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ORIGINAL ARTICLE

WILEY Cell Proliferation

Upregulated miR-1258 regulates cell cycle and inhibits cell proliferation by directly targeting E2F8 in CRC

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Abstract

Objectives: MicroRNAs (miRNAs) as small noncoding RNA molecules function by regulating their target genes negatively. MiR-1258 was widely researched in multi-cancers, but its role remains unclear in colorectal cancer (CRC).

Methods: The expression of miR-1258 and its specific target gene were detected in human CRC specimens and cell lines by miRNA RT-PCR, qRT-PCR and Western blot. The effects of miR-1258 on CRC proliferation were evaluated using CCK-8 assays, EdU incorporation, colony formation assays and cell-cycle assays; in vitro and the in vivo effects were investigated using a mouse tumorigenicity model. Luciferase reporter and RIP assays were employed to identify interactions between miR-1258 and its specific target gene.

Results: MiR-1258 was downregulated in CRC tissues and CRC cell lines, and upregulated miR-1258 was proved to inhibit proliferation and arrest cell cycle at G0/G1 in vitro and vivo. Luciferase reporter, RIP and western blot assays revealed E2F8 to be a direct target of miR-1258. The effects of miR-1258 in proliferation and cell cycle regulation can be abolished by E2F8 through rescue experiments. By directly targeting E2F8, miR-1258 influenced the expression of several cell-cycle factors, including cyclin D1 (CCND1) and cyclin dependent kinase inhibitor 1A (p21).

Conclusion: MiR-1258 may function as a suppressive factor by negatively controlling E2F8, thus, highlighting the potential role of miR-1258 as a therapeutic target for human CRC.

1 | INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide. Although researches have made great developments in diagnosis and treatment of CRC during these years, CRC still owes a high incidence and mortality, as estimated, CRC may kill 50 000 people per year in the United States and its 5-year survival rate is slightly greater than 10%.[1,2] One main reason for such poor survival of CRC is that the mechanism remains unclear and lacks efficient treatment. Hence, it is indispensable to reveal the molecular mechanism of CRC and find some potential therapy targets of CRC.

MicroRNAs (miRNAs) containing 20-24 nucleotides are kind of noncoding small RNA molecules which can bind to the 3′UTR of the target genes and further regulate the expression of messenger RNAs (mRNAs).[3-5] MiRNAs have been reported to play crucial roles in various fields of cellular, including proliferation, apoptosis, migration, invasion and differentiation. Dysregulated miRNAs can activate or silence some oncogenes or tumour

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suppressors and further cause the tumorigenicity in human. As reported, differentially expressed miRNAs have contributed a lot in diverse kinds of cancers.[6-8] Mechanism about the interactions between aberrantly expressed miRNAs and their targets mRNAs in cancer is under urgent priority to be revealed.

MiR-1258 which located on chromosome 2q31.3 was first shown to inhibit breast cancer brain metastasis by targeting heparanase.[9] Later, other studies demonstrated miR-1258 can function in non-small-cell lung cancer, liver cancer and gastric cancer[10-12]; however, the researches about miR-1258 in the field of CRC are still blank. In the present study, we tried to elucidate the roles of miR-1258 in CRC. We discovered that miR-1258 is downregulated in CRC, after experiments both in vivo and vitro, we found overexpressed miR-1258 can inhibit the proliferation and cause cell-cycle arrest at G0/G1 in CRC.

Through bioinformatic prediction and experimental confirmation, we identified E2F transcription factor 8 (E2F8) as the putative direct target of miR-1258. E2F8 is a family of transcription factors which regulate the expression of genes required for progression through the cell cycle.[13] Former studies focused on E2F8 and revealed lots of valuable findings, and Sun et al discovered that E2F8 promotes papillary thyroid cancer progression *via* regulating cell cycle.[14] Deng et al found that E2F8 may constitute a potential therapeutic target hepatocellular carcinoma.[15] However, mechanism of E2F8 in CRC has not been clarified. In our study, we indicated E2F8, which can be controlled by miR-1258 directly, was upregulated in CRC tissues and CRC cell lines and can abolish the effects caused by miR-1258 in inhibiting cell proliferation and regulating cell cycle.

In conclusion, we investigated the functions of miR-1258 and its correlations with E2F8 in CRC. Our findings demonstrated that miR-1258 downregulation has vital effects in regulating cell cycle and inhibiting proliferation of CRC, providing miR-1258 the role as a potential novel therapeutic target in CRC.

2 | MATERIALS AND METHODS

2.1 | Tissues specimens

We obtained 60 paired tumour and adjacent normal tissues from patients who were diagnosed with CRC and accepted the resection surgery between 2014 and 2015 at the Department of General Surgery, First Affiliated Hospital, Nanjing Medical University, China. The pathological diagnosis was made in the Department of Pathology in First Affiliated Hospital of Nanjing Medical University. Before the collection of specimens, we got the consent from the patients or their relatives. The study was approved by the Institutional Ethical Board of the First Affiliated Hospital of Nanjing Medical University. Samples were stored in −70°C conservation right after the resection and prior to the RNA extraction.

2.2 | CRC cell lines and cell culture

CRC cell lines including LoVo, HCT116, SW480, DLD-1, HT29 and normal epithelial colon cell NCM460 were purchased from American Type Culture Collection (ATCC, USA). The culture condition follows Dulbecco's modified Eagle's medium (DMEM; Winsent, Canada) supplemented with 10% foetal bovine serum (Wisent, Canada), 100 U/mL penicillin and 100 μg/mL streptomycin in a moist incubator (stabilize at 5% CO_2 and 37°C).

2.3 | Vector construction, lentivirus production and cell transfections

LV2-hsa-miR-1258-mimic vector (miR-1258-mimics) and the LV2-hsa-miR-1258-inhibitor vector (miR-1258-inhibitor) were constructed by lentiviral vectors (GenePharma, Shanghai, China). We also constructed a negative control with LV2 empty lentiviral. We infected DLD-1 and HCT116 grown to 40%-50% confluence by using lentiviral vectors at an appropriate multiplicity of infection (MOI). Stable transfected cells were screened by puromycin according to protocols. CRC cell lines were transfected with the E2F8 overexpressing recombinant vector pcDNA3.1-E2F8 (GenePharma, China) to overexpress E2F8 and empty plasmid as negative control (NC). The primer to overexpress E2F8 were designed as follows: 5′-CGG GATCCGAGGAATTTACAGAATGGAGAAC-3′ (forward); 5′-CCCG CTCTAGATTAATGGACATCCTCTGTTGAGACTTC-3′ (reverse). To knockdown E2F8, small interfering RNAs (siRNA) targeting the encoding region of E2F8 was purchased from GenePharma (Shanghai, China), and we used siRNA transfection reagent (Invitrogen) according to the protocols. Nontargeting control siRNA was used as negative control. The sequences used were siRNA for E2F8: 5′-GGCCA AAGACUGUAUACACTT-3′(sense), 5′-GUGUAUACAGUCUUUGGC CTT-3′(antisense); nontargeting control siRNA (NC): 5′-UUCUCCG AACGUGUCACGUTT-3′(sense), 5′-ACGUGACACGUUCGGAGAATT -3′(antisense).

2.4 | Quantitative real-time PCR (qRT-PCR) and miRNA RT-PCR

Following the manufacturer's instructions, total RNA was extracted from CRC tissues and cells with TRIzol reagent (Invitrogen, USA). For qRT-PCR of mRNA, total RNA was further reverse transcribed into cDNA through PrimeScript RT reagent kit (Takara, Dalian, China). For miRNA RT-PCR, we used Hairpin-it™ miRNA qPCR Quantitation Kit (GenePharma, China) to perform Target-specific reverse transcription and the TaqMan microRNA assay. The reactions were processed using a 7500 Realtime PCR System (Applied Biosystems, Carlsbad, CA, USA) with SYBR Premix Ex Taq Kit (TaKaRa). The specific primers of target mRNA/miRNA and internal control were designed as following: E2F8 forward, 5′-CCAACCCTGCTGTGAATA-3′ and E2F8 reverse 5′-TTTCTGGCTCATTACCCT-3′; β-actin forward, 5′-GCATCGTCACCAACTGGGAC-3′ and β-actin reverse, 5′-AC CTGGCCGTCAGGCAGCTC-3′, hsa-miR-1258 forward, 5′-AGTTAGG

ATTAGGTCGTGGAA-3′; Universal, 5′-GCGAGCA CAGAATTAAT ACGAC-3′; U6 forward, 5′-CTCGCTTC GGCAGCACA-3′ and U6 reverse, 5′-AACGCTTCACGAATTTGCGT-3′. Expression level was normalized to internal controls (β-actin or U6) and results were shown in form of relative expression calculated by $2^{-\Delta\Delta CT}$ method.

