non-carcinoma adjacent tissues (Fig. 1A). Besides, the level of miR- 203a- 3p in CRC tissues was lower than that of non-carcinoma tissues (Fig. 1B), indicating that LINC00704 and miR- 203a- 3p might be related to CRC progression.

LINC00704 promotes cell proliferation and inhibits cell apoptosis

Furthermore, the effects of LINC00704 on cell proliferation and apoptosis were investigated. First, the overexpression vector and siRNA were constructed and transfected into SW480 cells separately to confirm the transfection efficiency. RT-PCR detection showed that the level of LINC00704 was upregulated after transfection of the overexpression vector, whereas the mRNA level of LINC00704 was significantly downregulated after si-LINC00704 - 3 transfection (Fig. 2A).

Cell proliferation was assessed using EdU. The results indicated that si-LINC00704 transfection resulted in a significant decrease in cell proliferation rate (Fig. 2B and C). Moreover, flow cytometry suggested that the cell apoptosis rate significantly decreased after LINC00704 overexpression, while LINC00704 knockdown promoted the apoptosis rate of cells (Fig. 2D and E). Therefore, LINC00704 promoted cell proliferation while inhibiting cell apoptosis, which might be essential for the progression of CRC.

LINC00704 regulates TLR4 expression via miR- 203a- 3p

To investigate the relationship between LINC00704 and miR- 203a- 3p, miR- 203a- 3p mimic and inhibitor were constructed for transfection. Following transfection with miR- 203a- 3p mimic, the level of miR- 203a- 3p in cells was increased while the level of miR- 203a- 3p was decreased after treatment with the inhibitor. These findings indicated that the constructures could be used for subsequent experiments (Fig. 3A). Besides, TLR4 siRNAs were used and the expression levels of TLR4 were downregulated after TLR4 siRNA transfection, especially for si-TLR4 - 2 (Fig. 3B). Therefore, si-TLR4 - 2 was used for subsequent experiments.

Furthermore, SW480 cells were subjected to various treatments, including siRNAs, overexpression vectors, mimics, and inhibitors. The RT-PCR results indicated that treatment with miR- 203a- 3p mimic and LINC00704 siRNA resulted in reduced mRNA levels of TLR4 in SW480 cells. In contrast, after miR- 203a- 3p inhibitor and LINC00704 overexpression vector transfection, TLR4 expression showed an upregulation trend. In addition, the treatment of miR- 203a- 3p mimic effectively reversed the upregulation of TLR4 expression mediated by overexpression of LINC00704 (Fig. 3C and D). Moreover, transfection of inhibitor and LINC00704 overexpression reversed the downregulation of TLR4 mediated by TLR4 siRNA (Fig. 3E and F). The effects of LINC00704 and miR- 203a- 3p on TLR4 were confirmed by western blot analysis, showing consistent results with the RT-PCR findings (Fig. 3G to K). Overall, LINC00704 was found to promote TLR4 expression, whereas miR-203a- 3p inhibited TLR4 expression. LINC00704 regulated TLR4 expression through miR- 203a- 3p.

LINC00704 modulates cell proliferation and apoptosis via miR- 203a- 3p

The above results revealed that miR- 203a- 3p mediated the effect of LINC00704 on TLR4 mRNA and protein expression levels. Next, the regulatory effects of LINC00704 on SW480 cell viability were determined. SW480 cells were transfected with control vectors (NC), miR- 203a- 3p mimic, miR- 203a- 3p inhibitor, and different siRNAs, and cell proliferation ability was evaluated by EdU detection. The results showed that the cell

(See figure on next page.)

Fig. 3 LINC00704 regulates TLR4 expression via miR- 203a- 3p. **A**, SW480 cells were transfected with negative control vector (NC), miR- 203a- 3p mimic (mimic) and miR- 203a- 3p inhibitor (inhibitor). The control group was not transfected with any vector. RT-PCR was performed (n = 3). ***$P < 0.001$, vs. NC **B**, SW480 cells were transfected with negative control siRNA (si-NC) and 3 different TLR4 siRNAs (si-TLR4). The control group was not transfected with any siRNA. RT-PCR was performed (n = 3). *$P < 0.05$, vs. si-NC, **$P < 0.01$, vs. si-NC, ***$P < 0.001$, vs. si-NC. **C**, **D**, **E**, and **F**, RT-PCR was used to detect the level of TLR4 in the corresponding groups (n = 3). **$P < 0.01$, ***$P < 0.001$. G, Western blot image of TLR4 and β-actin of indicated groups (n = 3). **H**, **I**, **J** and **K**, Data analysis of Fig. 3G (n = 3). ***$P < 0.001$

