

RESEARCH ARTICLE

Hsa_circ_0001550 facilitates colorectal cancer progression through mediating microRNA-4262/nuclear casein kinase and cyclin-dependent kinase substrate 1 cascade

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Abstract

Background: Circular RNAs (circRNAs) play important roles in various malignancies, such as colorectal cancer (CRC). However, the function of hsa_circ_0001550 in CRC remains to be elucidated.

Methods: The expression levels of hsa_circ_0001550, microRNA (miR)-4262, and nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1) were determined by real-time qPCR. Cell biological behaviors were evaluated via colony formation assay, transwell assay, flow cytometry, and sphere formation assays. The target relationship was validated via dual-luciferase reporter and RNA pull-down assays. Protein expression was analyzed by western blot. Xenograft tumor model was adopted to evaluate hsa_circ_0001550 function in vivo.

Results: Hsa_circ_0001550 enrichment was enhanced in CRC tissue specimens and cell lines. Hsa_circ_0001550 absence hindered CRC cell proliferation, metastasis, stemness, and caused apoptosis. Hsa_circ_0001550 targeted miR-4262, and hsa_circ_0001550 absence-caused impacts were diminished by anti-miR-4262. MiR-4262 targeted NUCKS1. Hsa_circ_0001550 had positive regulation on NUCKS1 expression. NUCKS1 overexpression overturned the influences of hsa_circ_0001550 silencing on CRC cell progression. Hsa_circ_0001550 interference notably blocked in vivo xenograft tumor growth.

Conclusion: Hsa_circ_0001550 facilitated CRC progression by binding to miR-4262 to positively regulate NUCKS1 abundance.

KEYWORDS

colorectal cancer, hsa_circ_0001550, miR-4262, NUCKS1

Ying Zhou and Qilin Zhang contributed equally to this work.

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1 | INTRODUCTION

The dismal clinical prognosis of colorectal cancer (CRC) patients is attributed to lack of clinical symptoms and high recurrence and metastatic rates.¹⁻³ Uncovering the molecular mechanism underlying CRC progression will provide more effective bio-markers and targets to enhance the outcomes of CRC patients.

Circular RNAs (circRNAs), with stable circular structures, can be divided into noncoding circRNAs and coding circRNAs.⁴ CircRNAs are found to exert important function in multiple malignancies, including CRC.^{5,6} As reported, circ_001680 facilitates CRC cell proliferation and metastasis and enhances cell chemoresistance.⁷ Circ_0136666 is reported to contribute to cell proliferation capacity and glycolysis and suppress cell apoptosis in CRC cells.⁸ Hsa_circ_0001550 abundance is reported to be enhanced in CRC tumor specimens.⁹ However, hsa_circ_0001550 function in tumorigenesis has never been elucidated. Here, we inquired into the function and mechanism of hsa_circ_0001550 in CRC pathology.

Accumulating documents have indicated that circRNAs can absorb microRNAs (miRNAs) to relieve the suppressive impacts of miRNAs on gene expression.^{10,11} Previous articles have demonstrated that circRNAs can modulate CRC progression through miRNA/mRNA axis. Circ-ITGA7 is reported to inhibit cell proliferation by enhancing ASXL1 abundance via sequestering miR-3187-3p in CRC cells.¹² Circ_0071589 is reported to sponge miR-600 to up-regulate EZH2, thereby contributing to CRC progression.¹³ Bioinformatics analysis predicted that hsa_circ_0001550 can target miR-4262. MiR-4262 is confirmed as an antineoplastic factor in CRC.¹⁴⁻¹⁶ Zhang et al.¹⁶ found that circ-AGFG1 aggravated CRC progression by enhancing YY1 abundance via sequestering miR-4262 and miR-185-5p. We analyzed the correlation of hsa_circ_0001550 and miR-4262 in CRC progression.

Through bioinformatics tool Starbase, nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1) was found to be a possible target of miR-4262. A previous article showed that NUCKS1 was a vital regulator for cell cycle progression.¹⁷ The aberrant up-regulation of NUCKS1 has been identified in several malignancies, including CRC.¹⁸⁻²¹ A previous article found that NUCKS1 overexpression was relevant to the undesirable outcomes of CRC cases.²¹ Therefore, our study explored the target relationship of miR-4262 and NUCKS1 in CRC tumorigenesis.

We evaluated hsa_circ_0001550 function in CRC progression. Subsequently, its functional association with miR-4262 and NUCKS1 in CRC cells was explored.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens

Colorectal cancer specimens ($n = 70$) and para-cancer specimens ($n = 70$) were harvested from patients who were diagnosed with CRC at Seventh People's Hospital of Shanghai University of Traditional

Chinese Medicine. The tissue specimens were preserved at -80°C . All patients signed written informed consent. Our research was approved by the ethics committee of the Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine.

2.2 | Cell lines

SW620, LOVO, SW480, HCT116, and NCM460 from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) were cultivated with DMEM (Gibco) plus 10% FBS (Gibco) at 37°C with 5% CO_2 .

2.3 | Real-time reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen). The cDNA of circRNAs and mRNAs was gained via a commercial cDNA kit, and SYBR Green mix (Invitrogen) was adopted for qPCR. For miRNAs, Bulge-loop™ miRNA primer (Ribobio) was adopted. The fold changes were evaluated by $2^{-\Delta\Delta\text{Ct}}$ method and GAPDH or U6 was regarded as reference. All primers were listed in Table 1.

2.4 | RNase R and actinomycin D (Act D) treatment

RNA (2 μg) was mixed with 3 U/ μg RNase R for 0.5 h. To suppress transcription, Act D (2 mg/ml) was pipetted to the media to incubate with CRC cells. RT-qPCR was implemented to evaluate RNA abundance.

