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ORIGINAL ARTICLE

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Long non-coding RNA FOXP4-AS1 is an unfavourable prognostic factor and regulates proliferation and apoptosis in colorectal cancer

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Abstract

Objectives: Despite improvements in diagnosis and treatment, colorectal cancer (CRC) remains the third most common malignancy, and fourth-leading cause of cancer-related death worldwide, and has a particularly high incidence in Western countries. Recent studies have suggested that long non-coding RNAs (lncRNAs) compose a novel class of regulators of cancer biological processes, such as proliferation, apoptosis and metastasis. Here, we report that lncRNA FOXP4-AS1 acts as a functional oncogene in CRC pathogenesis. Moreover, we have attempted to investigate the effects of FOXP4-AS1 on tumour progression, both *in vitro* and *in vivo*.

Materials and methods: In this study, bioinformatic analyses and qPCR were performed to investigate FOXP4-AS1 expression in CRC tissue samples and CRC cell lines. We inhibited FOXP4-AS1 expression *via* FOXP4-AS1-specific siRNA transfection. Cell proliferation was assessed using cell viability and colony formation assays, as well as by flow cytometry and ethynyl deoxyuridine (Edu) analyses. Apoptosis was assessed using flow cytometry. Animal tumour xenografts were generated, and immunohistochemistry (IHC) was performed to evaluate effects of FOXP4-AS1 on CRC tumour growth *in vivo*.

Results: We found that FOXP4-AS1 was up-regulated in CRC tissues and cell lines and that its overexpression positively correlated with advanced pathological stages and larger tumour size. Additionally, we found that FOXP4-AS1 knockdown inhibited cell proliferation and induced apoptosis. Furthermore, FOXP4-AS1 knockdown induced marked increase in number of cells in G0/G1 phase and reduction in number of cells in S phase, in DLD-1, HT-29 and HCT116 cell lines. Consistent with these findings, FOXP4-AS1 silencing inhibited tumour growth *in vivo*.

Conclusion: These findings suggest that FOXP4-AS1 plays a crucial role in CRC progression and may be a new biomarker in patients with CRC.

1 | INTRODUCTION

There have been dramatic improvements in the early detection and treatment of colorectal cancer (CRC); however, it remains the fourth-leading cause of cancer-related death in both men and women.[1,2] In 2016, an estimated 71,830 men and 65,000 women will be diagnosed with CRC, and 26,270 men and 24,040 women will die of this disease in the United States.[1] Thus, it is urgent to identify novel biomarkers and therapeutic targets that are correlated with CRC tumourigenesis and progression.

*These authors contributed equally to this work.

Over the past decade, human genome sequencing studies and the GENCODE project have demonstrated that only approximately 2% of the human genome comprises protein-coding genes, while the majority of the genome comprises non-coding genes encoding numerous non-coding transcripts, including microRNAs, circular RNAs and long non-coding RNAs (lncRNAs).[3,4] Among these, long non-coding RNAs are emerging as crucial players in various biological processes via distinct mechanisms.[5,6] They are commonly defined as transcripts greater than 200 nt in length but have no protein-coding capacity.[7] Recent experiments confirmed that lncRNA dysregulation plays a role in various malignancies.[8–10] For instance, lncRNA HOTAIR overexpression facilitates breast cancer metastasis by recruiting the PRC2 complex to specific target genes genome-wide, leading to H3K27 trimethylation and epigenetic silencing of metastasis suppressor genes.[11] In contrast, lncRNA maternally expressed gene 3 (MEG3) may function as a tumour suppressor partly by activating p53 in colorectal cancer.[12] Therefore, a powerful suggestive link exists between lncRNAs and cancer, and lncRNAs can act as oncogenes or tumour suppressors, although the biological functions of the majority of lncRNAs remain to be elucidated.

Many studies have demonstrated that lncRNAs contribute to cancer cell phenotypes by silencing tumour suppressors or activating oncogenes.[13,14] In addition, many cell cycle-related genes (including p15, p21, p27 and KLF2) act as tumour suppressors in various cancers by inhibiting the activity of kinases involved in the G1/S transition.[15–17] Our previous studies have demonstrated that lncRNA BANCR (BRAF activated non-coding RNA) promotes CRC cell proliferation partly by down-regulating p21 expression.[18]

In the present study, we report for the first time that a novel lncRNA, FOXP4-AS1, was significantly up-regulated in colorectal cancer tissues. The association between FOXP4-AS1 overexpression and clinicopathological characteristics was also studied. Additionally, FOXP4-AS1 knockdown inhibited cell proliferation and induced apoptosis both in vitro and in vivo. Moreover, the expression of cell cycle-related genes (including p15, p21, p27 and KLF2) was also analysed after FOXP4-AS1 knockdown. Our findings suggest that lncRNA FOXP4-AS1 plays an important role in colorectal cancer progression and may be a therapeutic target in patients with CRC.

2 | MATERIALS AND METHODS

2.1 | Expression profiling data retrieval and analysis of lncRNAs in CRC

Colorectal cancer lncRNA expression profiling data were retrieved and analysed. Raw microarray data were downloaded from Gene Expression Omnibus (GEO) data sets (GSE21510 and GSE9348). We analysed the lncRNA FOXP4-AS1 expression profiles of colorectal cancer patients included in the GSE21510 and GSE9348 data sets. Paired t-tests based on specific experimental designs were employed to validate probe significance. We used BLAST+ to map these probing sequences to human lncRNA sequences contained in the RefSeq database at NCBI. FDR was calculated by the Benjamini-Hochberg method. The expression levels of FOXP4-AS1 and GAPDH in normal human colorectal tissues were downloaded from BIOGPS data sets (http://biogps.org).

2.2 | Tissue samples and clinical data collection

A total of 48 paired CRC and adjacent non-tumour tissue samples were obtained from patients with CRC who underwent surgery at the Second Affiliated Hospital, Nanjing Medical University, between 2013 and 2016. All patients were confirmed to have CRC via histopathological evaluations. None of the patients had been treated before surgery. Detailed information pertaining to clinicopathological characteristics was recorded. All tissue samples were rapidly snap-frozen in liquid nitrogen and stored at −196°C until RNA extraction. Informed consent was obtained from all patients. Our study was approved by the Research Ethics Committee of Nanjing Medical University, China.