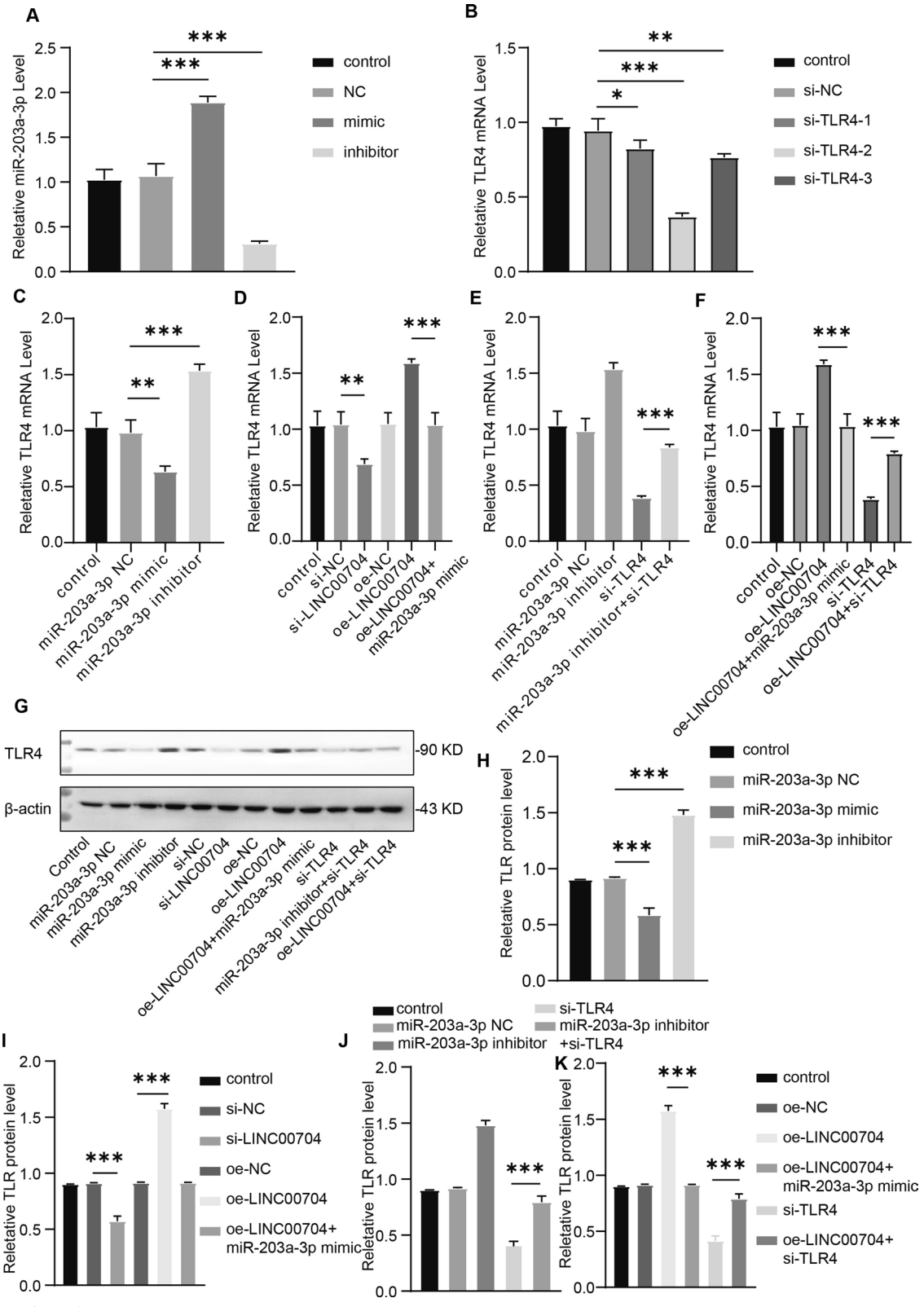

Fig. 3 (See legend on previous page.)

