

RETRACTED ARTICLE: Long Non-Coding RNA LEF1-AS1 Promotes Migration, Invasion and Metastasis of Colon Cancer Cells Through miR-30-5p/SOX9 Axis

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Introduction: Aberrant expression of long non-coding RNAs (lncRNAs) has been implicated in the tumorigenesis and progression of colon cancer. Lymphoid enhancer-binding factor 1 antisense RNA 1 (LEF1-AS1), a highly conserved and newly discovered long non-coding RNA, has been reported to be overexpressed and correlated with poor prognosis in colon cancer, but the exact role of it remains uncertain.

Materials and Methods: In our study, the biological functions of LEF1-AS1 in colon cancer were analyzed by cell viability assay, colony formation assay, scratch wound healing assay, transwell cell invasion assay, soft agar assay, luciferase reporter assay, pull down assay, tumor xenograft model and Western blot.

Results: We found that LEF1-AS1 was upregulated in colon cancer patients and correlated with poor overall survival and recurrence-free survival. Besides, enforced expression of LEF1-AS1 in HT29 and T84 cells promoted migration, invasion, anchorage-independent growth, tumor xenograft formation and lung metastasis, while knockdown of LEF1-AS1 in COLO320 cells suppressed cell migration, invasion, anchorage-independent growth and tumor xenograft formation. In addition, LEF1-AS1 was directly interacted and inversely correlated with miR-30-5p in colon cancer, and SOX9 was a downstream target for miR-30-5p. LEF1-AS1 overexpression increased the expression level of SOX9, and restoration of SOX9 attenuated the effects caused by LEF1-AS1 knockdown in cell migration, invasion, anchorage-independent growth and tumor xenograft formation.

Conclusion: Our results indicated that LEF1-AS1 promoted migration, invasion and metastasis of colon cancer cells partially through miR-30-5p/SOX9 axis. The oncogenic LEF1-AS1 could be a potential prognostic biomarker for colon cancer.

Keywords: LEF1-AS1, colon cancer, miR-30-5p, SOX9

Introduction

Colon cancer is the third most common malignancy in men and the second in women worldwide, with nearly 1.2 million new cases and 600 000 deaths every year.¹ The incidence of colon cancer almost doubles in the past few decades, accompanied by rising mortality. Colon cancer is more frequent in industrialized areas such as Europe and North America, whereas it is relatively uncommon in some countries in central Asia and Africa.² Rapid increases in the incidence and mortality of colon cancer in Asia-Pacific region have been noted, which has been ascribed to growing industrialization and urbanization. The 5-year survival rates of early-stage colon patients are approximately 90% but decline to less than 10% in patients with distant metastases.³

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Traditional prognostic biomarkers such as tumor size, tumor grade or presence of metastasis have been used for decades, but they have limitations in predicting patient outcome.⁴ Thus, it is important to find new prognostic and predictive biomarkers to assist colon cancer treatment.

Long non-coding RNAs (lncRNAs) are a widespread class of RNA transcripts longer than 200 nucleotides in length but without any protein-coding potential. They are typically transcribed and spliced like other protein-coding genes, though in lower expression levels and more tissue-specific manner.⁵ Initially, lncRNAs were simply regarded as a result of transcriptional noise, but recent studies have demonstrated that lncRNAs are involved in many cellular process such as epigenetic modification, transcription and translation.⁶ Dysregulation of lncRNAs plays an important role in tumor initiation and progression of colon cancer. For example, lncRNA *SNHG15* was overexpressed and highly correlated with poor survival in colon cancer patients. Transcription of *SNHG15* was regulated by *MYC* oncogene, and overexpression of *SNHG15* promoted proliferation, invasion and drug resistance of colon cancer by interacting with AIF.⁷ Downregulation of *LINC00675* was identified in both colon cancer tissues and cell lines, and ectopic expression of *LINC00675* inhibited proliferation, invasion and migration of colon cell lines by sponging miR-942. Lymphoid enhancer-binding factor 1 (LEF1) antisense RNA 1 (LEF1-AS1) is a highly conserved and newly discovered long non-coding RNA encode in the plus strand of LEF1 at chromosome 4q25. Many studies have demonstrated that LEF1-AS1 is enrolled in the tumorigenesis of a variety of cancer, such as glioblastoma,⁸ oral squamous cell carcinoma,⁹ non-small cell lung cancer¹⁰ and prostate cancer.¹² Furthermore, several recent studies had indicated that LEF1-AS1 was upregulated and correlated with the overall and recurrence-free survival of colon cancer patients, but the exact role of LEF1-AS1 in colon cancer was uncertain.¹²⁻¹⁴