2.3 | RNA extraction and qPCR assays

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. RNA quantity and quality were determined by a NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). For qRT-PCR, 1 μg of RNA was reverse-transcribed to cDNA using a reverse transcription kit (Takara, Dalian, China). qPCR assays were conducted on an ABI 7500. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The following primers were used for target amplification: FOXP4-AS1 (forward): 5′-GTGAGCTTCTGGGTTCGACA-3′ and FOXP4-AS1 (reverse): 5′-ATTGAGGGTTAGGGCAGCAC -3′; GAPDH (forward): 5′-GGGAGCCAAAAGGGTCAT-3′ and GAPDH (reverse): 5′-GAGTCCTTCCACGATACCAA-3′; p15 (forward): 5′-GGACTAGTGGAGAAGGTGCG-3′ and p15 (reverse): 5′-GGGCGCTGCCCATCATCATG-3′; p21 (forward): 5′-GTCCACTGGGCCGAAGAG-3′ and p21 (reverse): 5′-TGCGTTCACAGGTGTTTCTG-3′; p27 (forward): 5′-TGCAACCGACGATTCTTCTACTCAA-3′ and p27 (reverse): 5′-CAAGCAGTGATGTATCTGATAAACAAGG-3′; and KLF2 (forward): 5′-CTGCACATGAAACGGCACAT-3′ and KLF2 (reverse): 5′-CAGTCACAGTTTGGGAGGGG-3′. All qPCR data were calculated and expressed relative to threshold cycle (shown as ΔCT) values and then converted to fold changes.

2.4 | Cell lines and culture conditions

Five CRC cell lines (DLD-1, HT-29, HCT116, SW480 and Lovo) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 or DMEM (GIBCO-BRL) medium supplemented with 10% foetal bovine serum (10% FBS), 100 U/mL penicillin and 100 mg/mL streptomycin in humidified air at 37°C with 5% CO_2.

2.5 | Cell transfection

CRC cells were transfected with siRNA oligonucleotides with plasmids using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. The siRNAs were purchased from Invitrogen (Invitrogen). The nucleotide sequences of the siRNAs used for the experiment were as follows: si-FOXP4-AS1 (UGUCGAACCCAGAAGCUCACUUUCC) and si-NC (UUCUCCGAACGUGUCACGUTT). After 48 h of transfection, the cells were harvested for further study.

2.6 | Cell viability and colony formation assay

Cell viability was monitored using Cell Proliferation Reagent Kit I (MTT; Roche Applied Science, Roche, Basel, Switzerland). The five CRC cell lines were transfected with si-FOXP4-AS1 or si-NC (3000 cells/well) and cultured in 96-well plates with six replicate wells. Cell viability was assessed according to the manufacturer's recommendations. For the colony formation assay, a total of 500 cells were seeded in a six-well plate and maintained in medium containing 10% FBS, which was replaced every 5 days. After 2 weeks, the cells were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). Visible colonies were counted manually. Wells were measured in triplicate in each treatment group.

2.7 | Flow cytometry

DLD-1, HT-29 and HCT116 cells transfected with si-FOXP4-AS1 or si-NC were harvested after 48 h, stained with PI using a Cycletest™ Plus DNA Reagent Kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol, and analysed with a flow cytometer (FACScan®; BD Biosciences) equipped with CellQuest software (BD Biosciences). The percentages of cells in G0/G1, S and G2/M phase were calculated and compared.

DLD-1, HT-29 and HCT116 cells transfected with si-FOXP4-AS1 or si-NC were harvested after 48 h for apoptosis analysis. The cells were then treated with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) in the dark at room temperature, according to the manufacturer's recommendations. The cells were subsequently analysed by FACScan® and identified as viable, dead, early apoptotic or late apoptotic cells.

2.8 | Ethynyl deoxyuridine (Edu) analysis

Proliferating cells were assessed using a 5-ethynyl-2-deoxyuridine labelling/detection kit (Ribobio, Guangzhou, China), according to the manufacturer's protocol. Briefly, DLD-1, HT-29 and HCT116 cells were cultured in 96-well plates at a density of 5×10^3cells per well and transfected with si-FOXP4-AS1 or si-NC for 48 h. Then, 50 μM Edu labelling medium was added to the cell culture, which was incubated for 2 h at 37°C with 5% CO_2. Then, the cultured cells were fixed with 4% paraformaldehyde (pH 7.4) for 30 min and treated with 0.5% Triton X-100 for 20 min at room temperature. After being washed with PBS, the samples were stained with anti-Edu working solution for 30 min at 25°C before being incubated with 100 μL of DAPI (5 μg/mL) for 30 min at 25°C and observed under a fluorescent microscope. The percentage of Edu-positive cells was calculated from five random fields in three wells.

2.9 | Tumour xenografts in animals

Four-week-old athymic male mice were purchased from the Animal Center of Nanjing University (Nanjing, China) and maintained in pathogen-free conditions. DLD-1 cells were transfected with sh-FOXP4-AS1 or empty vector and harvested from six-well plates, washed with phosphate-buffered saline (PBS), and resuspended at a density 2×10^7 cells/mL. Each mouse was subsequently injected in the lower right flank with 100 μL of suspended cells. Tumour growth was examined every 3 days, and tumour volumes were measured as the product of length×width^2×0.5. At 15 days post-injection, the mice were sacrificed via CO_2 asphyxiation, and tumour growth was examined.

2.10 | Immunohistochemistry (IHC)

Xenograft tumours were immunostained for H&E and Ki-67. Signals were amplified and visualized with 3′-diaminobenzidine chromogen, and the tumours were subsequently counterstained with haematoxylin. Expression was considered positive when 60% or more of the tumour cells were stained. The IHC staining results were independently scored by the author and a pathologist to minimize subjectivity and then compared, and final comprehensive results were obtained. Anti-Ki-67 (1:100) antibodies were purchased from R & D.