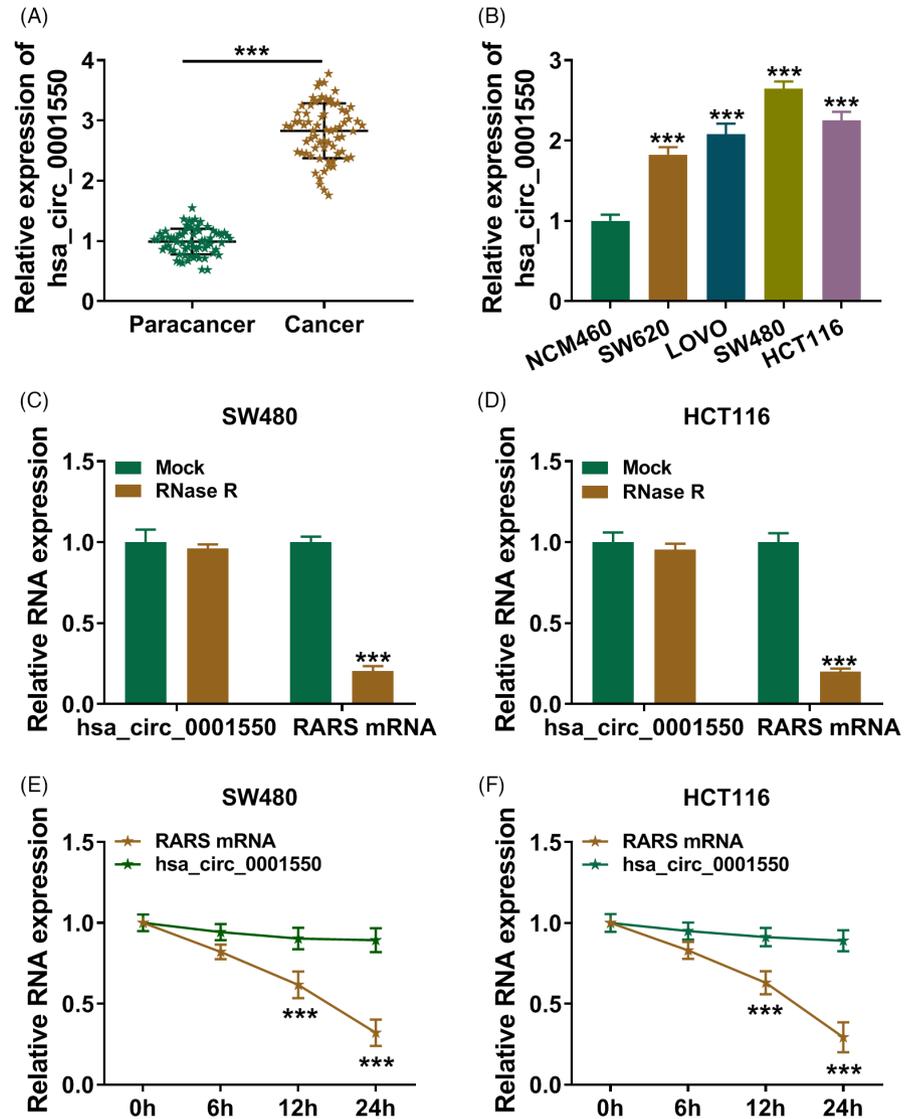
2.5 | Cell transfection

The sh-hsa_circ_0001550 and sh-NC were constructed by Genechem. MiR-4262 mimics, miR-NC, anti-miR-4262, and anti-NC

TABLE 1 Primer sequences in RT-qPCR assay

Gene	Sequence (5'-3')
Hsa_circ_0001550	Forward: TCAACTACCTGCTCTGGGAGA
	Reverse: TTTTCTTCTTGTAACTGCTCCAAA
RARS	Forward: TTTGCAGGGTATGACGTGCT
	Reverse: TTGTCTTGCAGGTGAGCGAT
miR-4262	Forward: GCCGAGGACATTCAGACTAC
	Reverse: CAGTGCAGGGTCCGAGGTAT
NUCKS1	Forward: CAGCCTGACTTTGTGGGAAC
	Reverse: CCAACAACAGGGATGGTCACT
GAPDH	Forward: TATGATGACATCAAGAAGGTGGT
	Reverse: TGTAGCCAAATTCGTTGTCATAC
U6	Forward: GCTTCGGCAGCACATATACTAAAAT
	Reverse: CGTTCACGAATTTGCGTGTCACT

FIGURE 1 Hsa_circ_0001550 abundance is enhanced in CRC. (A) Hsa_circ_0001550 abundance in CRC tissues and paracancer tissues was evaluated via RT-qPCR. (B) Hsa_circ_0001550 enrichment in CRC cells and NCM460 cells was examined via RT-qPCR. (C and D) After RNase R treatment, hsa_circ_0001550 expression and RARS mRNA expression were measured by RT-qPCR. (E and F) The stability of hsa_circ_0001550 was evaluated using Act D assay. *** $p < 0.001$



were purchased from GenePharma. NUCKS1 overexpression plasmid and pcDNA were acquired by Sangon Biotech. Transfection was implemented via Lipofectamine™ 3000 (Invitrogen).

2.6 | Colony formation assay

Colorectal cancer cells were dispersed in 12-well plates and were grown for 14 days. The colonies were immobilized via 4% paraformaldehyde and dyed via 0.5% crystal violet. The number of colonies (more than 50 cells) was then analyzed.

2.7 | Transwell assays

The commercial transwell compartment covered with or without Matrigel diluent (BD Biosciences) was adopted for invasion or migration assay. CRC cells were plated onto the above compartments in serum-free medium, with the lower compartments

supplemented with 10% FBS. Invaded and migrated cells were immobilized via 4% paraformaldehyde, dyed via 0.5% crystal violet, and then counted.

2.8 | Flow cytometry (FCM) analysis

A double-staining apoptosis kit (Beyotime, Beijing, China) was adopted to evaluate cell apoptosis. CRC cells were mixed with 10 μ l of Annexin V-FITC and 10 μ l of PI for 15 min. The apoptotic rate was then assessed.

2.9 | Sphere formation assay

Colorectal cancer cells were seeded onto commercial 96-well Clear Round Bottom Ultra Low Attachment Microplate (Corning Incorporated). After incubation for 7 days, the number of tumor spheres was analyzed under a microscope. Sphere formation efficiency was analyzed as the proportion of cells which could form tumor spheres.