2.5 | Immunohistochemistry

All specimens were fixed in 4% formalin and then embedded in paraffin. Sections (thickness, 4 μm) were incubated overnight at 4°C in primary antibodies with endogenous peroxides and proteins blocked for specific detection of E2F8 or Ki-67 (Abcam). Sections washed by PBS were incubated with HRP-Polymer-conjugated secondary antibody at 37°C for 1 hour. Subsequently, we stained sections with 3,3-diaminobenzidine solution for 3 minutes and counterstained the nuclei were with haematoxylin. The tumour section was examined in a blinded manner. The percentage of positive tumours and the intensity of the cell staining were determined based on three randomly selected regions per section. Staining was scored according to intensity and percentage of positive cells. The staining intensity was scored according to 4 grades: 0 (No staining), 1 (weak staining), 2 (intermediate staining), or 3 (strong staining). The product (percentage of positive cells and respective intensity scores) was used as the final staining score (a minimum value of 0 and a maximum of 300).

2.6 | Cell proliferation assay

We used Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) to detect the cell proliferation according to the manufacturer's instructions; 2000 cells/well were seeded into 96-well plates and cultured as previously described; 10 uL CCK-8 solution mixed with serum-free medium were added every 24 hours. After 2-hour incubation, we used a microplate reader to detect the absorbency at a test wavelength of 450 nm and a reference wavelength of 630 nm.

2.7 | Colony formation assay

Under the culture condition described before, we seeded 500 cells/well in 6-well plate. After a week, each well was processed the following procedure: washed with phosphate buffer saline (PBS) for three times in room temperature, then fixed by ethyl alcohol for 30 seconds and finally stained with crystal violet. Colonies were counted by naked eye after rinsed by PBS.

2.8 | 5-Ethynyl-2′-deoxyuridine (EdU) assay

We measured cell proliferation using the EdU assay kit (RiboBio, China). Cells were seeded into 24-well plates (2×10^4 cells/well) and cultured with DMEM (10% FBS) for 24 hours before the addition of EdU (50 μmol/L). According to the protocols, cells were then incubated for 2 hours at 37°C, fixed in 4% formaldehyde for 30 minutes and permeabilized with 0.5% TritonX-100 for 10 minutes at room temperature. After washing with PBS, 1× ApolloR reaction cocktail (400 μL) was added to react with the EdU for 30 minutes. Subsequently, Hoechest33342 (400 μL) was added for 30 minutes to visualize the nuclei. Images of cells were obtained under a Nikon microscope (Nikon, Japan). Proliferation was analysed using the mean number cells in three fields for each sample.

2.9 | Cell-cycle analysis

Transfected cells were firstly digested by trypsin and then centrifuged at 300 ***g*** for 5 minutes. After washed by PBS twice, cells were fixed in 75% ethanol under −20°C overnight. Before detection, cells were washed twice by PBS, and then cells were fixed with 500 uL PI staining solution and incubated for 15 minutes in the dark at room temperature. We used a BD FACSCanto II (BD Biosciences, USA) flow cytometry with FlowJo software to analyse the cell cycle.

2.10 | Western blot

According to the manufacturer's instructions, we used a RIPA kit (Beyotime, Shanghai, China) to extract proteins from CRC cells and tissues. Protein was separated based on their molecular weight on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). We blocked the membranes with 5% nonfat powdered milk in Tris-buffered saline for 2 hours, and then membranes were incubated at 4°C overnight with specific primary antibodies. After rinsed in TBST for three times (10 minutes each time), membranes were incubated in secondary antibodies (Rabbit or Mouse) at room temperature for 2 hours and then were washed again (three times, 10 minutes each time). Protein expression levels were detected by ECL Plus (Millipore, Billerica, MA, USA) with a Bio-Imaging System. The primary antibodies follow: E2F8 (Abcam, 1:1000), CCND1, p21, p27, CDK2 and β-actin were used as an internal control.

2.11 | Luciferase report assay

Sequences corresponding to the 3′-UTR of E2F8 mRNA and containing the wild-type or mutated miR-1258 binding sequence were synthesized by GeneScript (Nanjing, China). We inserted these sequences into the XbaI and SacI site of pmirGLO dual-luciferase miRNA target expression vector (Promega, USA). They were co-transfected with vectors and miR-1258-mimics/miR-1258-inhibitor or miR-NC by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Luciferase activity was measured by Luciferase Reporter Assay System (Promega, USA).

2.12 | RNA immunoprecipitation assay

The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) was used to conduct RIP assays according to the manufacturer's instructions. Cells were collected and lysed by the NP-40 lysis buffer containing 1 mmol/L PMSF, 1 mmol/L DTT, 1%

Protease Inhibitor Cocktail (Sigma-Aldrich) and RNase inhibitor (Invitrogen). The cells were then incubated with RIP buffer containing magnetic beads bound with human anti-Argonaute2 (Ago2) antibody (Millipore) or normal mouse IgG (Millipore) as a negative control for 4 hours at 4°C. Precipitate was digested with Proteinase K buffer, and then co-immunoprecipitated RNA was isolated for qRT-PCR and miRNA RT-PCR analysis, respectively.

2.13 | Tumour xenograft in nude mouse model

This study was approved by Animal Ethics Committee of Nanjing Medical University (NJMU). Male BALB/c nude mice (aged 4 weeks) were purchased from the Animal Center of NJMU. 2×10^6 transfected cells were injected bilaterally and subcutaneously into the flanks of the nude mice. Tumours were measured by Vernier callipers every 5 days, and the mice were euthanized after 3 weeks. The formula used to calculate the tumour volume follows: volume = (width^2 × length)/2.

2.14 | Statistical analysis

All experiments in this research were performed in triplicate independently. All analysis was performed using SPSS 19.0 software. Chi-squared test was used to detect differences in the data of clinicopathological findings. Pearson correlation was used to analyse correlation of expression between miR-1258 and E2F8. The data were shown as mean ± standard deviation (SD). Student's unpaired *t* test was used to determine the significant differences of other results. The data were considered statistically significant; "*" indicates "$P < 0.05$," "**"indicates "$P < 0.01$," and "***" indicates "$P < 0.001$."

3 | RESULTS

3.1 | miR-1258 is downregulated in CRC tissues and CRC cell lines

In order to investigate the expression level of miR-1258 in CRC, we obtained 60 paired CRC tissues and adjacent normal tissues. By miRNA RT-PCR, we identified miR-1258 is downregulated in CRC tissues compared with adjacent normal tissues (Figure 1A). We also detected the expression of miR-1258 in CRC cell lines (LoVo, HCT116, SW480, DLD-1, HT29) as well as normal epithelial colon cell NCM460. As shown in Figure 1B, expression level of miR-1258 in CRC cell lines was lower than NCM460. To further research the correlation between miR-1258 expression level and clinical features (gender, age, stage, T grade, lymph node metastasis, distant metastasis, tumour size), patients were, respectively, allocated to High and Low expression group based on expression level of miR-1258 higher and lower than median. Table 1 demonstrated that expression of miR-1258 is negatively correlated with tumour size larger than 3 cm. Using Ki-67, a proliferation marker, we stained the High and Low expression groups to analyse the correlation between miR-1258 and proliferation. Figure 1C indicated that higher expression of miR-1258 owns a lower proliferation index.