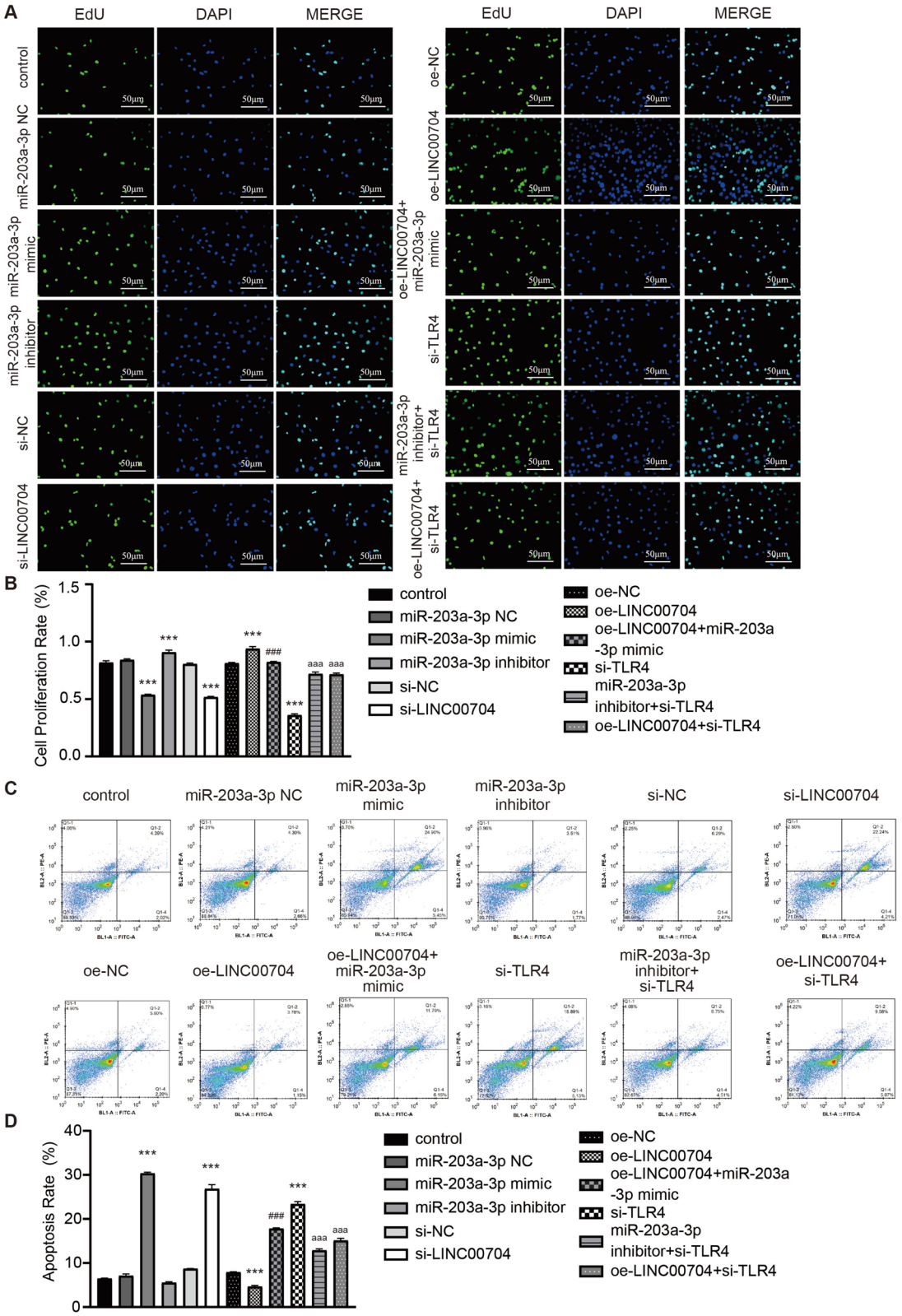

Fig. 4 LINC00704 regulates cell proliferation and apoptosis through miR- 203a- 3p. **A**, EdU images taken by microscopy (200 ×). **B**, Data analysis of Fig. 4 A (n = 3) ***$P < 0.001$ vs. Control, $^{###}P < 0.001$ vs. miR- 203a- 3p mimic group, $^{aaa}P < 0.001$ vs. si-TLR4 group. **C**, The level of apoptosis was tested by flow cytometry. **D**, Data analysis of Fig. 4 C (n = 3). ***$P < 0.001$ vs. Control, $^{###}P < 0.001$ vs. miR- 203a- 3p mimic group, $^{aaa}P < 0.001$ vs. si-TLR4 group