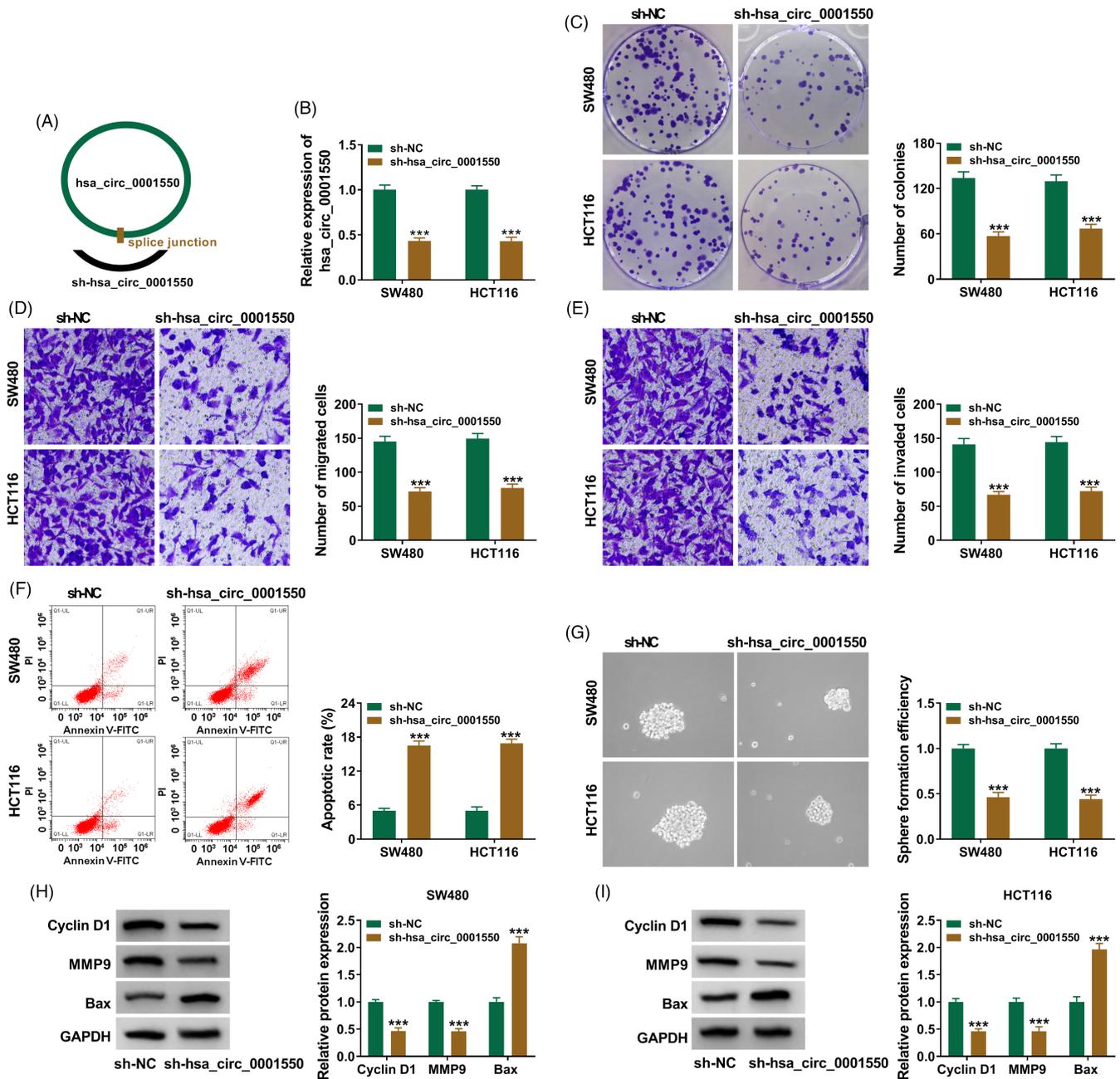


FIGURE 2 Hsa_circ_0001550 absence suppresses CRC progression in vitro. (A) The sh-hsa_circ_0001550 was designed to target the splice junction of hsa_circ_0001550. (B–I) CRC cells were transfected with sh-NC or sh-hsa_circ_0001550. (B) RT-qPCR was implemented to evaluate silencing efficiency of sh-hsa_circ_0001550. (C) Cell proliferation was evaluated via colony formation assay. (D and E) Transwell assays were implemented to assess CRC cell metastasis. (F) FCM analysis was implemented to detect cell apoptotic rate. (G) Sphere formation assay was implemented to evaluate cell stemness. (H and I) The enrichment of Cyclin D1, MMP9, and Bax was determined via western blot. *** $p < 0.001$

2.10 | Western blot assay

Protein was extracted by RIPA lysis buffer (Beyotime). Protein samples were separated by separating gel, and then shifted onto a PVDF membrane. The membrane was mixed with primary antibodies at 4°C. The primary antibodies were listed as below: anti-Cyclin D1 (ab16663; Abcam), anti-MMP9 (ab76003; Abcam), anti-Bax (ab32503; Abcam), anti-NUCKS1 (ab80425; Abcam), and anti-GAPDH (ab8245; Abcam).

The membrane was mixed with the secondary antibody (Abcam) for 2 h and was detected by ECL system (Bio-Rad).

2.11 | Dual-luciferase reporter assay

The sequence of hsa_circ_0001550 or NUCKS1 3'UTR with the putative or mutant complementary sites of miR-4262 was inserted into