3.2 | MiR-1258 inhibits the proliferation and arrests cell cycle at G0/G1 phase

We selected two CRC cell lines according to their expression pattern, HCT116 and DLD-1, which owned lowest and highest expression level of miR-1258 among these five CRC cell lines, demonstrating

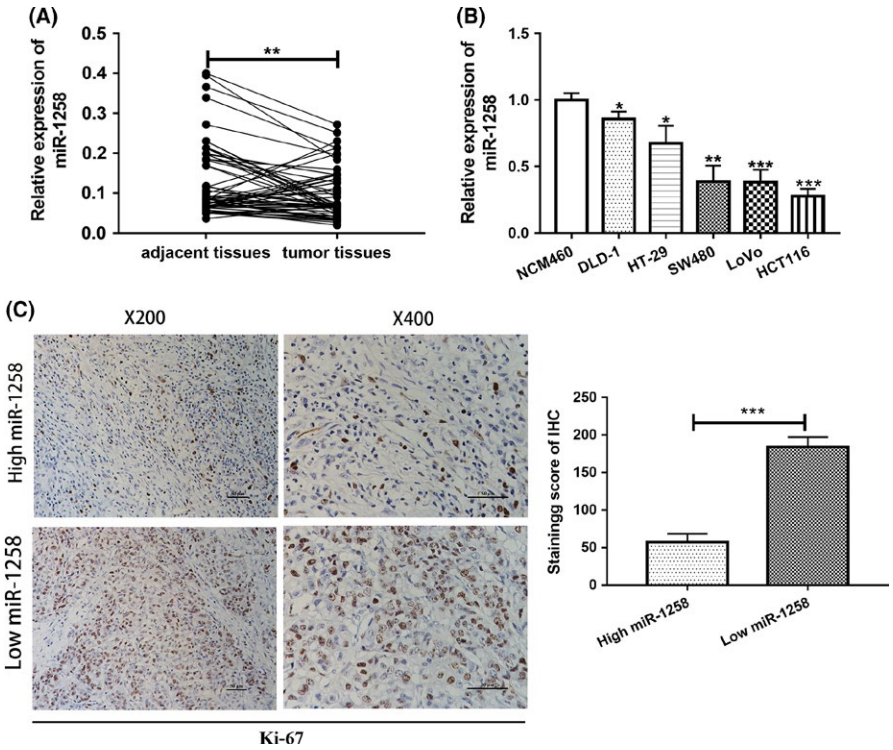

FIGURE 1 Expression of miR-1258 in CRC tissues and CRC cell lines. A, Expression of miR-1258 in 60 paired CRC tissues and adjacent normal tissues were detected by miRNA RT-PCR. B, Expression of miR-1258 in CRC cell lines and normal epithelial colon cell NCM460 were detected by miRNA RT-PCR. C, Immunohistochemical of CRC patient samples showed a negative correlation between the expression of miR-1258 and Ki-67. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

TABLE 1 Expression of miR-1258 and E2F8 according to patients' clinical features

Characteristics	Number	miR-1258 expression		*P* value	E2F8 expression		*P* value
		High group	Low group		High group	Low group	
Age (y)							
<50	9	4	5	0.72	6	3	0.28
>50	51	26	25		24	27	
Gender							
Female	32	15	17	0.60	16	16	1
Male	28	15	13		14	14	
Lymph node metastasis							
No	37	18	19	0.79	17	20	0.43
Yes	23	12	11		13	10	
Tumour stage							
Stage I, II	33	14	19	0.19	17	16	0.80
Stage III, IV	27	16	11		13	14	
TNM staging system							
T1 + T2	32	14	18	0.30	15	17	0.60
T3 + T4	28	16	12		15	13	
Distant metastasis							
No	52	25	27	0.45	28	24	0.13
Yes	8	5	3		2	6	
Tumour size (cm)							
<3	34	21	13	0.04[a]	12	22	0.01[a]
>3	26	9	17		18	8	

[a]$P < 0.05$.

better typicality and representation, to transfect the miR-1258-mimics or inhibitor lentivirus, respectively, and analyse the function of miR-1258 in CRC cells. MiRNA RT-PCR confirmed the transfection efficiency. The results were shown in Figure 2A, relative expression of miR-1258 in HCT116-micmcis and DLD-1-inhibitor was significant higher and lower, respectively, than the control group. We carried out CCK-8, colony formation and EdU assay to evaluate the influence of miR-1258 in proliferation. As shown in Figure 2B, results of CCK-8 showed that upregulated miR-1258 inhibited the proliferation of HCT116 while downregulated miR-1258 tends out the opposite effects in DLD-1. As the same, upregulated miR-1258 decreased the ability of colony formation in HCT116; in contrast, downregulated miR-1258 promoted the colony formation in DLD-1 (Figure 2C). Results of EdU were shown in Figure 2D, the number of HCT116 cells incorporating EdU in the miR-1258-mimics-treated group was distinctly decreased compared with the number in the control group, while DLD-1 cells transfected with the miR-1258-inhibitor revealed a significant increase in cell proliferation compared with that of the control group. We used flow cytometry to analyse the effects of dysregulated miR-1258 in cell cycle, results were shown in Figure 2E, overexpressed miR-1258 in HCT116 presented a significant increase in the percentage of cells in the G0/G1 phase while downregulated miR-1258 in DLD-1 indicated cell cycle was arrest in S phase. These findings demonstrated that overexpressed miR-1258 inhibited CRC cell proliferation and induced cell-cycle arrest in G0/G1 phase.

3.3 | MiR-1258 suppresses E2F8 expression *via* interacting directly with a hypothetic binding site of E2F8-3′-UTR

To predict the potential target of miR-1258, we used bioinformatics tools in public database including TargetScan and mirTarBase. A putative binding site of E2F8-3′-UTR for miR-1258 was identified. We further utilized dual luciferase reporter assays to verify our prediction. Mutant-type (MUT) and wild-type (WT) E2F8-3′-UTR sequences (the former containing site-directed mutations in the putative miR-1258 target sites) were cloned into reporter plasmids. Luciferase activity significantly decreased in HCT116 and DLD-1 cells co-transfected with miR-1258-mimics and the pmirGLO-WT-E2F8-3′-UTR. In contrast, luciferase activity increased in HCT116 and DLD-1 cells co-transfected with miR-1258-inhibitor and the pmirGLO-WT-E2F8-3′-UTR. However, no significant reduction was observed about luciferase activity in group co-transfected with pmirGLO-MUT-E2F8-3′-UTR and miR-1258-mimics/miR-1258-inhibitor (Figure 3A). We further performed RIP to explore the binding status between E2F8 and miR-1258 in CRC cell lines. Results showed that miR-1258 and E2F8 were both enriched in