Sex-determining Region Y box 9 (SOX9) is a member of SRY-related high-mobility group box (SOX) transcription factors that controls cell fate by directing cell differentiation and maintaining tissue homeostasis.¹⁵ Mutation of SOX9 was firstly identified as the cause of campomelic dysplasia, a severe skeletal malformation syndrome with defective chondrogenesis and variable 46+XY sex reversal in 1994.¹⁶ In addition, SOX9 was found to play important roles in the development of testis, pancreas, intestine, brain and kidney.¹⁷ During the development of intestine, SOX9 was expressed in the progenitor cells at the bottom of the

intestinal crypts, and the expression level of SOX9 seemed to control the proliferation and differentiation of these cells.¹⁸ SOX9 is also dysregulated in many cancers and implicated in tumor growth, invasion and metastasis.^{19,20} Knockout SOX9 in mouse models repressed tumorigenesis of prostate and pancreatic cancer,^{19,21} while overexpression of SOX9 in prostate cancer cell lines enhanced tumor growth and invasion.²⁰ In colon cancer, SOX9 was overexpressed and high expression of SOX9 promoted migration, invasion and epithelial mesenchymal transition of colon cancer cell lines.²²

In our study, we found that LEF1-AS1 promoted migration, invasion and anchorage-independent growth of colon cancer cells in vitro and facilitated tumor xenograft growth and lung metastasis in vivo. In addition, LEF1-AS1 mediated SOX9 expression by serving as a molecular sponge for miR-30-5p, and SOX9 restoration abolished the effects caused by LEF1-AS1 knockdown in colon cancer cells. Our results suggested that LEF1 exerted an oncogenic role in colon cancer via miR-30-5p/SOX9 axis. Thus, LEF1-AS1 could be a potential prognostic biomarker for colon cancer.

Materials and Methods

Patient Samples

Written informed consent was obtained from all participants in our study. The use and collection of tissue samples were reviewed and approved by the ethics committee of Cancer Hospital of China Medical University. A total of 50 pairs of colon cancer samples and matched tumor-adjacent tissues were provided by Cancer Hospital of China Medical University from February 2014 to September 2015. All tissue samples were fresh frozen and stored at -80°C . The demographic and clinicopathological features of these patients were retrieved from database and the follow up was continued for 48 months after surgery for survival analysis.

Cell Culture

Colon cancer cell lines COLO320, SW480, SW1417, SW948, T84, HT29 and human HEK293T cell line were obtained from American Type Culture Collection (ATCC). Colon cancer cell line COLO678 and CL11 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). SW480, COLO678 and HEK293T cells were cultured with RPMI-1640 medium (Invitrogen, USA). COLO320, SW1417, SW948, T84, HT29 and CL11

cells were cultured with DMEM: F12 (1:1) medium (Thermo Fisher Scientific, USA). All cells were supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% PenStrep (100 U/mL Penicillium and 100 µg/mL Streptomycin) in a humidified incubator containing 5% CO₂ at 37°C.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA of tissue samples and culture cells was extracted by TRIzol reagent (Invitrogen, USA) as the protocol described. RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used to generate Complementary DNA. The expression of miR-30-5p family was quantified by TaqMan microRNA Reverse Transcription Kit (Applied Biosystem, USA) as protocol described. The expression of LEF1-AS1 and SOX9 was analyzed using SYBR Premix Ex Taq kit (Takara, Japan) in ABI 7900 Real Time PCR system according to the manufactures' instruction. GAPDH or snRNAU6 was used as an internal control. The relative expression levels were calculated by the equation $2^{-\Delta\Delta CT}$. The primers used for qRT-PCR were: LEF1-AS1, forward: 3'-TGT GAC TCC AGA GGC GGA CCA C-5'; reverse: 3'-AGG AGG CGG CAA GAA GAA G-5'; SOX9: 3'-CGT CAA CGG CTC CAG CAA C-5', reverse: 3'-CCG CTT CTC GCT CTC GTT CA-5'.