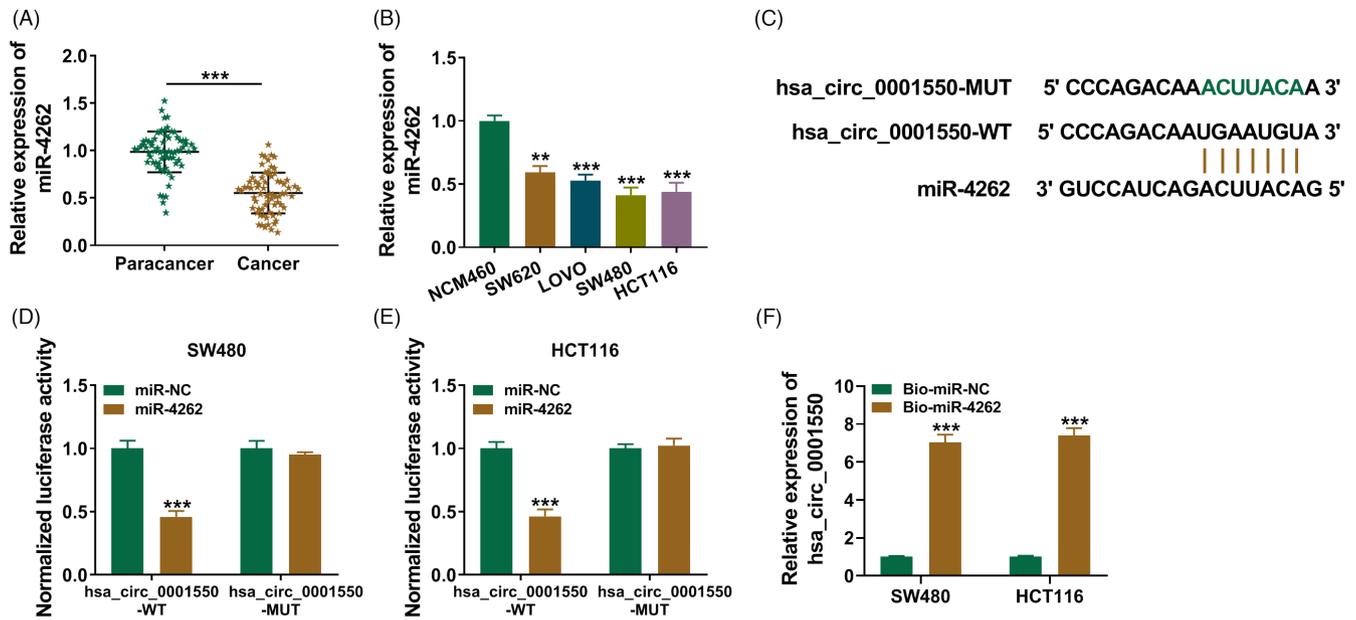


FIGURE 3 Hsa_circ_0001550 directly absorbs miR-4262. (A) RT-qPCR was conducted to examine miR-4262 enrichment in CRC tissues and paracancer tissues. (B) The miR-4262 enrichment in CRC cells and NCM460 cells was analyzed via RT-qPCR. (C) Starbase database predicted the binding sequence of hsa_circ_0001550 and miR-4262. Dual-luciferase reporter assay (D and E) and RNA pull-down assay (F) were implemented to validate associated relationship of hsa_circ_0001550 and miR-4262. ** $p < 0.01$, *** $p < 0.001$

pmirGLO vector (Promega) to obtain hsa_circ_0001550-WT/MUT and NUCKS1-3'UTR-WT/MUT vectors. CRC cells were introduced with miRNAs and reporter plasmids. The fluorescence intensities were assessed via a commercial kit (Promega).

2.12 | RNA pull-down assay

The probe for miR-4262 with biotin labeling (Bio-miR-4262) was constructed by GenePharma. The probe was mixed with cell extracts and beads (Invitrogen). Hsa_circ_0001550 abundance in precipitated complex was analyzed via RT-qPCR.

2.13 | Xenograft tumor model

Transfected SW480 cells were injected into BALB/c mice (Vital River Laboratory Animal Technology). Tumor dimension was analyzed every week as $\text{length} \times \text{width}^2 \times 0.5$. Tumor weight was recorded 5 weeks later. Ethical approval was acquired from the Ethics Committee of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine.

2.14 | Statistical analysis

Data processed by GraphPad software were shown in $\text{mean} \pm \text{SD}$, with $p < 0.05$ as the threshold of significance. Student's t test and ANOVA were adopted to evaluate the differences.

3 | RESULTS

3.1 | Hsa_circ_0001550 abundance is enhanced in CRC

Hsa_circ_0001550 abundance was increased in CRC specimens ($n = 70$) and cells (Figure 1A,B). SW480 and HCT116 cell lines were selected for the follow-up assays. Hsa_circ_0001550 could resist to RNase R digestion (Figure 1C,D). In CRC cells treated with Act D, hsa_circ_0001550 exhibited higher stability than its linear form RARS mRNA (Figure 1E,F). Overall, these results verified the circular feature and high abundance of hsa_circ_0001550 in CRC.

3.2 | Hsa_circ_0001550 absence suppresses CRC progression in vitro

We designed sh-hsa_circ_0001550 targeting the splice junction of hsa_circ_0001550 to silence it (Figure 2A). The knockdown capacity of sh-hsa_circ_0001550 was validated in SW480 and HCT116 cells (Figure 2B). Hsa_circ_0001550 absence prominently decreased colony number (Figure 2C), proving that hsa_circ_0001550 knockdown suppressed CRC cell proliferation. Transwell assays displayed that hsa_circ_0001550 absence caused a marked reduction in migrated and invaded cell numbers (Figure 2D,E), demonstrating that hsa_circ_0001550 silencing inhibited cell metastasis. FCM analysis presented that the apoptosis of CRC cells was notably induced by hsa_circ_0001550 knockdown (Figure 2F). Subsequently, we analyzed CRC cell stemness through sphere formation assay.