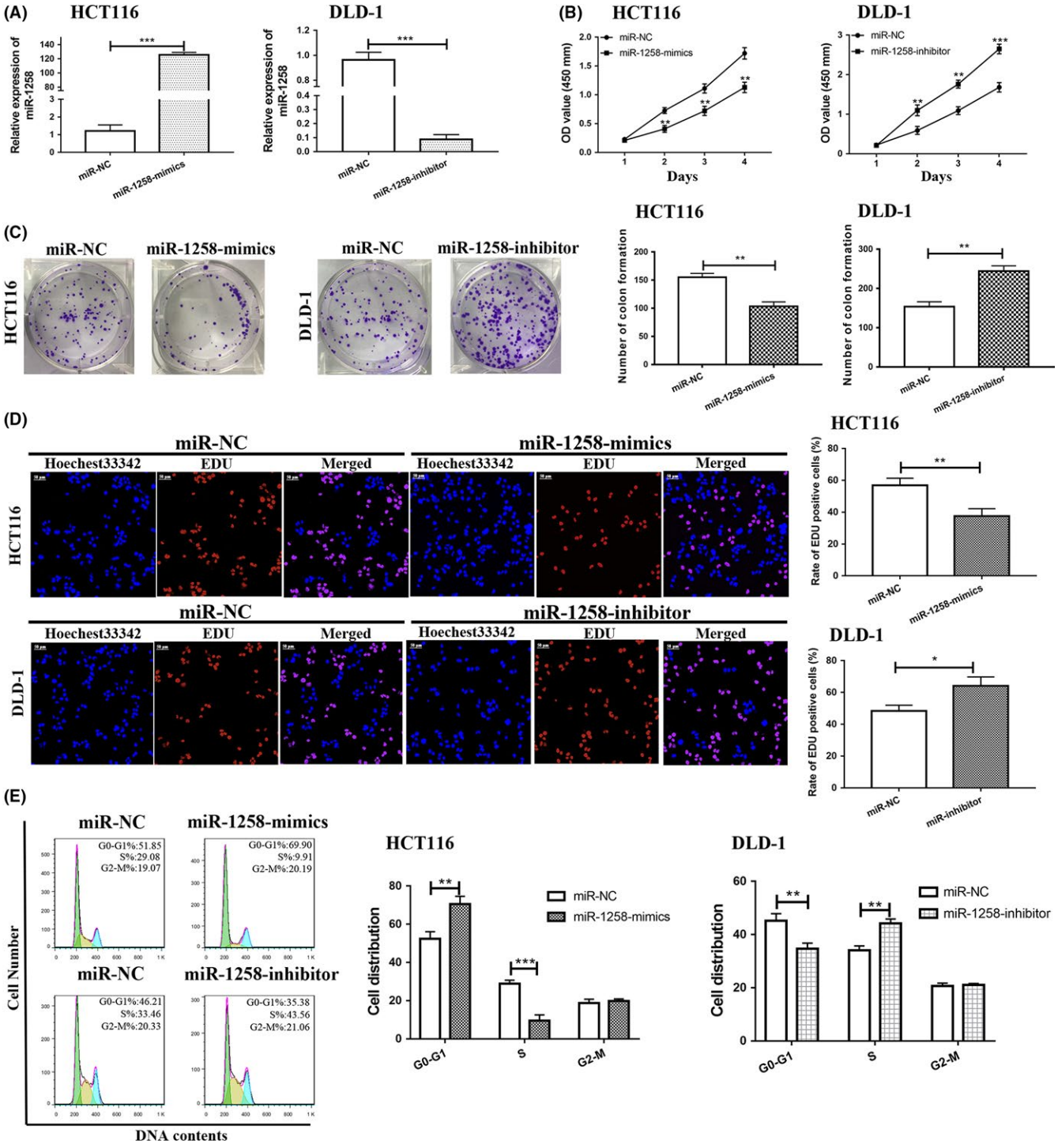

FIGURE 2 MiR-1258 inhibits proliferation and arrests cell cycle at G0/G1 in CRC cell lines. A, MiRNA RT-PCR was used to confirm the expression of miR-1258 in cells transfected with miR-1258-mimics and miR-1258-inhibitor lentivirus in turn. (B, C, D) Effects of miR-1258 on proliferation in CRC cell lines were detected by CCK-8, colony formation and EdU. E, Effects of miR-1258 on regulating cell cycle in CRC cell lines. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Ago2-coating beads relative to IgG control group. In HCT116 and DLD-1 transfected with miR-1258-mimics, we identified E2F8 is upregulated compared with miR-NC treated group through RIP assay. The reverse changes of E2F8 were also observed in HCT116 and DLD-1 transfected with miR-1258-inhibitor (Figure 3B). Expression of E2F8 and its correlation with miR-1258 were also detected. In 60 paired CRC tissues and adjacent normal tissues, expression level of E2F8 was upregulated in CRC tissues compared with adjacent normal tissues and negatively associated with miR-1258 according to qRT-PCR (Figure 4A). We used western blot and IHC to detect expression of E2F8 in 6 paired CRC tissues and adjacent normal tissues (Figure 4B). We also research the

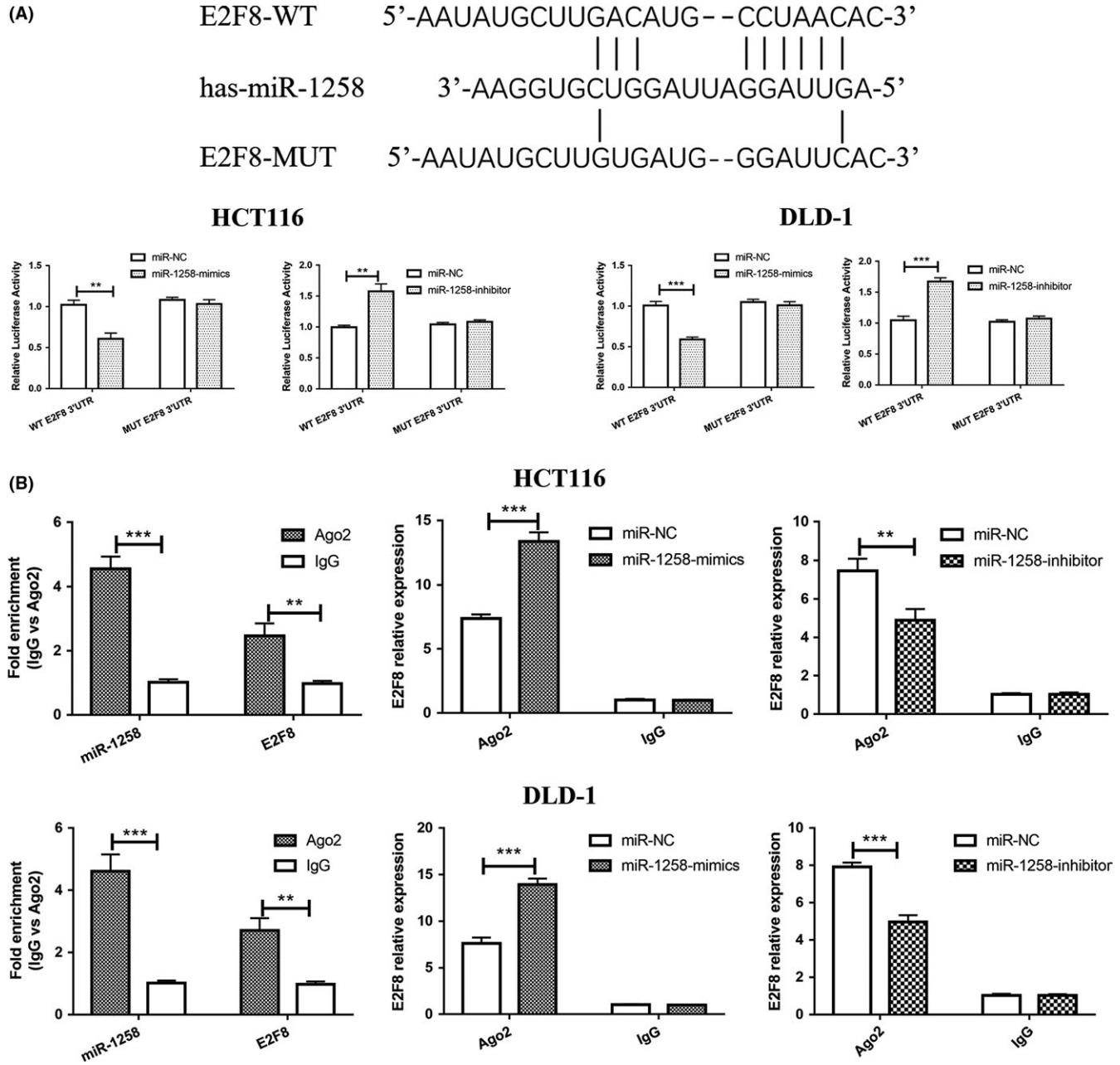

FIGURE 3 E2F8 was proved to be a potential target of miR-1258. A, Luciferase reporter assay was conducted to verify that miR-1258 bound to the 3′-UTR region of E2F8 directly. Luciferase activity was analysed in cells co-transfected with miR-1258-mimics or negative control with pGL3-E2F8-WT or pGL3-E2F8-MUT. B, RIP assays confirmed the binding status between miR-1258 and E2F8 in untreated and treated CRC cell lines, respectively. (**$P < 0.01$, ***$P < 0.001$)

correlation between expression pattern and clinical features (gender, age, stage, T grade, lymph node metastasis, distant metastasis, tumour size), and we found that high expression level of E2F8 was associated with bigger tumour size (Table 1). In CRC cell lines, E2F8 was upregulated in CRC cell lines compared with NCM460 (normal epithelial colon cell) (Figure 4C). In transfected CRC cells, we discovered that expression level of E2F8 is lower in HCT116-miR-1258-mimics and higher in DLD-1-miR-1258-inhibitor compared with the negative control in tools of qRT-PCR and western blot (Figure 4D). These findings demonstrated that miR-1258 can directly negatively control E2F8.