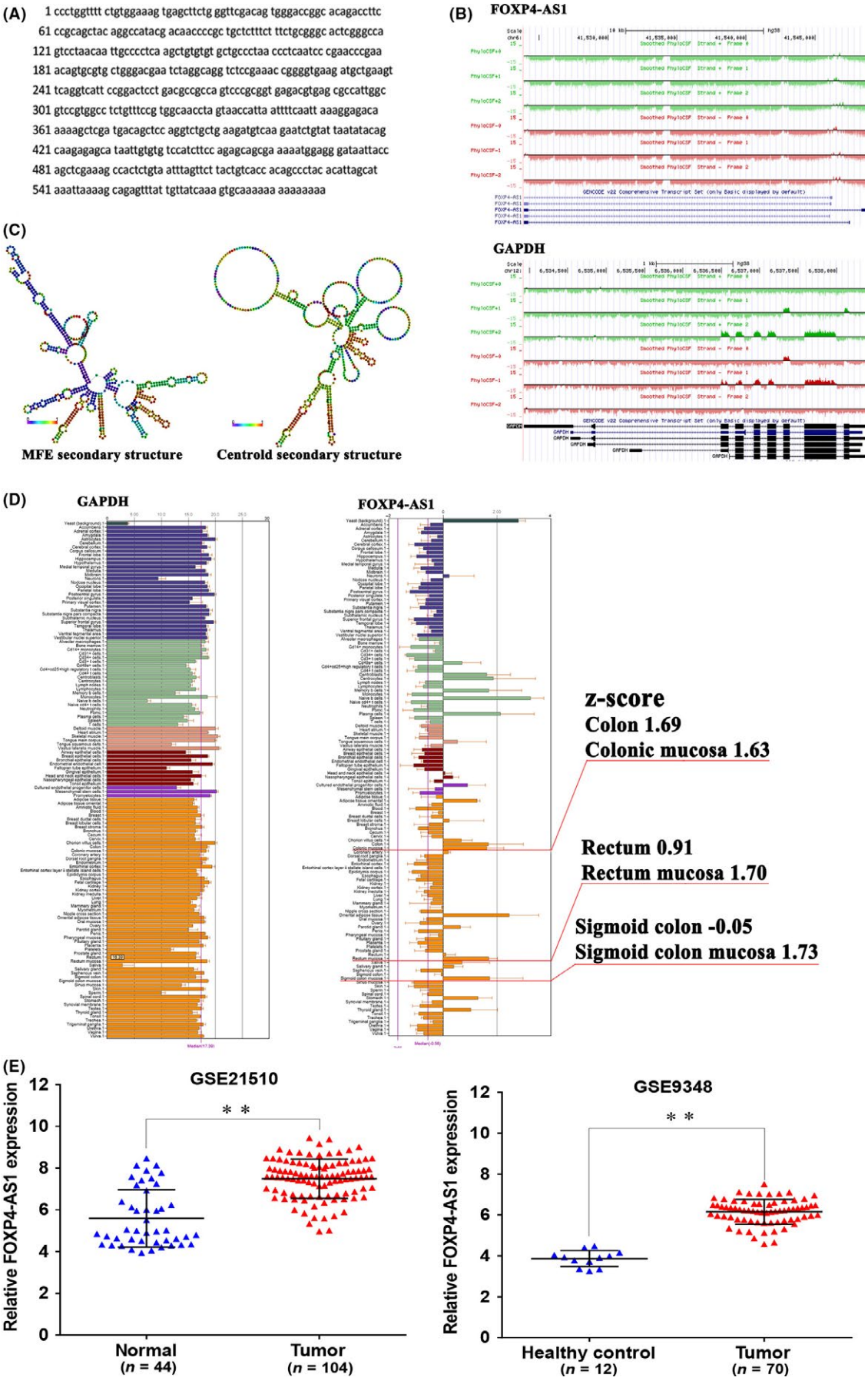
2.11 | Statistical analysis

All statistical analyses were performed using SPSS software, version 22.0 (SPSS, Chicago, IL, USA). Student's *t* test or a chi-square test was used to evaluate significant differences between groups of data. All data are represented as the mean±SD. $P<.05$ was considered significant. $^*P<.05$; $^{**}P<.01$.

3 | RESULTS

3.1 | lncRNA FOXP4-AS1 expression levels in CRC

In an attempt to identify novel oncogenic lncRNAs that are involved in CRC progression, two microarray data sets (GSE21510 and GSE9348) were used to analyse lncRNAs that were differentially expressed in colorectal tumours samples and corresponding non-tumour samples. We found that lncRNA FOXP4-AS1 was consistently up-regulated in both the GSE21510 and the GSE9348 data sets (Figure 1E). The FOXP4-AS1 gene is located at the chromosomal locus 6p21.1 and encodes a 588 bp transcript (Figure 1A). In addition, BioGPS data set analysis indicated that the FOXP4-AS1 gene is not expressed in normal human colorectal tissues (A z-score >5 suggests that a gene is expressed in a particular tissue)


(A)
1 ccctggtttt ctgtggaaag tgagcttctg ggttcgacag tgggaccggc acagaccttc
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481 agctcgaaag ccactctgta atttagttct tactgtcacc acagccctac acattagcat
541 aaattaaaag cagagtttat tgttatcaaa gtgcaaaaaa aaaaaaaa
(B)
FOXP4-AS1
GAPDH
(C)
MFE secondary structure
Centrold secondary structure
(D)
GAPDH
FOXP4-AS1
z-score
Colon 1.69
Colonic mucosa 1.63
Rectum 0.91
Rectum mucosa 1.70
Sigmoid colon -0.05
Sigmoid colon mucosa 1.73
(E)
GSE21510
Relative FOXP4-AS1 expression
Normal (n = 44)
Tumor (n = 104)
GSE9348
Healthy control (n = 12)
Tumor (n = 70)

FIGURE 1 Higher FOXP4-AS1 expression levels in CRC and coding potential analyses of FOXP4-AS1 transcripts. A, The full sequence of FOXP4-AS1 is published in the NCBI database (NR_126415.1). B, PhyloCSF predicted that FOXP4-AS1 has no protein coding potential. The tracks showed the PhyloCSF scores for each codon in each of six frames. Regions with a score greater than 0 were predicted to be coding regions, while regions with a score less than 0 were predicted to be non-coding regions. The protein-coding gene GAPDH was used as a control. C, Prediction of the structure of FOXP4-AS1 based on minimum free energy (MFE) and partition function. The colour scale indicates the confidence for the prediction for each base, with shades of red indicating strong confidence (http://rna.tbi.univie.ac.at/). D, Low FOXP4-AS1 expression levels in normal human colorectal tissues. The protein-coding gene GAPDH was used as a control. E, Higher FOXP4-AS1 expression levels in the profiles of CRC patient tissue samples from Gene Expression Omnibus