RNA Fluorescent in situ Hybridization

Cy3-labeled LEF1-AS1 probe was purchased from Ribobio (Guangzhou, China). Subcellular localization of LEF1-AS2 was detected by the RNA Fluorescent in situ hybridization kit (Ribobio# R1106, Guangzhou) as the manufacturer's instruction described. In brief, COLO320 cells were fixed by 4% paraformaldehyde, then incubated with LEF1-AS1 lncRNA-Fish probe mix at 37°C overnight. Washed with hybridization buffer and PBS for 5 min, then cells were incubated with DAPI for 10 min avoiding night. The fluorescent signal was detected by LSM 5Pa Laser Scanning Microscope (Zeiss Germany, Oberkochen, Germany).

Plasmid Constructs, Lentivirus Packaging and Infection

LEF1-AS1 and SOX9 expression plasmids were constructed by cloning the cDNA sequence into the pCDH-CMV-MCS-EF1-Puro (System Biosciences #CD510B-1) lentiviral vector. The pCDH-CMV-MCS-EF1-Puro empty plasmid was used as empty vector control (EV). The primer sequences for cloning the cDNA sequence of LEF1-AS1 or SOX9 are shown in Table 1. Two short-hairpin RNA targeting LEF1-AS1 (sh-LEF1-AS1-1 and sh-LEF1-AS1-2) and non-targeting control (sh-ctrl) were designed (Table 2) and synthesized by GenePharma (Shanghai, China). The expression plasmids of miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p and miR-30e-5p were constructed by cloning the mature sequence of each microRNA into the pCMV-MIR lentiviral plasmid (CnGene #PCMVIR). The empty pCMV-MIR lentiviral plasmid was used as miR-ctrl. Lentivirus packaging was conducted by introducing the lentiviral expression plasmids plus with lentivirus packaging vectors $\delta 8.9$ and Ψ SVG into HEK293T cells using Lipofectamine 3000 (Life Technologies #L3000015) as the protocol described. Lentiviral particles were collected and stored at -80°C. For virus infection, cells were incubated with virus particles overnight with 8 µg/mL polybrene (Sigma-Aldrich, USA).

Cell Viability Assay

CellTiter-Glo Luminescent Cell Viability Assay kit (Promega #G7572) was used to evaluate cell viability in accordance with the manufacturer's instruction. In brief, the cells and CellTiter-Glo reagent were balanced to room temperature, then mixed thoroughly on an orbital shaker. Incubated the plates avoiding light for 10 min at room temperature, then the luminescence signal was recorded. All assays were done in triplicates.

Colony Formation Assay

HT29, T84 or COLO320 cells were seeded in 6-well plates at 1500 cells per well, then cultured for 3 weeks. Colonies were fixed by 4% paraformaldehyde for 15 min, and stained

Table 1 Primer Sequences for LEF1-AS1 or SOX9

Name	Forward Primer (5'>3')	Reverse Primer (5'>3')
LEF1-AS1 expression vector	CCCTTTGTGTGGCCTGGACT	TTCCTGATGCTCTAGGTTGGTT
SOX9 expression vector	GCGTATGAATCTCCTGGA	AACACAAATGTCCAAAGGG
3'UTR of SOX9	CCAGAACTCCAGCTCCTA	CTCCTCCTTAAATCGCAAAG

Table 2 LEF1-AS1 shRNA Sequences

shRNA	Sequence (5'>3')
sh-LEF1-AS1-1	CCUGGGUGGAUAUGGUAUU
sh-LEF1-AS1-2	GGAGAUUAAUGCAGAACAA
sh-ctrl	ACGGAGGCTAAGCGTCGCAA

with crystal violet for 1.5 h at room temperature. Images were obtained by a scanner (Microtek, TMA 1600III). All assays were done in triplicates.

Soft Agar Assay

Soft agar assay was performed as previously described.²³ HT29 (8000/well), T84 (8000/well) or COLO320 (10,000/well) cells were seeded in 0.4% top agar in 6-well plates and cultured for 3~4 weeks. Stained the colonies with 1 mg/mL thiazolyl blue tetrazolium bromide (MTT) for 1.5 h. Took photos with a scanner (Microtek, TMA 1600III). All assays were carried out in triplicates.