proliferation rate decreased after miR- 203a- 3p mimic, si-TLR4, and si-LINC00704 transfection. Consistently, transfection with inhibitor and LINC00704 overexpression vector upregulated the cell proliferation rate. Besides, LINC00704 overexpression reversed the inhibition function of miR- 203a- 3p mimic on proliferation. The si-TLR4 inhibited cell proliferation ability, while transfection of miR- 203a- 3p inhibitor and LINC00704 overexpression vector could reverse this inhibition effect (Fig. 4A and B).

Cell apoptosis was also detected using flow cytometry. Similarly, the apoptosis rate of SW480 cells increased following transfection with miR- 203a- 3p mimic, si-TLR4, and as si-LINC00704. The miR- 203a- 3p inhibitor led to a decreasing trend in cell apoptosis rate, but the difference was not significant. The cell apoptosis rate was significantly inhibited after transfection with the LINC00704 overexpression vector. Overexpression of LINC00704 effectively inhibited the increase in cell apoptosis rate induced by miR- 203a- 3p mimic following transfection of miR- 203a- 3p inhibitor; moreover, the LINC00704 overexpression vector could reverse the increase in apoptosis rate caused by TLR4 interfering RNA (Fig. 4C and D). Overall, the results revealed that LINC00704 promoted cell proliferation and inhibited cell apoptosis. miR- 203a- 3p mediated the effect of LINC00704 on proliferation and apoptosis.

LINC00704 promotes immune escape of CRC cells by controlling TLR4 expression through miR- 203a- 3p

To explore the effect of LINC00704 on immunologic escape, SW480 cells were co-cultured with monocytes according to different effect/target ratios (monocytes: SW480 cells = 20:1, 10:1 and 5:1, respectively). After transfection with si-LINC00704, CCK8 was used to detect SW480 cell viability, indicating that after LINC00704 knockdown, the cell viability of SW480 cells was downregulated when the effect/target ratio was 5:1 (Fig. 5A), suggesting that LINC00704 regulated cell viability. The supernatant of SW480 cells was collected and ELISA revealed increased protein levels of IFN-γ, TGF-β1, and IL- 10 after LINC00704 si-RNA transfection (Fig. 5B). Moreover, flow cytometry indicated that the ratio of CD4 +/CD8 + T cells was upregulated by LINC00704 knockdown (Fig. 5C).

Finally, whether LINC00704 regulated TLR4 expression by targeting miR- 203a- 3p was explored. First, the wild type (WT) and mutant dual luciferase vectors of LINC00704 were constructed and then co-transfected with miR- 203a- 3p. Luciferase activity was detected to verify the interaction between LINC00704 and miR-203a- 3p. The relative luciferase activity was inhibited when wild-type LINC00704 was transfected with miR-203a- 3p, revealing an interaction between the two molecules (Fig. 5D). Next, the wild type and mutant vectors of the 3'noncoding region of the TLR4 gene were constructed and co-transfected with miR- 203a- 3p into cells. The luciferase activity was restrained when wild-type TLR4 was co-transfected with miR- 203a- 3p mimic, indicating that miR- 203a- 3p interacted with TLR4 mRNA (Fig. 5E). Therefore, LINC00704 promoted CRC cell immune escape by regulating TLR4 expression through miR- 203a- 3p.

Discussion

The present study indicated that the mRNA level of LINC00704 increased in CRC tissues while miR- 203a-3p was downregulated. First, LINC00704 facilitates the immune escape of CRC cells by upregulating TLR4 by binding with miR- 203a- 3p. LINC00704 promotes cell proliferation while inhibiting cell apoptosis.