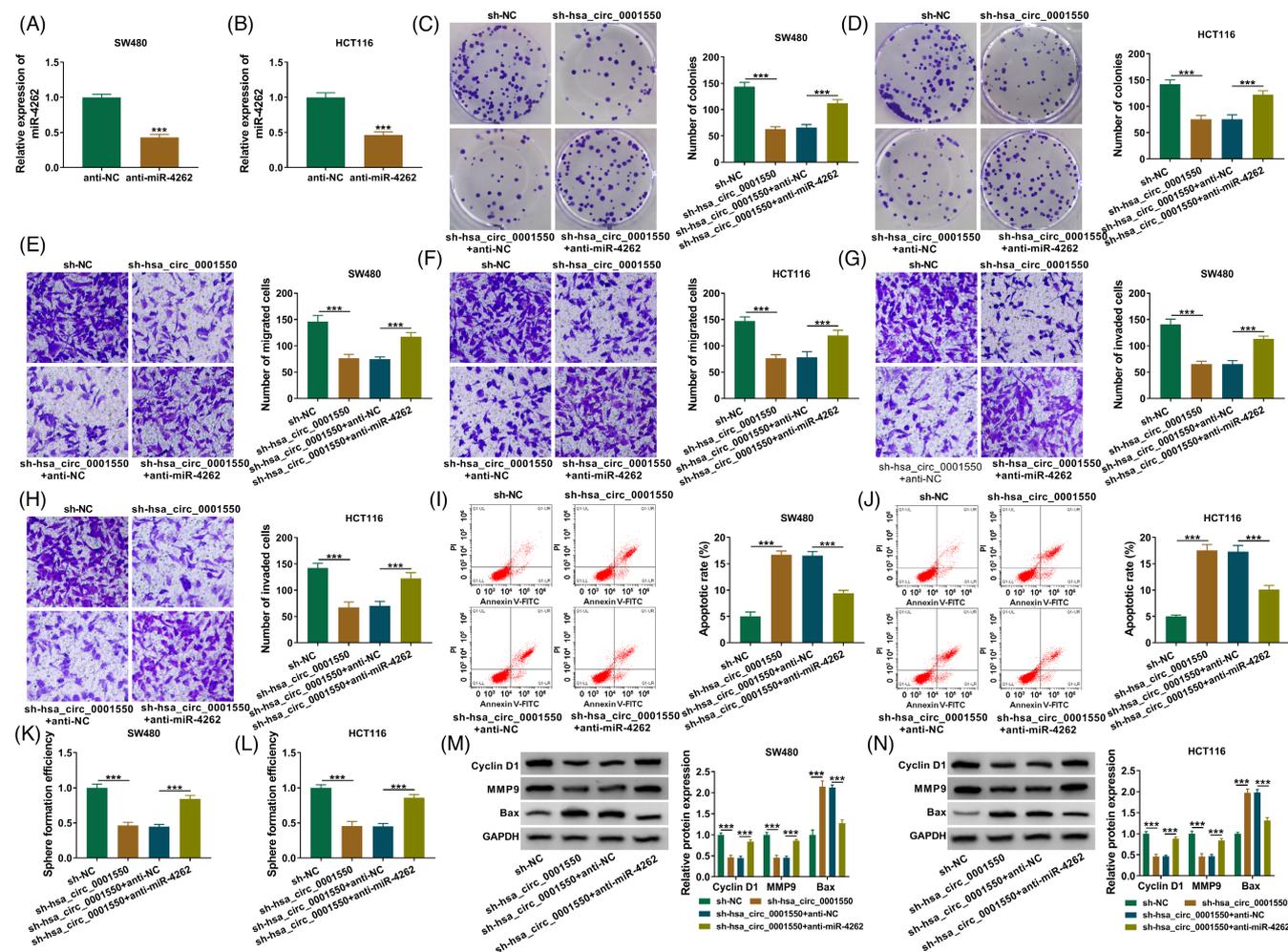


FIGURE 4 Hsa_circ_0001550 absence hinders CRC progression by partly up-regulating miR-4262. (A and B) The interference capacity of anti-miR-4262 was analyzed via RT-qPCR. (C–N) CRC cells were introduced with sh-hsa_circ_0001550 or sh-hsa_circ_0001550 + anti-miR-4262 along with their negative controls. (C and D) Colony formation assay was implemented to determine cell proliferation. (E–H) Transwell assays were performed to analyze cell metastasis. (I and J) Cell apoptotic rate was assessed via FCM analysis. (K and L) Sphere formation assay was implemented to evaluate cell stemness. (M and N) The protein abundance was examined via western blot. *** $p < 0.001$

Hsa_circ_0001550 depletion blocked CRC cell sphere formation efficiency (Figure 2G). The expression of proliferation-associated protein (Cyclin D1), metastasis-associated protein (MMP9), and pro-apoptotic protein (Bax) was determined in hsa_circ_0001550-silenced CRC cells by western blot assay. Hsa_circ_0001550 interference reduced Cyclin D1 and MMP9 abundance, while increased Bax abundance (Figure 2H,I). These results together demonstrated that hsa_circ_0001550 interference hindered CRC cell proliferation, metastasis, and stemness, whereas induced cell apoptosis.

3.3 | Hsa_circ_0001550 directly absorbs miR-4262

MiR-4262 abundance was prominently declined in CRC specimens and cells (Figure 3A,B). With the employment of Starbase tool, miR-4262 was found to harbor the complementary sequence with hsa_circ_0001550 (Figure 3C). To validate the associated relationship of hsa_circ_0001550 and miR-4262, the wild-type

binding sites (5'-UGAAUGU-3') in hsa_circ_0001550 were mutated to 5'-ACUACA-3'. The luciferase intensity of hsa_circ_0001550-WT vector was prominently decreased by miR-4262 mimic, while that of the hsa_circ_0001550-MUT vector had no significant change (Figure 3D,E), proving that hsa_circ_0001550 could sponge miR-4262. RNA pull-down assay displayed that hsa_circ_0001550 could be enriched by biotin-labeled miR-4262 probe (Figure 3F), which further demonstrated the binding relationship between hsa_circ_0001550 and miR-4262. Overall, miR-4262 was sponged by hsa_circ_0001550.

3.4 | Hsa_circ_0001550 absence inhibits CRC progression by partly up-regulating miR-4262

RT-qPCR validated the interference efficiency of anti-miR-4262 (Figure 4A,B). Anti-miR-4262 largely restored cell proliferation and metastasis in hsa_circ_0001550-silenced CRC cells (Figure 4C–H). FCM analysis suggested that hsa_circ_0001550 knockdown-induced