Wound Healing Assay

HT29, T84 or COLO320 cells were seeded in 6-well plates and cultured until cell confluence reached 90%. Scratched the cell monolayer with a sterile plastic tip, then washed away the cell debris with culture medium. Images were obtained at 0, 24 and 48 h by microscope (IX71, Olympus, Japan). The width of the scratched gaps at 0, 24 and 48 h was measured by Digimizer software system (MedCalc software, Belgium). Wound closure was calculated by the equation: Wound closure (%) = (1 - width at 24 or 48 h / width at 0 h) × 100%.

Transwell Cell Invasion Assay

Matrigel invasion chamber (8 µm, BD Bioscience, USA) was used to evaluate cell invasion. Briefly, cells (1×10^5) were seeded in the upper chamber without serum. The lower chamber was filled with RPMI1640 medium with 10% FBS. Then cultured for 24 h, and the invasion cells were stained with Giemsa dye. All experiments were carried out in triplicates.

Luciferase Reporter Assay

LEF1-AS1 or the 3'-UTR of SOX9 were cloned into the pMIR-REPORT plasmid, then the potential SOX9 and LEF1-AS1 binding sites for miR-30-5p were mutated by Quick-change site-directed mutagenesis kit (Agilent Technologies, USA), and verified by sanger sequencing. The primer sequences for cloning LEF1-AS1 or the 3'-UTR of SOX9 are shown in Table 1. For luciferase reporter assay, HEK 293T cells were co-transfected with the pMIR-REPORT

plasmid containing LEF1-AS1 or the 3'-UTR of SOX9, the miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p or miR-ctrl expression plasmid, and a renilla luciferase plasmid with a ratio of 2:2: 1. Dual Luciferase reporter assay system (Promega#E1910) was used to evaluate luciferase activity. All assays were done in triplicates.

Pull-Down Assay with Biotinylated miR-30-5p

COLO320 cells were transfected with biotinylated wild-type or mutant miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p, or negative control (NC). Wild-type or mutant SOX9 transduced HT29 cells were transfected with biotinylated miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p or negative control (NC). Cell lysates of these cells were collected 48 h post-transfection, then incubated with Dynabeads M-280 Streptavidin (Invitrogen, USA) overnight at 4°C as previously described. The beads were washed three times with ice-cold lysis buffer and once with high salt buffer as previously reported.²⁴ The bound RNAs were extracted by TRIzol reagent and evaluated for LEF1-AS1 or SOX9 expression by qRT-PCR.

Western Blot

Collected protein lysates by digesting cells in RIPA buffer containing protease inhibitors (Sigma-Aldrich, USA). BCA protein assay kit (Thermo Scientific, USA) was used to evaluate protein concentration. A total of 30 µg protein was electrophoresed on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Then, the membranes were incubated with specific first antibodies and corresponding second antibody. The specific antibodies were list as below: SOX9 (Cell signaling #82630; 1:1000), GAPDH (Cell signaling #2118; 1:1000). The second antibody was goat anti-rabbit IgG HRP-linked antibody (Cell signaling #7074; 1:4000).

Tumor Xenograft Growth and Lung Metastasis Model

All animal experiments were approved by the Animal Care and Experimental Committee of Cancer Hospital of China Medical University. HT29 cells (2×10^6) transduced with EV or LEF1-AS1, or COLO320 cells (2×10^6) transduced with sh-LEF1-AS1-1, sh-LEF1-AS1-2 or sh-ctrl were subcutaneously injected in nude mice. Tumor volume was measured every three days by calipers and calculated by the formula: Tumor volume = (length × width²)/2. All

mice were sacrificed five weeks after tumor cells injection, then tumors were dissected out and weighed. HT29 cells (3×10^6) transduced with EV or LEF1-AS1 were injected into the tail vein of nude mice, then tumor lung metastasis was calculated 4 weeks post-injection.

Statistical Analysis

Data were analyzed by GraphPad Prism 6 software (GraphPad Inc., USA). The difference between two groups was analyzed by Student's *t*-test. The difference between multiple groups was analyzed by two-way ANOVA. Overall survival and recurrent-free survival were plotted by the Kaplan–Meier method and estimated by the Log-rank test. All data were shown as mean \pm standard deviation ($\bar{x} \pm s.d$) or mean \pm standard Error of Mean ($\bar{x} \pm s.e.m$) with at least three replicates. $P < 0.05$ was considered statistically significant.