Malignant cells can evade immune surveillance, playing a crucial role in controlling tumor growth [23, 24]. TLRs are a member of pattern recognition receptors regulating the immune system. Studies have demonstrated that the activation of TLR4 promotes tumor immune escape and inhibits tumor cell apoptosis [25–28]. Therefore, exploring novel strategies to overcome tumor immune escape holds significance. Targeting the TLR4-related signaling pathway has been shown to be an immunotherapeutic method in several types of tumors like lung cancer [29], colitis-associated cancer [30], cervical cancer [31], and some others. TLR4 exerts both pro- or anti-tumor effects based on different tumor types and the activation

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Fig. 5 LINC00704 promotes cell immune escape by modulating TLR4 level through miR- 203a- 3p. **A** SW480 cells were co-cultured with monocytes according to different effect/target ratios (monocytes: SW480 cells = 20:1, 10:1, and 5:1). After transfection, the SW480 cell viability in each group was detected by CCK8 (n = 5). ***$P < 0.001$ vs. Control 20:1 group. **B**, Protein levels of IFN-γ, TGF-β1, and IL- 10 from the supernatant of SW480 cells were examined by ELISA (n = 3). ***$P < 0.001$ vs. Control 20:1 group, $^{###}P < 0.001$ vs. Control 10:1 group, $^{aaa}P < 0.001$ vs. Control 5:1 group. **C**, Flow cytometry was conducted to detect the ratio of CD4 +/CD8 + T cells in the indicated groups. ***$P < 0.001$ vs. Control 20:1 group, $^{###}P < 0.001$ vs. Control 10:1 group. **D**, Luciferase activity was detected to verify the interaction between LINC00704 and miR- 203a- 3p (n = 3). ***$P < 0.001$ vs. Control 20:1 group. **E** The wild type and mutant dual luciferase vectors of the 3'noncoding region of the TLR4 gene were constructed and co-transfected with miR- 203a- 3p. Luciferase activity was detected to verify the interaction between TLR4 and miR- 203a- 3p (n = 3). For Fig. 5 A to C, ***$P < 0.001$ vs. Control 20:1 group