3.4 | MiR-1258 inhibits proliferation and regulates cell cycle by targeting E2F8

To further investigate the influence of interaction between miR-1258 and E2F8 in CRC, we conducted the rescue experiments. In our rescue experiments, we designed two comparisons, the first comparison: HCT116 cells co-transfected with miR-1258-mimics and NC vs. HCT116 cells co-transfected with miR-1258-mimics and E2F8, the second comparison: DLD-1 cells co-transfected with miR-1258-inhibitor and si-control vs. DLD-1 cells co-transfected with miR-1258-inhibitor and si-E2F8. In HCT116 and DLD-1 cells,

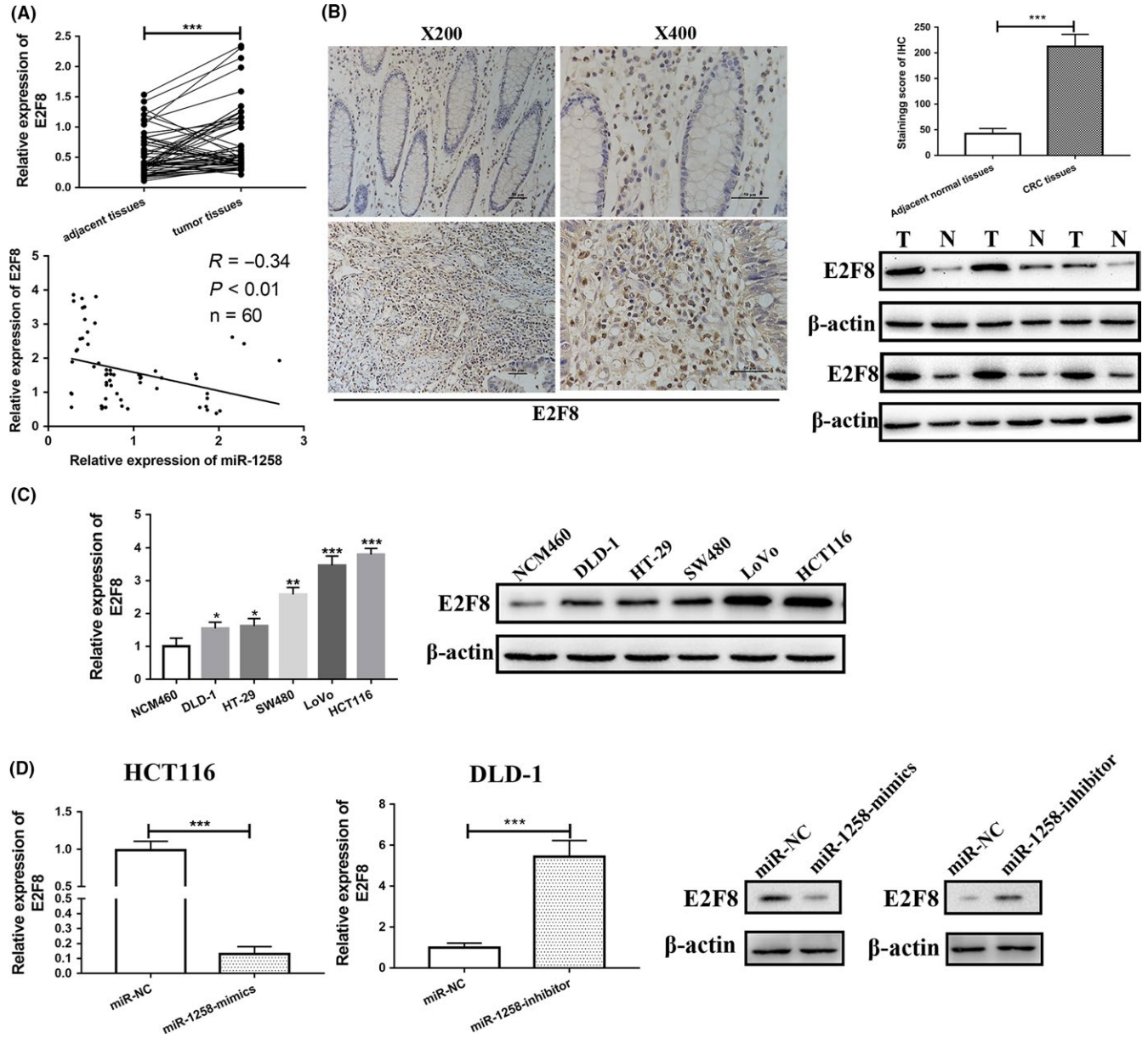

FIGURE 4 Expression pattern of E2F8 in CRC. A, Expression of E2F8 was upregulated in 60 paired CRC tissues compared with adjacent normal tissues and was negatively correlated with miR-1258. B, Immunohistochemistry showed the expression level of miR-1258 in CRC tissues and paired adjacent normal tissues, western blot showed the expression level of miR-1258 in 6 paired CRC tissues and adjacent normal tissues. C, Expression of E2F8 in CRC cell lines and normal epithelial colon cell NCM460 were detected by qRT-PCR and western blot. D, Expression of E2F8 in CRC cell lines transfected with relevant lentivirus was detected by qRT-PCR and western blot. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

we used pcDNA3.1-E2F8 and siRNA-E2F8 to upregulate or downregulate E2F8, respectively. Expression was detected both in qRT-PCR and western blot (Figure 5A). Compared with upregulated miR-1258 HCT116 cells, expression level of E2F8 was higher in cells co-upregulated E2F8 and miR-1258. In DLD-1, co-downregulated E2F8 and miR-1258 cells own a lower expression of E2F8 compared with downregulated miR-1258 group (Figure 5B). Subsequently, the colony formation assay, EdU assay and CCK-8 assay were applied. As shown in Figure 5C-E, upregulated E2F8 abolished the effect of upregulated miR-1258 in suppressing HCT116 proliferation. Similarly, effect of promoting DLD-1 proliferation made by downregulated miR-1258 can be counteracted by downregulated E2F8. We also used flow cytometric to explore whether the effects of miR-1258 on cell cycle were mediated by the regulation of E2F8. It turned out that upregulated E2F8 can abolish cell-cycle arrest caused by upregulated miR-1258 in HCT116 cells. Likewise, downregulated E2F8 can cause cell-cycle arrest in the G0/G1 phase in downregulated miR-1258 DLD-1 cells (Figure 5F). These findings suggested that miR-1258 inhibits proliferation and regulates cell cycle by directly targeting E2F8.