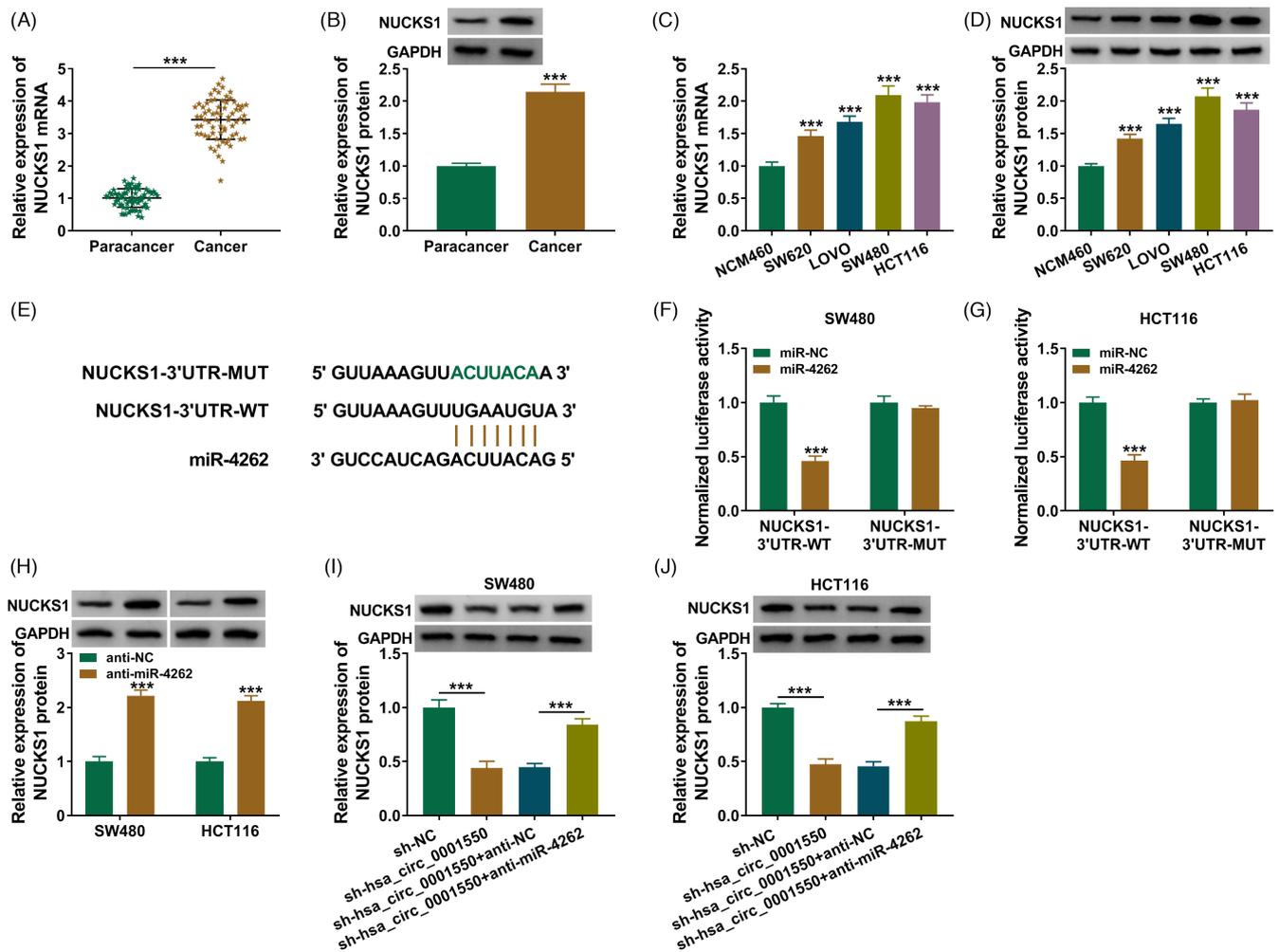


FIGURE 5 NUCKS1 is a downstream molecule of miR-4262. (A and B) NUCKS1 mRNA and protein enrichment was examined via RT-qPCR and western blot in CRC tissues and paracancer tissues. (C and D) RT-qPCR and western blot assay were conducted to detect NUCKS1 abundance in CRC cells and NCM460 cells. (E) The associated relation of miR-4262 and NUCKS1 was predicted via Starbase database. (F and G) The target relation of miR-4262 and NUCKS1 was validated via dual-luciferase reporter assay. (H) NUCKS1 enrichment was examined via western blot assay in CRC cells transfected with anti-NC or anti-miR-4262. (I and J) NUCKS1 protein enrichment was detected by western blot assay in CRC cells transfected with sh-hsa_circ_0001550 and anti-miR-4262. *** $p < 0.001$

apoptosis was attenuated by silencing miR-4262 (Figure 4I,J). CRC cell stemness regulated by sh-hsa_circ_0001550 was rescued by miR-4262 interference (Figure 4K,L). Hsa_circ_0001550 absence-caused effects on the abundances of Cyclin D1, MMP9 and Bax were all largely counteracted by miR-4262 interference (Figure 4M,N). Taken together, hsa_circ_0001550 depletion-caused suppressive influences on CRC cell phenotypes were partly dependent on miR-4262 up-regulation.

3.5 | NUCKS1 is a downstream target of miR-4262

Nuclear casein kinase and cyclin dependent kinase substrate 1 abundance was enhanced in CRC tissue specimens and cell lines (Figure 5A–D). With the help of Starbase bioinformatics database, we found that NUCKS1 3'UTR harbored the complementary sequence with miR-4262 (Figure 5E). The luciferase intensity was notably decreased in NUCKS1-3'UTR-WT reporter plasmid by miR-4262 mimic (Figure 5F,G),

confirming the interaction between miR-4262 and NUCKS1. The protein abundance of NUCKS1 was elevated in CRC cells by silencing miR-4262 (Figure 5H). Hsa_circ_0001550 depletion decreased NUCKS1 protein abundance, while anti-miR-4262 largely restored NUCKS1 protein abundance (Figure 5I,J). Taken together, hsa_circ_0001550/miR-4262 modulated NUCKS1 abundance in CRC cells.