Results

LEF1-AS1 Is Upregulated and Correlated with Poor Prognosis in Colon Cancer

In a previous study, LEF1-AS1 was found to be upregulated in colorectal cancer patients in The Cancer Genome Atlas (TCGA) dataset and the Gene Expression Omnibus (GEO) datasets (GSE50760, GSE70880), and high expression of LEF1-AS1 predicted worse overall and recurrent-free survival.¹³ Besides, another study demonstrated that high LEF1-AS1 expression was associated with lymph node metastasis and shorter overall survival in metastatic colon cancer patients.¹⁴ To further prove this, the expression level of LEF1-AS1 in 50 colon cancer samples and matched tumor-adjacent tissues was determined by qRT-PCR. As shown in Figure 1A, we found that LEF1-AS1 was obviously overexpressed in colon cancer tissues (Tumor) compared with tumor-adjacent tissues (Normal) ($P < 0.001$) corresponding with previous study. We further analyzed the correlation of LEF1-AS1 expression with clinicopathological characteristics of colon cancer patients (Table 3). In our study, the colon cancer patients were divided into Low LEF1-AS1 expression group and High LEF1-AS1 expression group by using the median of the cohort as cut-off value. We found that high LEF1-AS1 expression was positively correlated with a higher Dukes staging ($P = 0.041$) and lymph node metastasis ($P < 0.001$) (Table 3). In addition, colon cancer patients with high LEF1-AS1 expression were prone to have distant metastasis, but this was not statistically significant ($P = 0.128$)

due to the small number of patients analyzed. Kaplan–Meier survival analysis showed that colon cancer patients with high LEF1-AS1 expression had a prominent poor overall-survival and recurrent-free survival than these with low LEF1-AS1 expression, corresponding with previous study (Figure 1B and C). In addition, the expression level of LEF1-AS1 was evaluated in a number of colon cancer cells. We found that LEF1-AS1 was overexpressed in COLO320 and CL11 cell lines, and low expressed in SW948, T84 and HT29 (Figure 1D). The subcellular localization of LEF1-AS1 was detected by RNA fluorescent in situ hybridization, and we found that LEF1-AS1 was mainly located in the cytoplasm of COLO320 cells (Figure 1E). Collectively, our data showed that LEF1-AS1 was upregulated in colon cancer patients, and high LEF1-AS1 expression was closely correlated with poor overall-survival and recurrent-free survival, corresponding with previous reports.

LEF1-AS1 Promotes Migration, Invasion and Anchorage-Independent Growth of Colon Cancer Cells

To study the biological functions of LEF1-AS1 in colon cancer, we enforced LEF1-AS1 expression in two colon cancer cell lines HT29 and T84 with low endogenous LEF1-AS1. By transducing with a LEF1-AS1 expression lentiviral vector, HT29 and T84 showed comparable LEF1-AS1 expression with COLO320 (Figure 2A). Next, cell viability assay was conducted, and we found that ectopic LEF1-AS1 expression in HT29 and T84 had no obvious influence on cell proliferation (Figure 2B). Moreover, overexpression of LEF1-AS1 had no evident impact on colony formation of HT29 and T84 cells in colony formation assay, yet (Figure 2C). Since colon cancer patients with high LEF1-AS1 expression were inclined to have a higher Dukes staging, lymph node metastasis and distant metastasis, we speculated that LEF1-AS1 might enroll in the migration, invasion or metastasis of colon cancer. In wound healing assay, we found that HT29 and T84 cells transduced with LEF1-AS1 expression vector had a significant increase in wound healing ability compared with cells transduced with empty vector (EV), indicating that LEF1-AS1 might accelerate cell migration of colon cancer cells (Figure 2D and E). Moreover, the number of invasion cells were apparently increased in LEF1-AS1 overexpressed HT29 and T84 cells compared with EV transduced cells in transwell cell invasion assay,