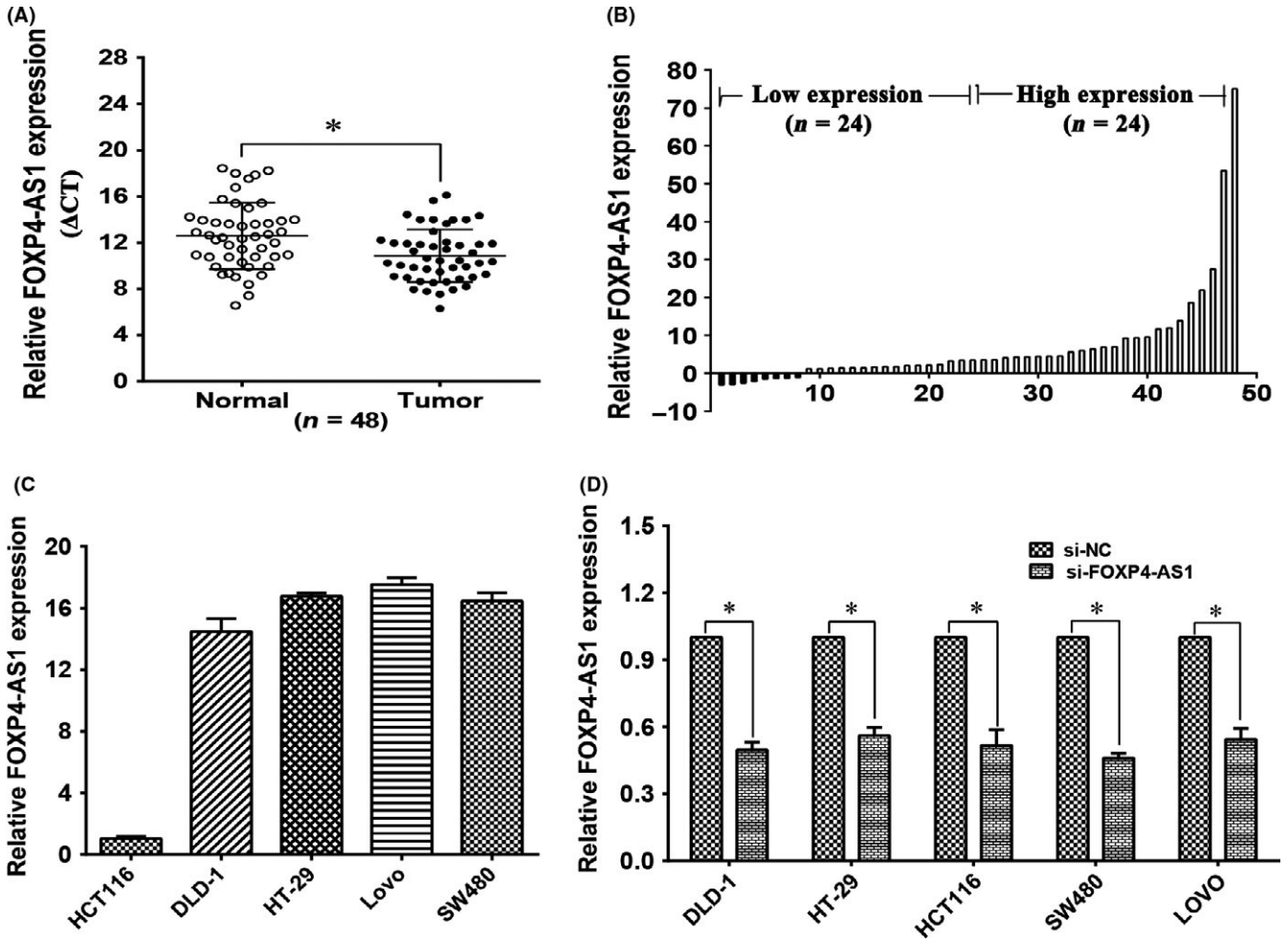

FIGURE 2 Relative FOXP4-AS1 expression levels in CRC tissues and CRC cell lines. A, FOXP4-AS1 expression levels in matched tumour tissues and adjacent normal tissues from 48 CRC patients were measured by qPCR. Relative gene expression determinations were made with the comparative delta CT normalized to GAPDH expression ($P<.05$). B, Patients were divided into two groups according to the median value of relative FOXP4-AS1 expression. C, qPCR analysis of FOXP4-AS1 expression levels in five CRC cell lines (HCT116, DLD-1, HT-29, Lovo and SW480). D, qPCR analysis of FOXP4-AS1 expression levels in five CRC cell lines transfected with siRNA. Bars: s.d., $^{*}P<.05$, $^{**}P<.01$, from three independent experiments

(Figure 1C). Moreover, PhyloCSF predicted that FOXP4-AS1 has no protein-coding potential. The tracks showed the PhyloCSF score for each codon in each of six frames. Regions with a score greater than 0 were predicted to be coding regions, while regions with a score less than 0 were predicted to be non-coding regions. The protein-coding gene GAPDH was used as a control (Figure 1B). These results indicate that lncRNA FOXP4-AS1 expression is increased in CRC tissues and that FOXP4-AS1 may serve as an oncogene in CRC progression.

3.2 | FOXP4-AS1 overexpression was positively correlated with advanced pathological stages and larger tumour sizes in patients with CRC

To confirm that FOXP4-AS1 was overexpressed in CRC tissues, we detected FOXP4-AS1 expression levels in 48 paired CRC samples and analysed adjacent normal tissues with qPCR. All samples were normalized to GAPDH. The results showed that FOXP4-AS1 expression levels were significantly higher in tumour tissues than in adjacent

TABLE 1 Correlation between FOXP4-AS1 expression and clinicopathologic characteristics of patients with CRC (n=48).