3.6 | NUCKS1 overexpression largely restores the malignant phenotypes of hsa_circ_0001550-silenced CRC cells

High efficiency of NUCKS1 plasmid was validated (Figure 6A,B). The proliferation and metastasis were restored by NUCKS1 plasmid in hsa_circ_0001550-silenced CRC cells (Figure 6C–H). NUCKS1 overexpression suppressed hsa_circ_0001550 absence-induced apoptosis in CRC cells (Figure 6I,J). NUCKS1 plasmid also restored

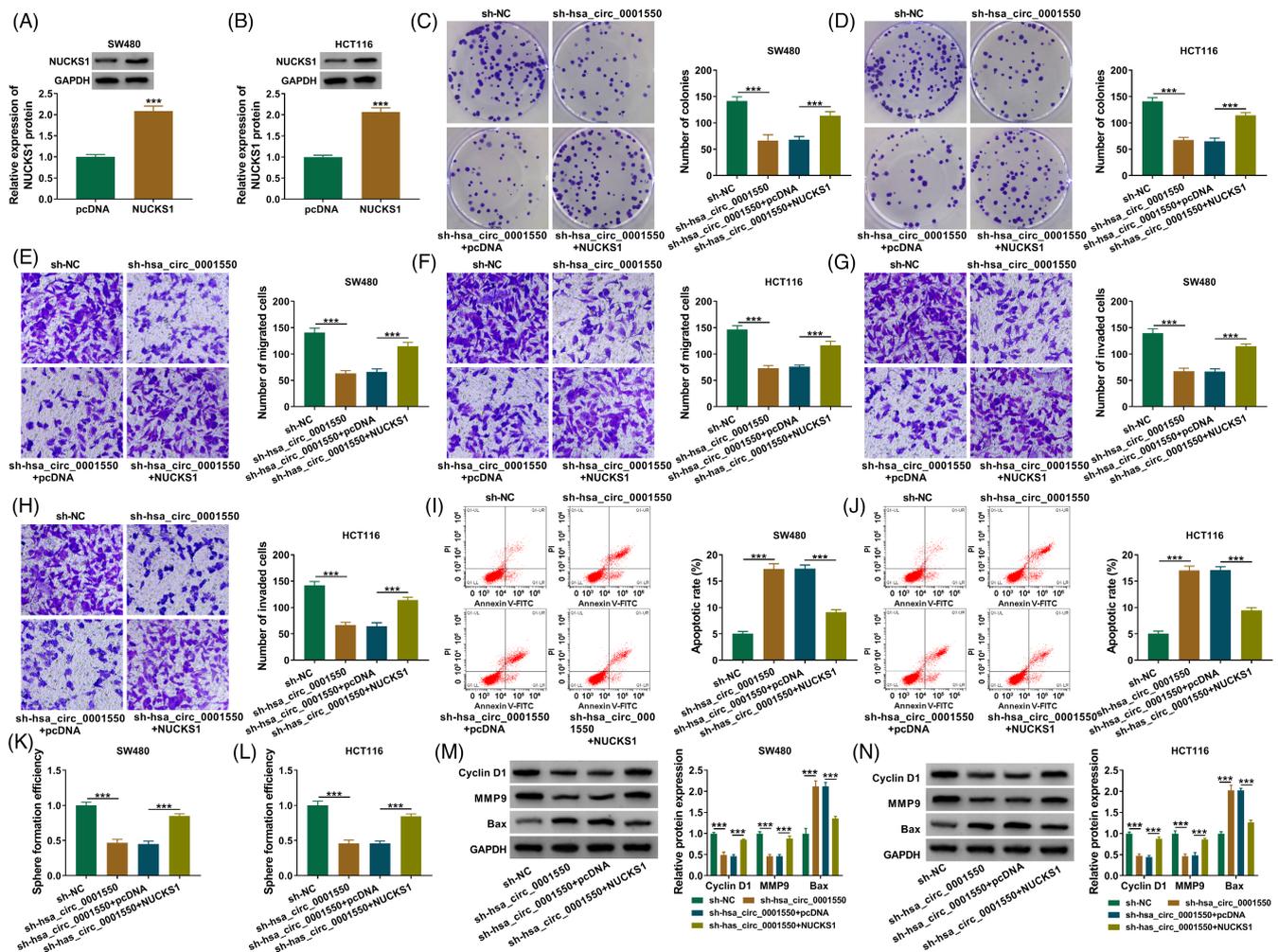


FIGURE 6 NUCKS1 overexpression largely restores the malignant phenotypes of hsa_circ_0001550-silenced CRC cells. (A and B) The overexpression ability of NUCKS1 plasmid was analyzed via western blot. (C–N) SW480 and HCT116 cells were introduced with sh-hsa_circ_0001550 alone or sh-hsa_circ_0001550+NUCKS1 along with their negative controls. (C and D) Cell proliferation was evaluated via colony formation assay. (E–H) Cell metastasis was determined via transwell assays. (I and J) FCM analysis was implemented to evaluate cell apoptotic rate. (K and L) Sphere formation assay was implemented to evaluate cell stemness. (M and N) The protein enrichment was examined via western blot. *** $p < 0.001$

cell sphere formation efficiency in hsa_circ_0001550-silenced CRC cells (Figure 6K,L). The protein abundance of Cyclin D1, MMP9, and Bax was rescued in sh-hsa_circ_0001550+NUCKS1 group (Figure 6M,N). Overall, these data showed that hsa_circ_0001550 depletion blocked CRC advancement partly by reducing the expression of NUCKS1.

3.7 | Hsa_circ_0001550 absence markedly suppresses xenograft tumor growth in vivo

The dimension and weight of xenograft tumors were decreased in hsa_circ_0001550-silenced group (Figure 7A,B). Furthermore, hsa_circ_0001550 and NUCKS1 abundance was reduced, while miR-4262 abundance was up-regulated in tumor tissues derived from sh-hsa_circ_0001550 group (Figure 7C,D). Hsa_circ_0001550 facilitated xenograft tumor growth in vivo.

4 | DISCUSSION

Plenty of evidence indicated that circRNAs can modulate CRC progression. Hsa_circ_0000218 has been reported to facilitate CRC development by absorbing miR-139-3p and enhancing RAB1A abundance.²² Hsa_circ_0007142 has been found to promote CRC advancement through mediating miR-122-5p/CDC25A signaling.²³ We focused on the function of a poorly studied circRNA, hsa_circ_0001550, in CRC. A previous study suggested that hsa_circ_0001550 abundance was enhanced in CRC tissue specimens.⁹ However, its function in CRC initiation and progression has never been elucidated. Consistently, hsa_circ_0001550 expression was increased in CRC tissues and cells. Hsa_circ_0001550 interference restrained CRC cell proliferation, metastasis, and stemness, while elevated cell apoptosis. More importantly, hsa_circ_0001550 interference notably blocked in vivo tumor growth. These results confirmed the oncogenic role of hsa_circ_0001550 in CRC.