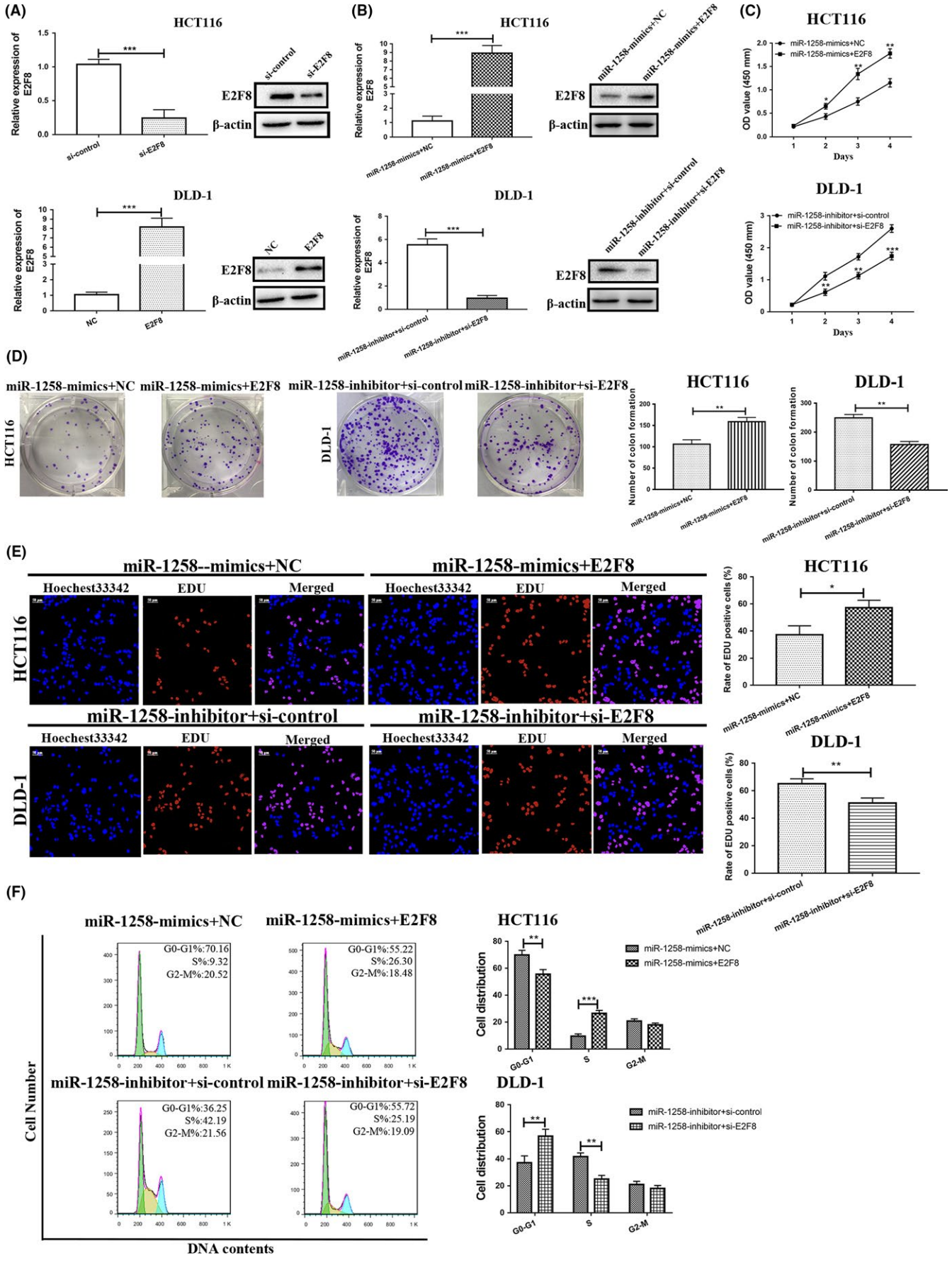
(A)
HCT116
Relative expression of E2F8
si-control
si-E2F8
E2F8
β-actin
DLD-1
NC
E2F8
(B)
HCT116
miR-1258-mimics+NC
miR-1258-mimics+E2F8
DLD-1
miR-1258-inhibitor+si-control
miR-1258-inhibitor+si-E2F8
(C)
HCT116
OD value (450 mm)
Days
DLD-1
(D)
miR-1258-mimics+NC
miR-1258-mimics+E2F8
miR-1258-inhibitor+si-control
miR-1258-inhibitor+si-E2F8
Number of colon formation
(E)
Hoechest33342
EDU
Merged
Rate of EDU positive cells (%)
(F)
G0-G1%:70.16
S%:9.32
G2-M%:20.52
G0-G1%:55.22
S%:26.30
G2-M%:18.48
G0-G1%:36.25
S%:42.19
G2-M%:21.56
G0-G1%:55.72
S%:25.19
G2-M%:19.09
Cell Number
DNA contents
Cell distribution
G0-G1
S
G2-M

FIGURE 5 MiR-1258 inhibits proliferation and regulates cell cycle by targeting E2F8. A, Expression of E2F8 was verified in transfected CRC cell lines by qRT-PCR and western blot. B, Expression of E2F8 was confirmed by qRT-PCR and western blot in co-transfected CRC cell lines. (C, D, E) CCK-8, colony formation and EdU showed the situations of proliferation in co-transfected and transfected CRC cell lines. F, Changes of cell cycle in co-transfected CRC cell lines. (*P < 0.05, **P < 0.01, ***P < 0.001)

3.5 | MiR-1258 influences CCND1/p21 through E2F8

Previous studies have revealed that E2F8 can influence the cell cycle by interacting with some key factors.[14,15] In our study, as described before, miR-1258 can function by directly targeting E2F8, we wonder whether miR-1258 could influence any key factors in cell cycle in CRC, we detected the expression of some key factors in cell cycle by qRT-PCR and western blot, including CCND1, p27, p21 and CDK2. As shown in Figure 6A, B, in HCT116-miR-1258-mimics group, E2F8 and CCND1 were decreased while p21 was increased, and no significant changes were observed in p27 and CDK2 compared with the control group. In contrast, the opposite results were discovered in DLD-1-miR-1258-inhibitor group. We further used rescue experiments to find if the effects on influencing CCND1/p21 caused by miR-1258 can be partially counteracted by E2F8, and the results confirmed our hypothesis (Figure 6C, D). Taken together, we determined that miR-1258 can regulate CCND1/p21 through regulating E2F8.

3.6 | MiR-1258 inhibits tumorigenicity in vivo

To reveal the effects of miR-1258 on tumorigenicity in vivo, HCT116 and DLD-1 cells transfected with vectors above were injected into the flanks of nude mice to generate tumours ectopically. As shown in Figure 7A, in HCT116 group, cells transfected with miR-1258-mimics generated smaller and lighter tumours, while it showed the inverse results in DLD-1 group. We used miRNA RT-PCR to confirm the expression of miR-1258 in mice tumour tissues, HCT116 group transfected with miR-1258-mimics owned a higher expression level, while DLD-1 group transfected with miR-1258-inhibitor showed a lower expression (Figure 7B). We used qRT-PCR, western blot and immunohistochemical to detect the expression of E2F8 in mice tumours, E2F8 was downregulated in HCT116 group transfected with miR-1258-mimics and upregulated in DLD-1 group transfected with miR-1258-inhibitor (Figure 7C, D). We also stained mice tumours with Ki-67, and results indicated that HCT116 group transfected with miR-1258-mimics showed a lower proliferation index and DLD-1 group presented the inverse trend (Figure 7E). These findings demonstrated that miR-1258 can inhibit the tumorigenicity in vivo.

4 | DISCUSSION

CRC has a high incidence and mortality rate worldwide; however, we do not have efficient ways to cure this disease due to the mechanism of CRC is still unclear. Many researches before demonstrated that miRNA can play vital role in the process of cancers.[16,17] It was shown that miRNA can function as either oncogenes or tumour suppressors by directly targeting mRNAs. MiRNA can act as a tumour suppressor by downregulating oncogene translation and further inhibit the progression and development of tumour. Similarly, increased miRNA which can function as oncogene can promote the tumorigenicity by influencing its relevant target.[18] Most recent researches discovered that miRNAs can interact with each other and further affect the target mRNA to contribute to the progression and developments in cancer. Zen et al provided the first evidence that one miRNA can control the biogenesis of other miRNAs by directly targeting their primary transcripts in the nucleus.[19] His team also found that miR-122 can directly regulate the biogenesis of cell survival oncomiR miR-21 at the posttranscriptional level.[20] These findings all proved that miRNAs have critical effects in tumorigenicity and its urgent for us to reveal the mechanism of miRNA in process of CRC.

MiR-1258 was identified as a tumour suppressor in multicancers, including breast cancer, liver cancer, non-small-cell lung cancer and gastric cancer[9-12]; however, the expression pattern and its role in CRC remains unclear and urgent to be elucidated. It is also necessary to research the potential mechanism of miR-1258 in CRC. In the present study, we discovered that miR-1258 is downregulated in tumour tissues and CRC cell lines compared with adjacent normal tissues and normal epithelial colon cell NCM460. Stepwise investigation revealed the function of miR-1258 in proliferation and cell cycle both in vivo and vitro. The results showed that upregulated miR-1258 can inhibit proliferation both in vivo and vitro and cause cell-cycle arrest in G0/G1 phase. In contrast, the cell arrest in S phase and other reverse effects can be observed in downregulated miR-1258 groups. These findings demonstrated that upregulated miR-1258 can serve as a tumour suppressor in the process of CRC.