Characteristics	FOXP4-AS1		*P*
	Low No. of cases	High No. of cases	Chi-squared test, *P* value
Age (years)			
≤60	8	4	0.318
>60	16	20	
Gender			
Male	15	17	0.760
Female	9	7	
Tumor size (cm)			
≤5	13	8	0.036
>5	11	19	
Histologic differentiation			
Well	9	5	0.341
Poorly	15	19	
TNM stage			
I/II	16	8	0.042
III/IV	8	16	
Lymph node metastasis			
Positive	13	9	0.385
Negative	11	15	
Primary tumor site			
Colon	10	16	0.147
Rectum	14	8	

normal tissues (*P*<.01; Figure 2A). Then, to investigate the relationship between FOXP4-AS1 expression and clinicopathological features, we divided the samples into high (above the median, n=24) and low (below the median, n=24) FOXP4-AS1 expression groups according to the median FOXP4-AS1 level (Figure 1B). A chi-square test was performed to compare the clinicopathological features of the two groups. As shown in Table 1, FOXP4-AS1 overexpression in CRC tissues was significantly correlated with larger tumour sizes (*P*=.036) and advanced TNM stages (*P*=.042). However, several other clinical parameters were found to be non-significantly associated with FOXP4-AS1 expression (Table 1).

3.3 | Modulation of FOXP4-AS1 expression in CRC cells

We next performed qPCR analysis to examine FOXP4-AS1 expression in a panel of CRC cell lines, including DLD-1, HT-29, HCT116, SW480 and Lovo. We found that FOXP4-AS1 expression levels were higher in DLD-1 (*P*<.01), HT-29 (*P*<.01), SW480 (*P*<.01) and Lovo (*P*<.01) cells than in HCT116 cells (Figure 2C). Then, to investigate the functional effects of FOXP4-AS1 dysregulation in CRC cells, we knocked down its expression via FOXP4-AS1 siRNA transfection. qPCR analysis of FOXP4-AS1 levels was performed 48 h post-transfection. The results showed that FOXP4-AS1 expression was significantly reduced by si-FOXP4-AS1 transfection compared with control cells (Figure 2D).

3.4 | FOXP4-AS1 promoted CRC cell proliferation

To assess the biological role of FOXP4-AS1 in CRC cells, we performed an MTT assay. The results showed that FOXP4-AS1 knockdown significantly inhibited cell viability all in five CRC cell lines compared with control cells (Figure 3A). Similarly, the colony formation assay results showed that clonogenic survival was strikingly decreased following FOXP4-AS1 inhibition all in five CRC cell lines (Figure 3B).

Next, to determine whether the effects of FOXP4-AS1 on CRC cell proliferation are the result of FOXP4-AS1-mediated changes in cell cycle progression, we performed flow cytometry assay using DLD-1, HT-29 and HCT116 cells. The results of si-FOXP4-AS1 or si-NC transfection for 48 h showed that FOXP4-AS1 knockdown increased the percentage of cells in G0/G1 phase and decreased the percentage of cells in S and G2/M phase compared to control cells (Figure 4A). EdU analysis yielded similar results (Figure 5A). Previous studies have demonstrated that cell cycle-related genes can be regulated by lncRNAs,[19,20] and our previous study showed that p21 serves as a tumour suppressor in CRC.[18,21] To determine whether the inhibition of cell cycle-related genes (including p15, p21, p27 and KLF2) is required for the above-mentioned FOXP4-AS1-mediated effects on CRC cell proliferation, we performed qPCR analysis. The results showed that FOXP4-AS1 inhibition leads to increases in p15, p21, p27 and KLF2 expression in four CRC cell lines (Figure 5B). These data indicate that FOXP4-AS1 promotes CRC cell proliferation by promoting cell cycle progression.

3.5 | FOXP4-AS1 knockdown induced CRC cell apoptosis

To determine whether the effects of FOXP4-AS1 on colorectal cancer cell proliferation are associated with cell apoptosis inhibition, we performed flow cytometry assays. The results showed that DLD-1, HT-29 and HCT116 cells transfected with FOXP4-AS1 siRNA had higher apoptosis rates than control cells (Figure 4B). These data demonstrate that FOXP4-AS1 promotes the proliferation phenotype and inhibits the apoptosis of colorectal cancer cells.

3.6 | FOXP4-AS1 knockdown inhibits CRC cell tumourigenesis in vivo

To confirm the impact of FOXP4-AS1 on CRC cell growth in vivo, DLD-1 cells transfected with sh-FOXP4-AS1 or empty vector were injected into male nude mice. The cells were transfected with empty vector as a control. At 15 days post-injection, tumour growth in the sh-FOXP4-AS1 group was markedly slower than that in the control group (Figure 6A). Correspondingly, tumour volumes and weights were obviously decreased in transfected cells compared with control cells (Figure 6B,C). Moreover, immunohistochemistry (IHC) analysis confirmed that tumours that formed from DLD-1/sh-FOXP4-AS1 cells exhibited lower Ki-67 staining than those that formed from