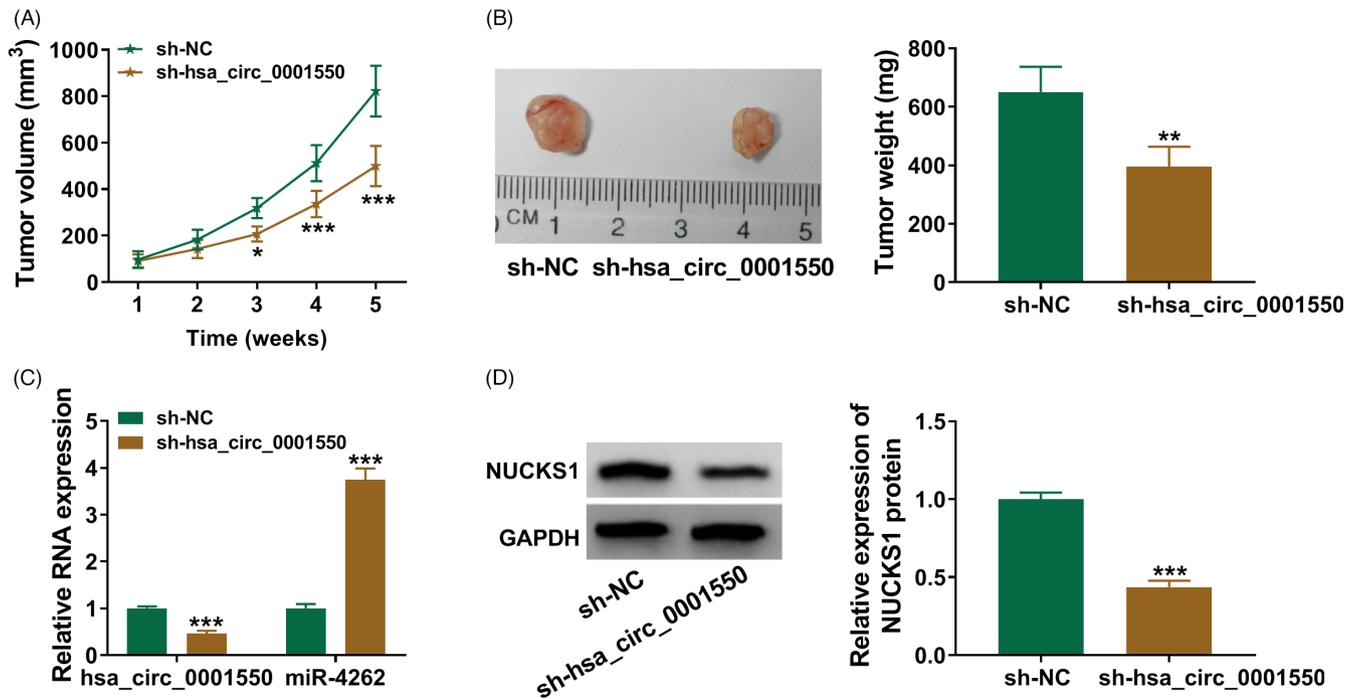


FIGURE 7 Hsa_circ_0001550 absence markedly suppresses xenograft tumor growth in vivo. (A) Tumor dimension was recorded as length \times width² \times 0.5. (B) After inoculation for 5 weeks, tumor weight was recorded. (C) RNA abundance was examined via RT-qPCR. (D) Western blot was implemented to determine NUCKS1 protein abundance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

CircRNAs can modulate gene expression by sequestering miRNAs.^{10,24} MiR-4262 was authenticated to be a downstream component of hsa_circ_0001550. MiR-4262 was validated as an anti-cancer molecule in several malignancies. MiR-4262 has been reported to suppress esophageal cancer cell proliferation and elevate cell apoptotic rate by reducing KLF6 abundance.²⁵ Besides, miR-4262 could restrain cell proliferation and invasion capacities in gastric cancer cells via suppressing proto-oncogene CD163.²⁶ Also, miR-4262 blocked cervical cancer cell proliferation and metastasis by modulating ZBTB33.²⁷ In CRC, circ-AGFG1 had been found to facilitate CRC advancement by enhancing YY1 abundance via sequestering miR-4262 and miR-185-5p,¹⁶ proving the anti-cancer activity of miR-4262 in CRC. To explore whether hsa_circ_0001550 regulated CRC progression by modulating miR-4262, the rescue assays were implemented. Hsa_circ_0001550 absence-caused inhibitory impacts on CRC cell biological behaviors were overturned by anti-miR-4262, demonstrating that hsa_circ_0001550 promoted CRC progression by sponging miR-4262.

To disclose the latent mechanism by which miR-4262 modulated CRC development, the downstream target of miR-4262 was predicted by bioinformatics database Starbase. NUCKS1 was authenticated as a target of miR-4262 in CRC cells. NUCKS1 has been identified as a proto-oncogene in many malignancies, including gastric cancer,²⁸ non-small cell lung cancer,²⁹ cervical squamous cell carcinoma,³⁰ and CRC.²¹ High NUCKS1 abundance was found to be prominently related to undesirable clinical outcomes of CRC cases.²¹ In this, NUCKS1 level was conspicuously increased in CRC. Additionally, it was observed that hsa_circ_0001550 positively modulated NUCKS1 enrichment by absorbing miR-4262. Subsequently, we implemented rescue assays

to explore whether hsa_circ_0001550 regulated CRC progression by mediating NUCKS1 expression. The results displayed that hsa_circ_0001550 knockdown-induced impacts were largely counteracted by NUCKS1 accumulation, indicating that hsa_circ_0001550 accelerated CRC progression by regulating NUCKS1 expression.

In summary, our study reveals a new regulatory axis that regulates the progression of CRC. We pointed out that hsa_circ_0001550 promoted CRC cell proliferation, metastasis, and stemness by regulating the miR-4262/NUCKS1 axis. Our research provides new ideas for targeted therapy of CRC.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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