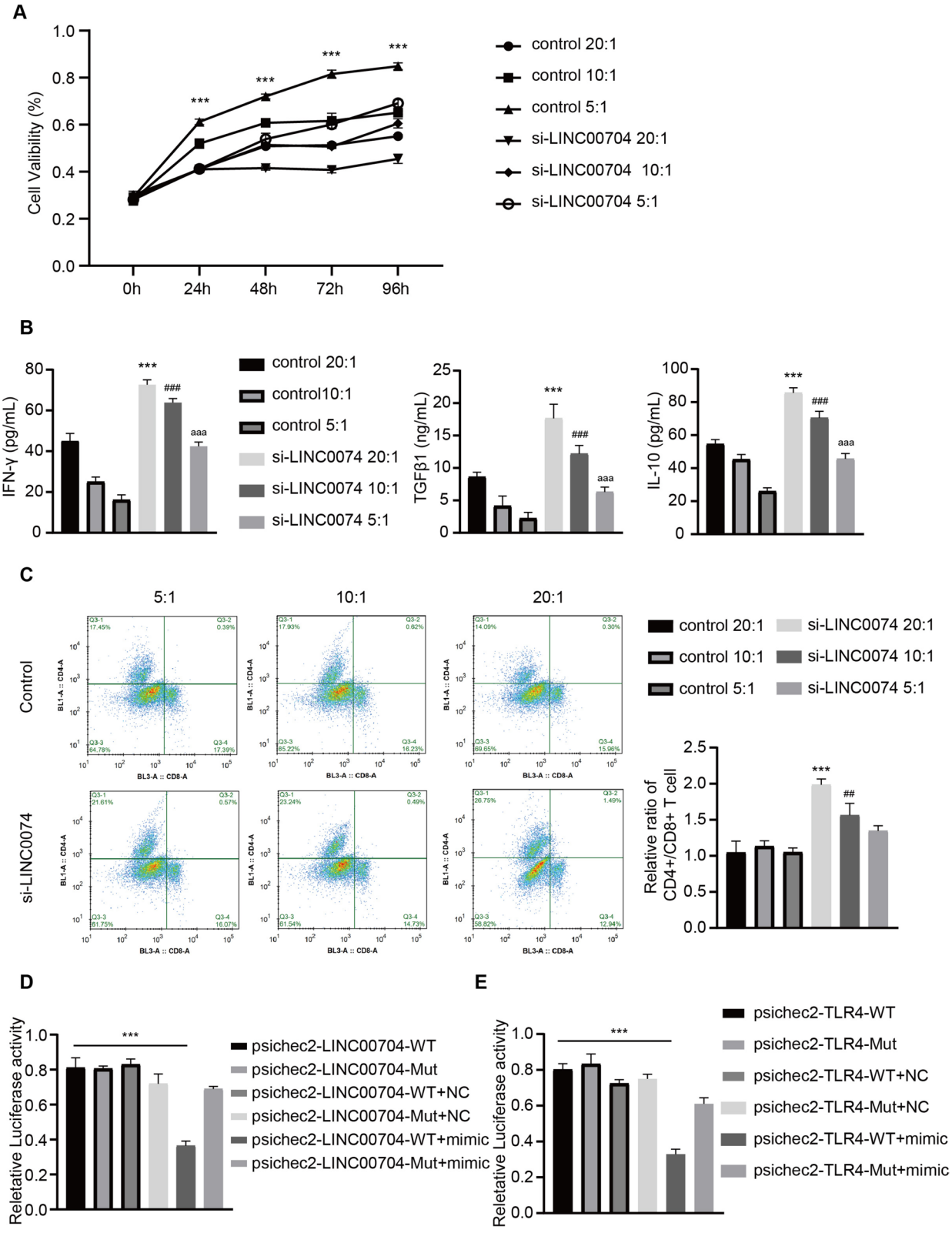

Fig. 5 (See legend on previous page.)

of TLR4 can be modulated in a TRIF-dependent or MyD88-dependent manner and result in different effects on tumor progression. This study revealed that TLR4 knockdown in SW480 cells promoted cell proliferation while inhibiting cell apoptosis, which facilitated tumor progression.

A variety of miRNAs participate in tumor progression and a previous study reported that miRNAs are closely correlated with diverse cancers [32]. Research on miRNAs suggests that biomarkers represent potential therapeutic methods. Therefore, the underlying mechanisms should be further studied to develop novel potential approaches. MiR- 203a- 3p is an essential miRNA regulating TLR4 expression, and miR- 203a- 3p has been found to be downregulated in CRC tissues. MiR-203a-3p is regarded as a candidate cancer suppressor, but the underlying mechanism has not been revealed yet [33]. Furthermore, miR- 203a- 3p suppressed TLR4 expression, thereby inhibiting downstream actions of TLR4, including cell proliferation and immune escape. Hence, small molecule drugs targeting miR- 203a- 3p may have therapeutic potential.

LINC00704 has been treated as a contributor to various types of tumors [34–36]. Studies have shown that LINC00704 overexpression could promote migration and invasion of cancer cells, whereas downregulation of LINC00704 could impair the proliferation of cancer cells. In diverse cancer types, LINC00704 exerts different roles and is deemed an essential regulator for cancer treatment. However, the biological mechanism in CRC remains unclear. Based on the predicted binding between LINC00704 and MiR-203a-3p from the RNA22 database, LINC00704 acted as a competitor of TLR4 to bind with MiR-203a-3p, which restrained the TLR4-inhibition effect of MiR-203a-3p and facilitated the expression of TLR4. Consequently, LINC00704 promotes cell proliferation, inhibits cell apoptosis, and contributes to immune escape.

In brief, LINC00704 facilitates the immune escape of CRC cells by upregulating TLR4 by binding with miR-203a- 3p, which provides a new theoretical basis for improving the treatment and prognosis of CRC in the future.

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Author contributions
The corresponding author is Xiaoyan Tang. She was responsible for project design, implementation , writing and revision. Yalei Jin and Hai Tao wrote the main manuscript text. Yuwei Liu and Sha Liu prepared Figs. 1, 2, 3, 4, 5 and the table. All authors reviewed the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The authors confirm that all experiments were performed in accordance with relevant guidelines and regulations. This study was conducted in accordance with the declaration of Helsinki and with relevant guidelines and regulations. This study was approved by the Ethics Committees of Wuhan University(No: 2023024k).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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