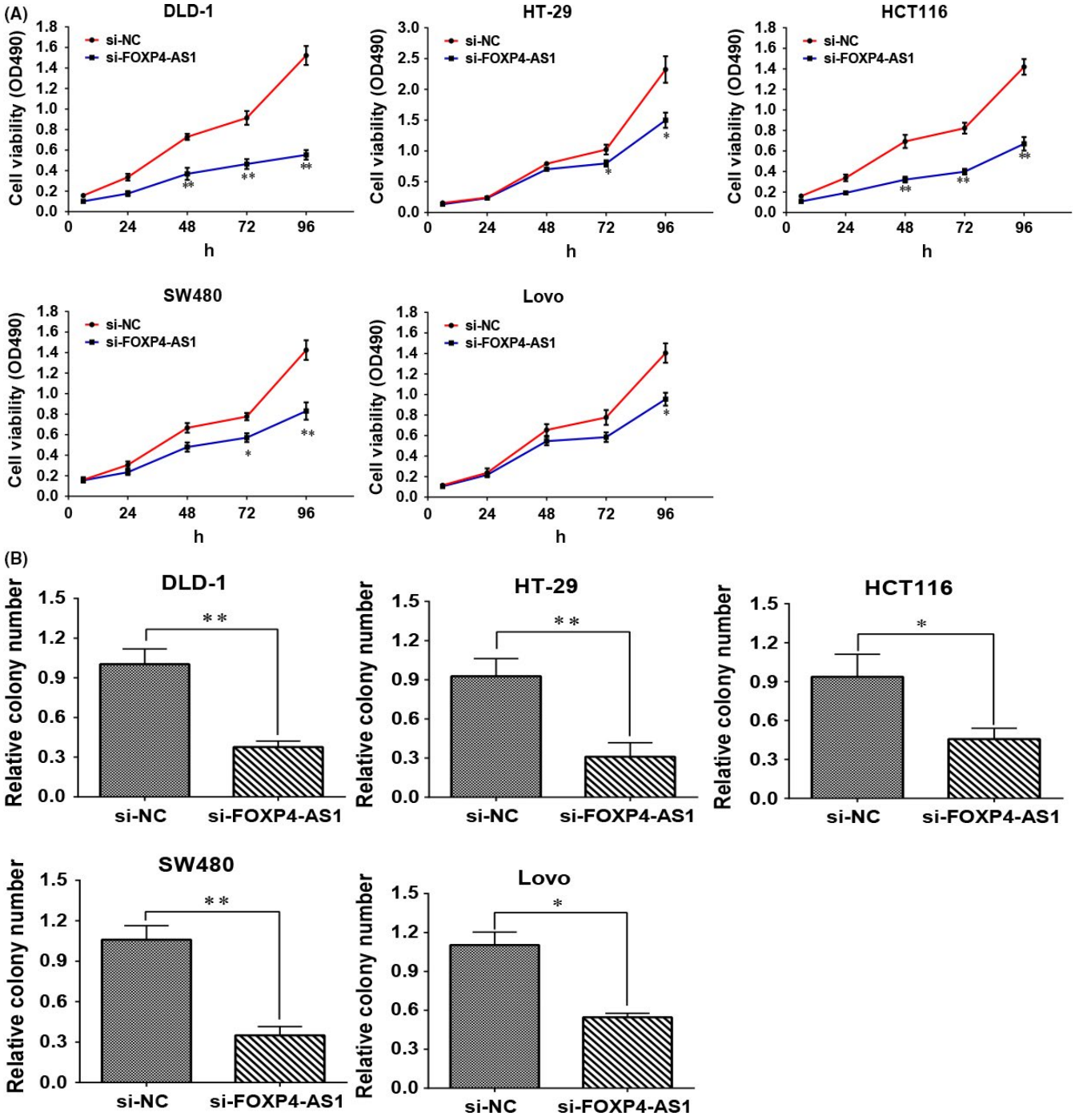

FIGURE 3 Effect of FOXP4-AS1 on CRC cell viability in vitro. A, MTT assays were used to determine the cell viability of five CRC cell lines transfected with siRNAs against FOXP4-AS1. B, Colony-forming assays were conducted to assess the proliferation of five CRC cell lines transfected with siRNAs against FOXP4-AS1. Bars: s.d., *P<.05, **P<.01, from three independent experiments

control cells (Figure 6E). Our results indicate that FOXP4-AS1 knockdown suppresses colorectal cancer cell tumour growth in vivo.

4 | DISCUSSION

Cancer is a disease of the genome caused by oncogene activation and tumour suppressor gene inhibition. In recent years, deep sequencing studies including large consortia (such as ENCODE project, TCGA and ICGC) and advances in technology (such as tiling arrays and RNA deep sequencing) have enabled the identification of numerous tumour-specific mutations not only in protein-coding sequences but also in non-coding sequences.[4,22] In particular, long non-coding RNAs (lncRNAs) have received increased attention. LncRNAs are strikingly similar to mRNAs. They are RNA polymerase II transcripts that are capped, spliced and polyadenylated and are devoid of an open reading frame