To further research the mechanism of miR-1258 in CRC, we used bioinformatics tools to predict the putative targets of miR-1258. Among these candidate target mRNAs, we chose to investigate E2F8. E2F8 which located on chromosome 11p15 encodes a member of a family of transcription factors which regulate the genes required for developments through the cell cycle. E2F8 can regulate progression from G1 to S phase by ensuring the nucleus divides at the proper time.[13] E2F8 was widely researched, and previous studies identified its crucial role in many biological processes. Among these biological processes, E2F8 functions most significantly in regulating cell cycle compared with other biological processes.[13] Moreover, E2F8 was also widely researched in different kinds of cancers, and its mechanism was often defined as oncogene by influencing the cell cycle. Jin et al discovered that by suppressing E2F8, metformin can induce cell-cycle arrest at G1 phase in lung cancer cells.[21] Sun et al found E2F8 can promote the papillary thyroid cancer progression *via* regulating cell cycle.[14] Ye

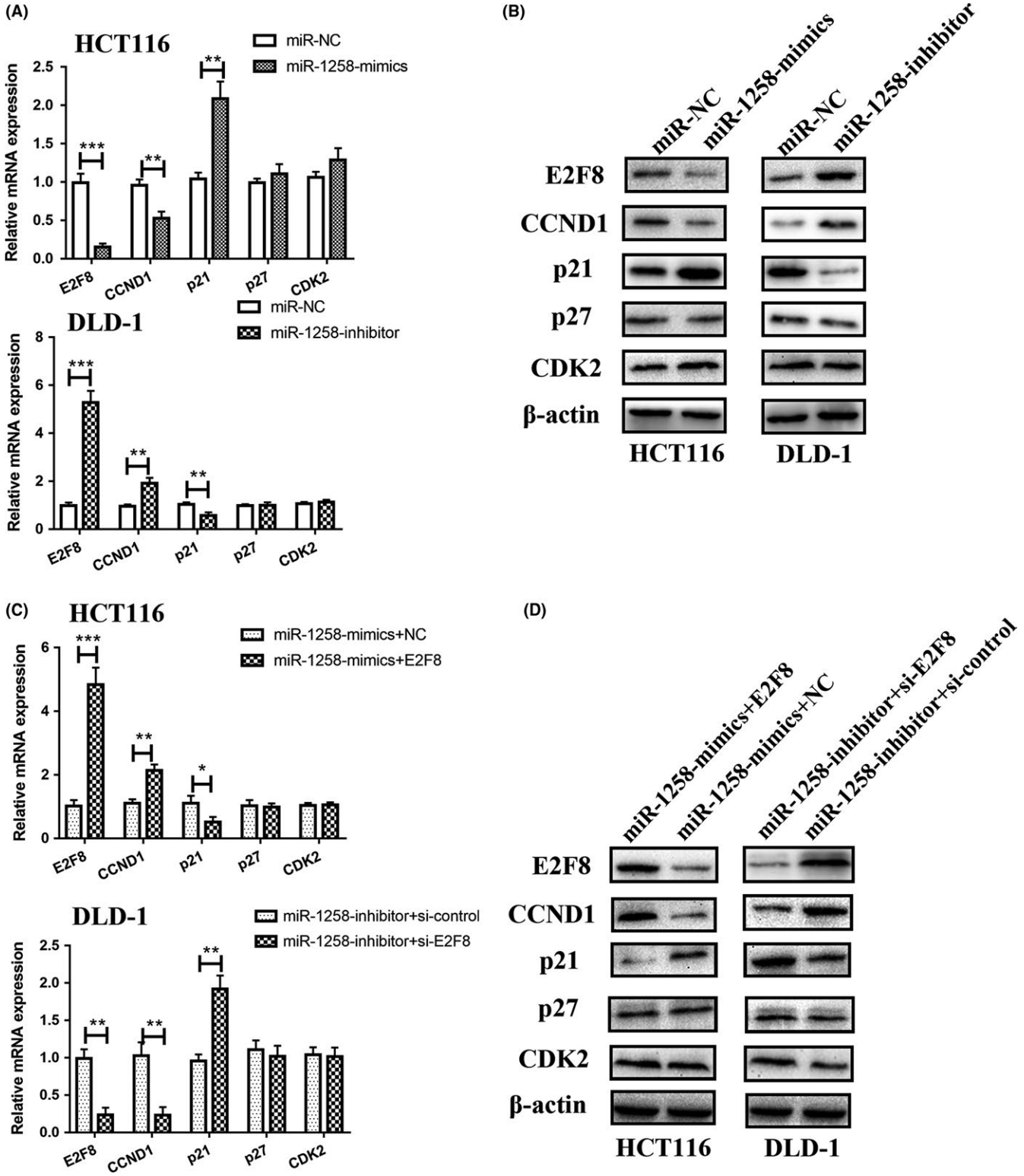

FIGURE 6 Expression of some key factors of cell cycle in transfected cells were detected by qRT-PCR and western blot. (A, B) Expression of mRNA in HCT116 cells transfected with miR-1258-mimics or miR-NC and DLD-1 cell transfected with miR-1258-inhibitor or miR-NC was detected by qRT-PCR and western blot. (C, D) Expression of mRNA in co-transfected and transfected CRC cells was detected by qRT-PCR and western blot. (**P < 0.01, ***P < 0.001)

et al indicated that upregulated E2F8 modulate G1/S phase transition and further promote the cell proliferation in breast cancer.[22] However, the researches about E2F8 in CRC remain rare and its mechanism needs to be clarified. In our study, we identified differential expression of E2F8 between CRC tissues and adjacent normal tissues. Expression pattern of E2F8 in CRC cell lines compared