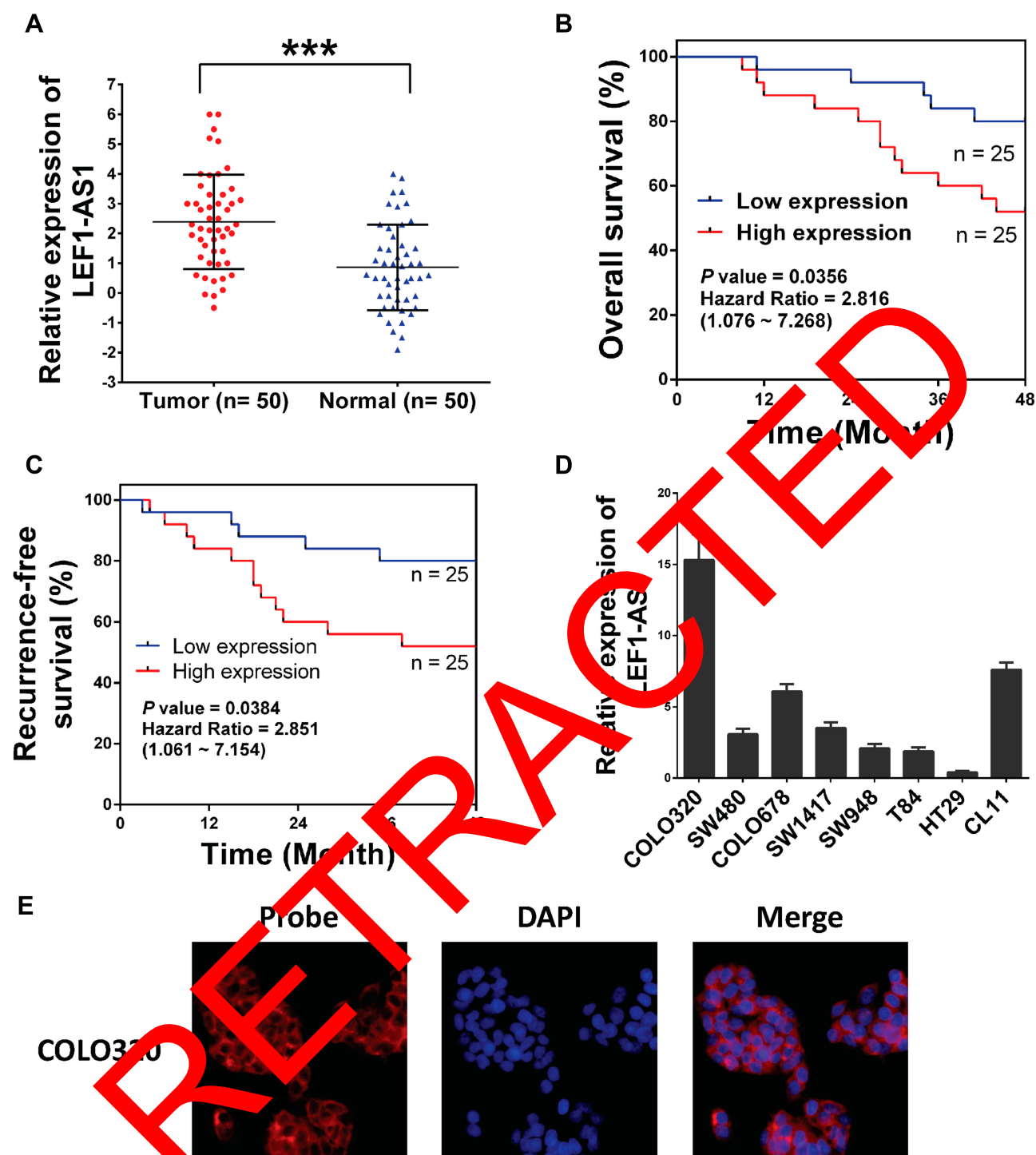


Figure 1 LEF1-AS1 is upregulated and correlated with poor prognosis in colon cancer: **(A)** the relative expression level of LEF1-AS1 in 50 colon cancer samples (Tumor) and matched tumor-adjacent tissues (Normal) was evaluated by qRT-PCR. **(B, C)** Kaplan-Meier survival analysis of colon cancer patients according to LEF1-AS1 expression. Overall survival **(B)** and recurrence-free survival **(C)** were shown. The median of the cohort was used as a cut-off value. **(D)** relative expression of LEF1-AS1 in colon cancer cell lines was evaluated by qRT-PCR. **(E)** Subcellular localization of LEF1-AS1 in COLO320 cells was detected by RNA fluorescent in situ hybridization. *** $P < 0.05$ comparing with matched tumor-adjacent tissues.

indicating that LEF1-AS1 enhanced invasion of colon cancer cells (Figure 2F). In soft agar assay, HT29 and T84 cells transduced with LEF1-AS1 expression vector formed more colonies than cells transduced with empty

vector, suggesting that LEF1-AS1 might increase anchorage-independent growth (Figure 2G).