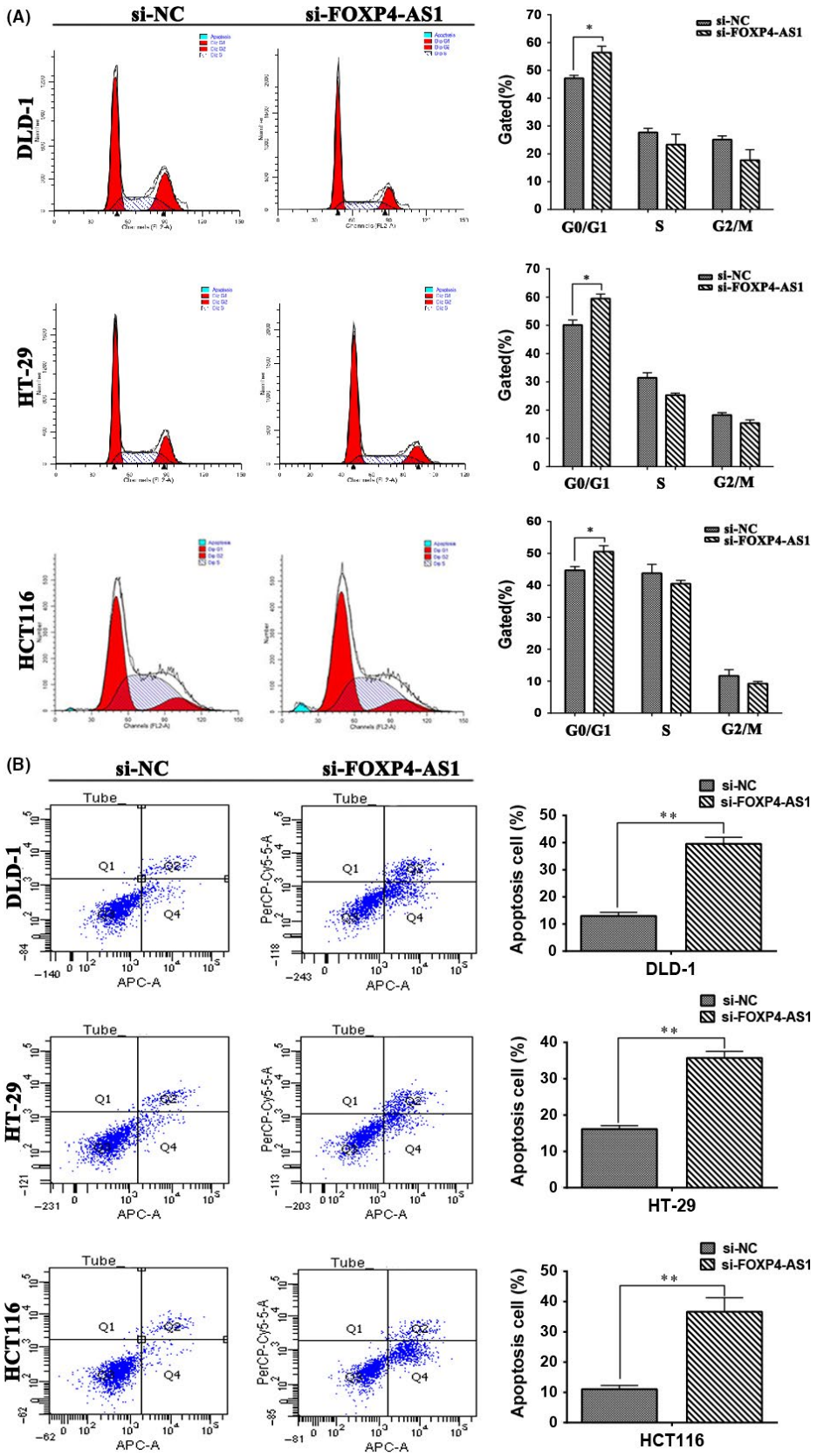

FIGURE 4 Effect of FOXP4-AS1 on CRC cell cycle progression and apoptosis in vitro. A, DLD-1, HT-29 and HCT116 cell cycle progression was analysed by flow cytometry. The bar chart represents the percentages of cells in G1–G0, S or G2–M phase, as indicated. B, DLD-1, HT-29 and HCT116 cells were stained and analysed by flow cytometry. Q2, early apoptotic cells. Q4, terminal apoptotic cells. Bars: s.d., **P*<.05, ***P*<.01, from three independent experiments

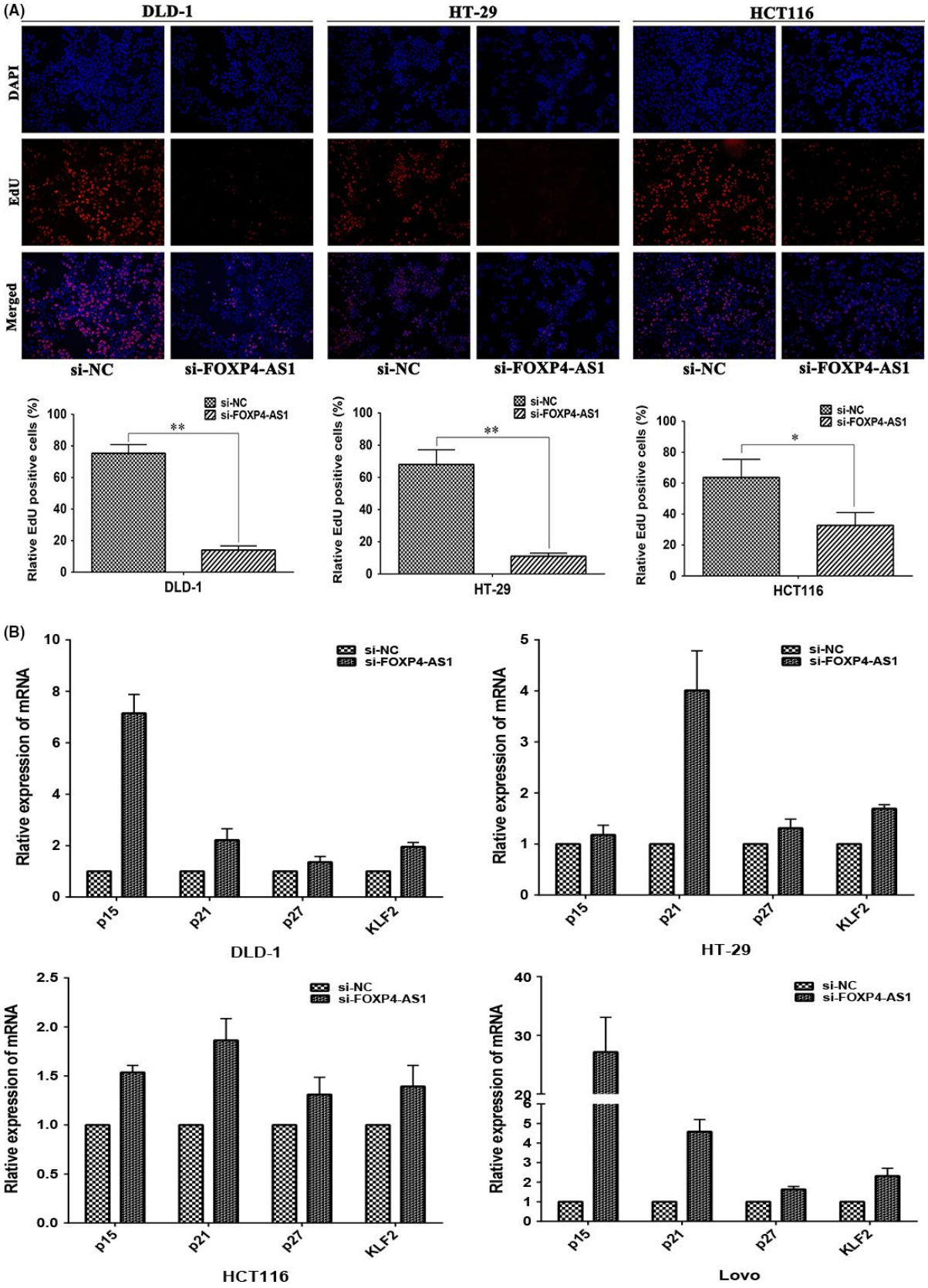

FIGURE 5 FOXP4-AS1 is required to suppress p15, p21, p27 and KLF2 expression and thus regulate the CRC cell cycle and CRC proliferation. A, Effect of FOXP4-AS1 on CRC cell proliferation, as shown by EdU assay. Proliferating CRC cells were labelled with Edu. The Click-it reaction revealed Edu staining (red). Cell nuclei were stained with DAPI (blue). Representative images and data based on three independent experiments. B, p15, p21, p27 and KLF2 mRNA expression levels were determined by qPCR following FOXP4-AS1 knockdown Bars: SD, **P*<.05, ***P*<.01