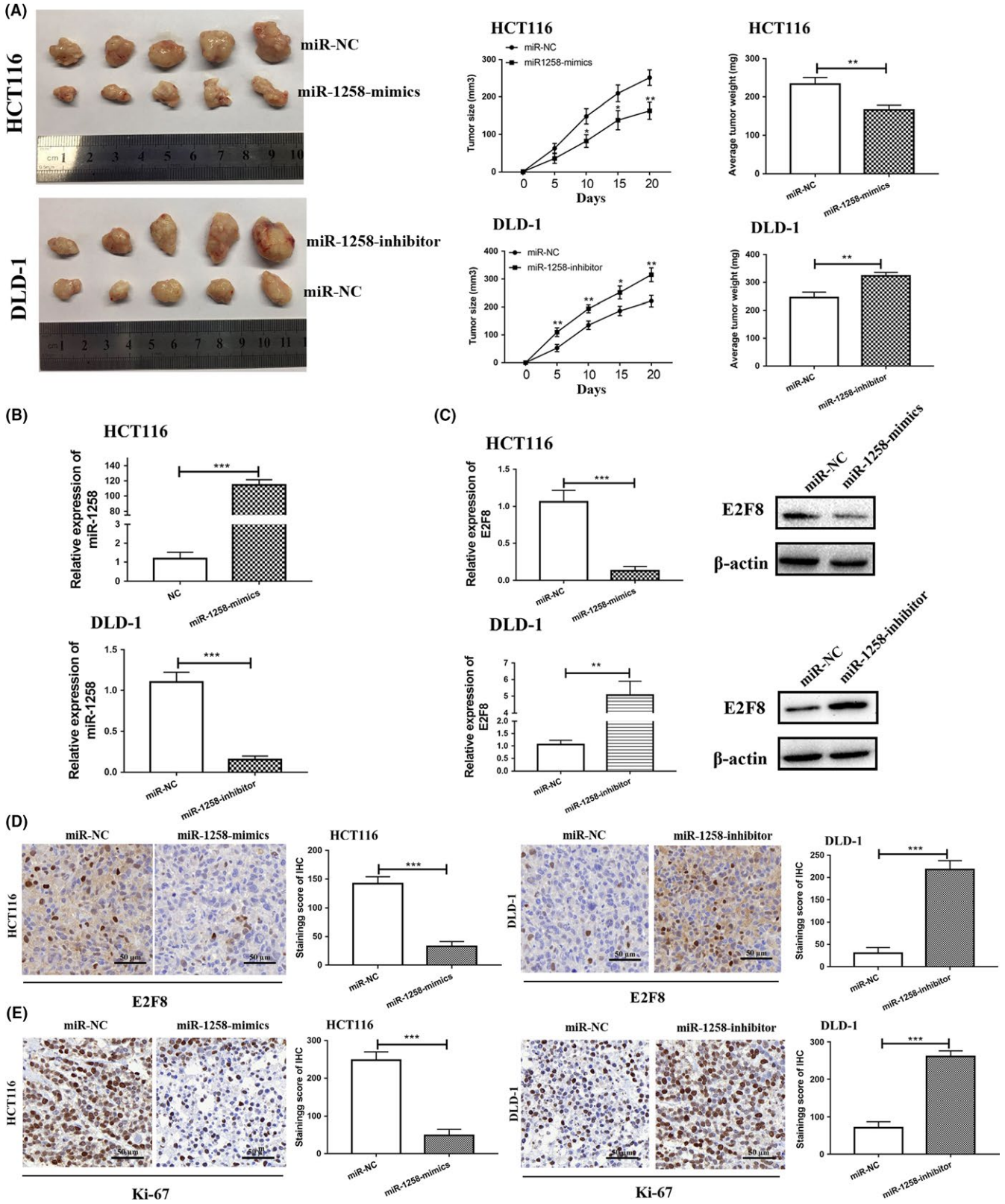

FIGURE 7 MiR-1258 inhibits tumorigenicity in vivo. A, Photographs of tumours obtained from the different groups of nude mice transfected with miR-1258-mimics, miR-1258-inhibitor and miR-NC. Tumours were observed by tumour size and average weight. B, Expression of miR-1258 was detected by miRNA RT-PCR (C, D) Expression of E2F8 was detected by qRT-PCR, western blot and immunohistochemical, respectively. E, Immunohistochemical of tumours showed a negative correlation between expression of miR-1258 and Ki-67. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

to epithelial colon cell NCM460 was consistent with that in tissues. E2F8 expression was also found to be associated with tumour size exceeding 3 cm in CRC patients. We further used luciferase reporter assays and RIP assays to confirm that miR-1258 negatively regulated E2F8 at the translational level by binding to a specific target site within the 3′-UTR. Upregulated miR-1258 in CRC cell lines can degrade E2F8 mRNA and ectopic expression of E2F8 significantly abolished the effects of the suppression of proliferation and cell-cycle arrest in G0/G1 phase caused by upregulated miR-1258. Similarly, transfection of si-E2F8 reversed the promotion of proliferation and cell-cycle arrest in S phase caused by miR-1258 downregulation. In summary, miR-1258 affects the tumorigenicity by directly targeting E2F8 and E2F8 can abolish the effects that miR-1258 caused.

Sustaining proliferation is considered to be a remarkable sign of cancer.[23] Generally, cell cycle is dysregulated in cancer cells, thereby breaking homeostasis of cell number and causing uncontrolled cell proliferation.[24] As former studies described, E2F8 can be a factor in regulating cell cycle; in our study, we revealed that dysregulated miR-1258 can play its role by directly targeting E2F8. By western blot, we found that miR-1258 can influence the p21 which can be a universal inhibitor of cyclin kinases[25] and CCND1 which was identified as a key factor in regulating cell cycle.[26] In miR-1258-mimics group, we detected downregulated E2F8, CCND1 and upregulated p21, and the reverse results were also observed in miR-1258-inhibitor group. Through rescue experiments, we found that E2F8 can partially reverse the expression changes of CCND1 and p21 caused by dysregulated miR-1258, indicating that miR-1258 may regulate cell cycle *via* CCND1/p21 through negatively controlling E2F8. However, the detailed mechanism of miR-1258 in regulating cell cycle still needs further investigation.

In conclusion, our study indicated that miR-1258 can suppress the progression and developments of CRC through influencing cell cycle by targeting E2F8 directly and provided miR-1258 the role as a novel therapeutic target for CRC.

5 | CONCLUSIONS

Taken together, miR-1258 was downregulated in CRC and E2F8 was upregulated. Upregulated miR-1258 can inhibit cell proliferation and regulate cell cycle by directly negatively controlling E2F8, providing a novel insight of miR-1258 as a therapeutic target for human CRC.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin*. 2015;65:87-108.
2. Brody H. Colorectal cancer. *Nature*. 2015;521:S1.
3. Bahubeshi A, Tischkowitz M, Foulkes WD. miRNA processing and human cancer: DICER1 cuts the mustard. *Sci Transl Med*. 2011;3:111 ps46.
4. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. 1993;75:843-854.
5. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell*. 1993;75:855-862.
6. Wang X, Yin H, Zhang H, et al. NF-kappaB-driven improvement of EHD1 contributes to erlotinib resistance in EGFR-mutant lung cancers. *Cell Death Dis*. 2018;9:418.
7. Ye YY, Mei JW, Xiang SS, et al. MicroRNA-30a-5p inhibits gallbladder cancer cell proliferation, migration and metastasis by targeting E2F7. *Cell Death Dis*. 2018;9:410.
8. Fang F, Huang B, Sun S, et al. miR-27a inhibits cervical adenocarcinoma progression by downregulating the TGF-betaRI signaling pathway. *Cell Death Dis*. 2018;9:395.
9. Zhang L, Sullivan PS, Goodman JC, Gunaratne PH, Marchetti D. MicroRNA-1258 suppresses breast cancer brain metastasis by targeting heparanase. *Can Res*. 2011;71:645-654.
10. Liu H, Chen X, Gao W, Jiang G. The expression of heparanase and microRNA-1258 in human non-small cell lung cancer. *Tumour Biol*. 2012;33:1327-1334.
11. Shi J, Chen P, Sun J, et al. MicroRNA-1258: an invasion and metastasis regulator that targets heparanase in gastric cancer. *Oncol Lett*. 2017;13:3739-3745.
12. Hu M, Wang M, Lu H, et al. Loss of miR-1258 contributes to carcinogenesis and progression of liver cancer through targeting CDC28 protein kinase regulatory subunit 1B. *Oncotarget*. 2016;7:43419-43431.
13. Christensen J, Cloos P, Toftegaard U, et al. Characterization of E2F8, a novel E2F-like cell-cycle regulated repressor of E2F-activated transcription. *Nucleic Acids Res*. 2005;33:5458-5470.
14. Sun J, Shi R, Zhao S, et al. E2F8, a direct target of miR-144, promotes papillary thyroid cancer progression via regulating cell cycle. *J Exp Clin Cancer Res*. 2017;36:40.
15. Deng Q, Wang Q, Zong WY, et al. E2F8 contributes to human hepatocellular carcinoma via regulating cell proliferation. *Can Res*. 2010;70:782-791.
16. Li J, Tan S, Kooger R, Zhang C, Zhang Y. MicroRNAs as novel biological targets for detection and regulation. *Chem Soc Rev*. 2014;43:506-517.
17. Paladini L, Fabris L, Bottai G, Raschioni C, Calin GA, Santarpia L. Targeting microRNAs as key modulators of tumor immune response. *J Exp Clin Cancer Res*. 2016;35:103.
18. Shenouda SK, Alahari SK. MicroRNA function in cancer: oncogene or a tumor suppressor? *Cancer Metastasis Rev*. 2009;28:369-378.
19. Tang R, Li L, Zhu D, et al. Mouse miRNA-709 directly regulates miRNA-15a/16-1 biogenesis at the posttranscriptional level in the nucleus: evidence for a microRNA hierarchy system. *Cell Res*. 2012;22:504-515.

20. Wang D, Sun X, Wei Y, et al. Nuclear miR-122 directly regulates the biogenesis of cell survival oncomiR miR-21 at the posttranscriptional level. *Nucleic Acids Res*. 2018;46:2012-2029.
21. Jin DH, Kim Y, Lee BB, et al. Metformin induces cell cycle arrest at the G1 phase through E2F8 suppression in lung cancer cells. *Oncotarget*. 2017;8:101509-101519.
22. Ye L, Guo L, He Z, et al. Upregulation of E2F8 promotes cell proliferation and tumorigenicity in breast cancer by modulating G1/S phase transition. *Oncotarget*. 2016;7:23757-23771.
23. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
24. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature*. 2001;411:342-348.
25. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature*. 1993;366:701-704.
26. Massague J. G1 cell-cycle control and cancer. *Nature*. 2004;432:298-306.

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