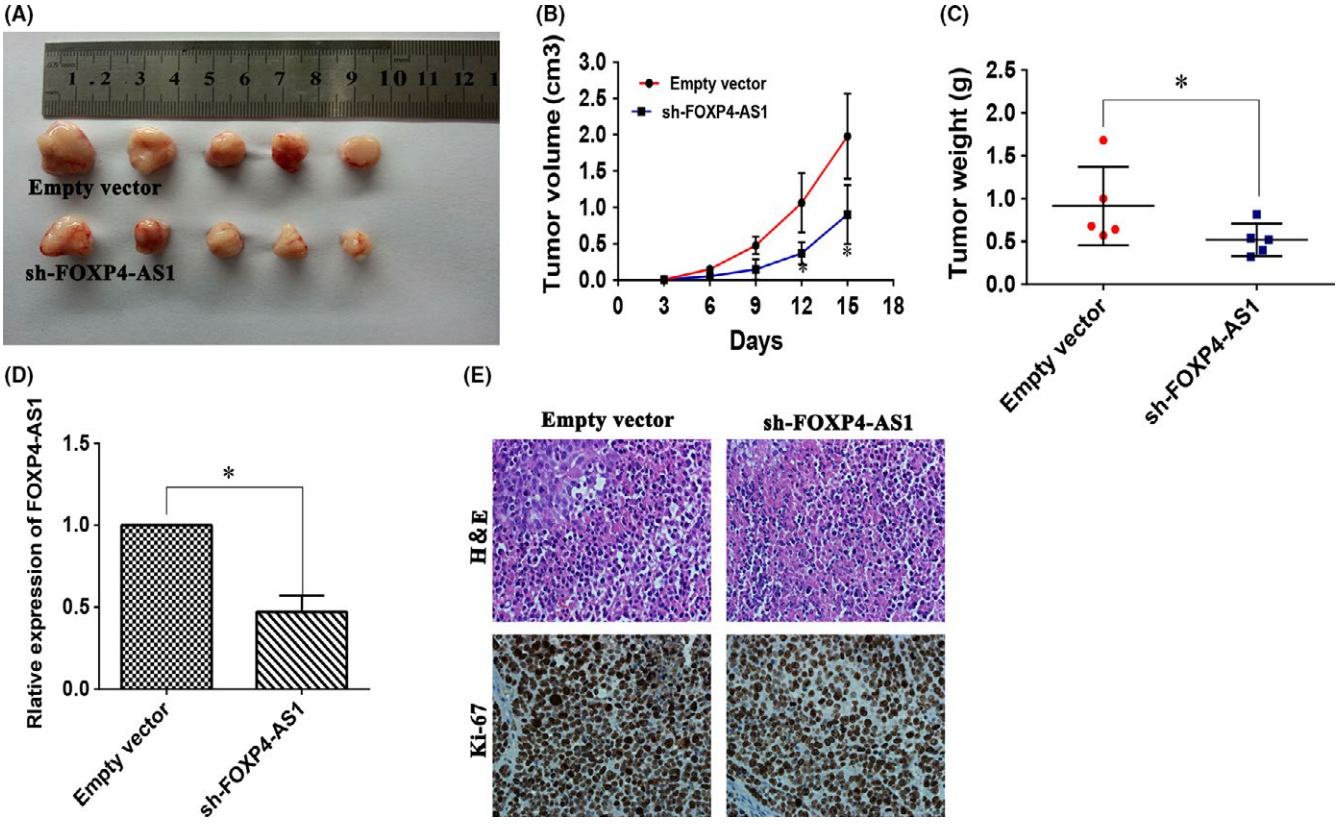

FIGURE 6 FOXP4-AS1 knockdown inhibits tumour growth in a xenograft mouse model (A) Tumours were harvested 15 days after the injection of DLD-1 cells transfected with sh-FOXP4-AS1 or empty vector. (B, C) Tumour volumes were calculated every 3 days after injection. Tumour weights were measured when the xenograft tumours were harvested. D, qPCR analysis of FOXP4-AS1 expression in xenograft tumour tissues. E, HE staining and immunohistochemistry images of the xenograft tumours. Representative Ki-67 protein levels in the xenograft tumours, as determined via IHC. Bars: SD, **P*<.05, ***P*<.01

(ORF) that can be translated into a protein sequence.[23] More recently, studies have reported that some lncRNAs are aberrantly expressed in and associated with the onset and progression of a variety of cancers, especially CRC.[24,25] These lncRNAs have gained great clinical value as new biomarkers of and novel therapeutic targets for this deadly disease.[26,27] For example, Huang et al. demonstrated that lncRNA CASC2 plays an important role in the pathobiology of human CRC and that it can function as a ceRNA by competitively binding miR-18a, thereby relieving the suppression of PIAS3 by miR-18a in CRC cells.[28] Wan et al. found that lncRNA HOTAIRM1 and CEA down-regulation is associated with CRC and that the combined assay of these two biomarkers appears to be promising with respect to the diagnosis of CRC (sensitivity was increased to 84%).[29] Our previous study demonstrated that HOTTIP promotes colorectal cancer cell proliferation partly via p21 silencing.[21]

In the present study, we investigated the potential role of FOXP4-AS1 as an oncogene in CRC development. First, two colorectal cancer gene expression data sets were obtained from the indicated publicly available GEO databases (GSE21510 and GSE9348). We found lncRNA FOXP4-AS1 was consistently up-regulated in both the GSE21510 and the GSE9348 data sets. Our subsequent studies of clinical samples and CRC cell lines demonstrated that FOXP4-AS1 is up-regulated and that FOXP4-AS1 overexpression is significantly associated with advanced pathological stages and larger tumour sizes in patients with CRC. As sustained proliferative signalling, growth suppressor evasion, and cell death resistance are the basic hallmark capabilities of malignancies, we evaluated the effects of FOXP4-AS1 on CRC cell proliferation and apoptosis.[30,31] The results showed that FOXP4-AS1 knockdown inhibited cell proliferation and tumour growth both in vitro and in vivo by extending the G0/G1-S phase transition. These findings suggest that FOXP4-AS1 plays an important role in modulating CRC progression. Moreover, flow cytometry assays demonstrated that the effects of FOXP4-AS1 on colorectal cancer cell proliferation were associated with cell apoptosis inhibition. We also analysed cell cycle-related gene expression after FOXP4-AS1 knockdown. Further investigation is required to understand the mechanism underlying the relationship between FOXP4-AS1 and cell cycle-related gene expression in colorectal cancer.

In summary, our study showed for the first time that lncRNA FOXP4-AS1 expression is up-regulated in CRC tissues and that FOXP4-AS1 overexpression is a poor prognostic factor in CRC patients. Furthermore, FOXP4-AS1 knockdown inhibited CRC cell proliferation and induced apoptosis both in vitro and in vivo. Therefore, FOXP4-AS1 functions as an oncogene in CRC tumourigenesis and thus may be an effective therapeutic target in and novel biomarker for CRC.

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