To further verify the biological functions of LEF1-AS1 in colon cancer cells, we knocked down LEF1-AS1

Table 3 Correlation Between LEF1-AS1 Expression and Clinicopathological Characteristics in Colon Cancer Patients

Characteristics	n	LEF1-AS1 Expression		P value
		Low	High	
Age (Years)				0.806
≤60	21	13	8	
>60	29	12	17	
Sex				0.621
Male	31	13	18	
Female	19	12	7	
Tumor size (cm)				0.515
≥5	22	14	8	
<5	28	11	17	
Dukes staging				0.041
A + B	23	17	6	
C + D	27	8	19	
Differentiation				0.695
Well	36	19	17	
Poor	14	6	8	
Lymph node metastasis				<0.001
Positive	32	11	21	
Negative	18	14	4	
Distant metastasis				0.001
Positive	12	3	9	
Negative	38	22	16	

expression in COLO320 cells by small hairpin RNA (shRNA), then cell viability assay, colony formation assay, wound healing assay, transwell cell invasion assay and soft agar assay were conducted to evaluate the changes. As shown in Figure 3A, the expression level of LEF1-AS1 in COLO320 cells was distinctly decreased after transducing with LEF1-AS1 shRNA (sh-LEF1-AS1-1 and sh-LEF1-AS1-2). Corresponding with previous results, we found that LEF1-AS1 knockdown had no evident influence on cell proliferation and colony formation of COLO320 cells (Figure 3B and C). In wound healing assay, knocked down LEF1-AS1 apparently impaired the wound healing potential of COLO320 cells, indicating that LEF1-AS1 knockdown might repress cell migration (Figure 3D). In transwell cell invasion assay, COLO320 cells transduced with sh-LEF1-AS1-1 or sh-LEF1-AS1-2 had small amount of invasion cells than COLO320 cells transduced with sh-ctrl, revealing that LEF1-AS1 knockdown also suppressed cell invasion (Figure 3E). Furthermore, knocked down LEF1-AS1 significantly inhibited colony formation of COLO320 cells in soft

agar assay, indicating a reduced anchorage-independent growth (Figure 3F and G). Taken together, these results suggested a role of LEF1-AS1 in increasing migration, invasion and anchorage-independent growth of colon cancer cells.

LEF1-AS1 Facilitates Tumor Xenograft Growth and Lung Metastasis of Colon Cancer Cells

We further analyzed the potential biological functions of LEF1-AS1 in colon cancer cells *in vivo*. In our study, LEF1-AS1 or EV transduced HT29 cells (2×10^6) were subcutaneously injected into nude mice, then tumor xenograft growth was monitored. As shown in Figure 4A, HT29 cells transduced with LEF1-AS1 had more rapid tumor growth than EV transduced cells. The tumor volume (Figure 4B) and tumor weight (Figure 4C) of LEF1-AS1 transduced HT29 cells were evidently increased, too. These results were further validated in COLO320 cells. Tumor xenograft growth of sh-LEF1-AS1-1 or sh-LEF1-AS1-2 transduced COLO320 cells was evaluated and our results suggested that knocked down LEF1-AS1 expression obviously impaired tumor growth (Figure 4D) and reduced tumor volume (Figure 4E) and weight (Figure 4F). Since LEF1-AS1 increased migration and invasion of colon cancer cell lines, we postulated that LEF1-AS1 might affect lung metastasis of colon cancer cells *in vivo*. Thus, LEF1-AS1 or EV transduced HT29 cells (3×10^6) were injected into the tail vein of nude mice and tumor lung metastasis was tested. As we expected, LEF1-AS1 transduced HT29 cells formed more tumors than EV transduced HT29 cells in the lungs of nude mice, indicating that LEF1-AS1 facilitated metastasis of colon cancer cells (Figure 4G and H). Above all, our results revealed that LEF1-AS1 might increase tumor growth and lung metastasis of colon cancer cells *in vivo*.

LEF1-AS1 Mediates SOX9 Expression by Serving as a Molecular Sponge for miR-30-5p Family

To disclose the underlying mechanism of LEF1-AS1 in colon cancer, we predicted 37 microRNAs containing the possible binding sites for LEF1-AS1 using the LncBase Predicted V.2 tools.²⁵ Among the predicted microRNAs, we found that knocked down LEF1-AS1 expression in COLO320 cells upregulated the expression level of miR-30-5p family (miR-30a-5p, miR-30b-5p, miR-30c-5p,

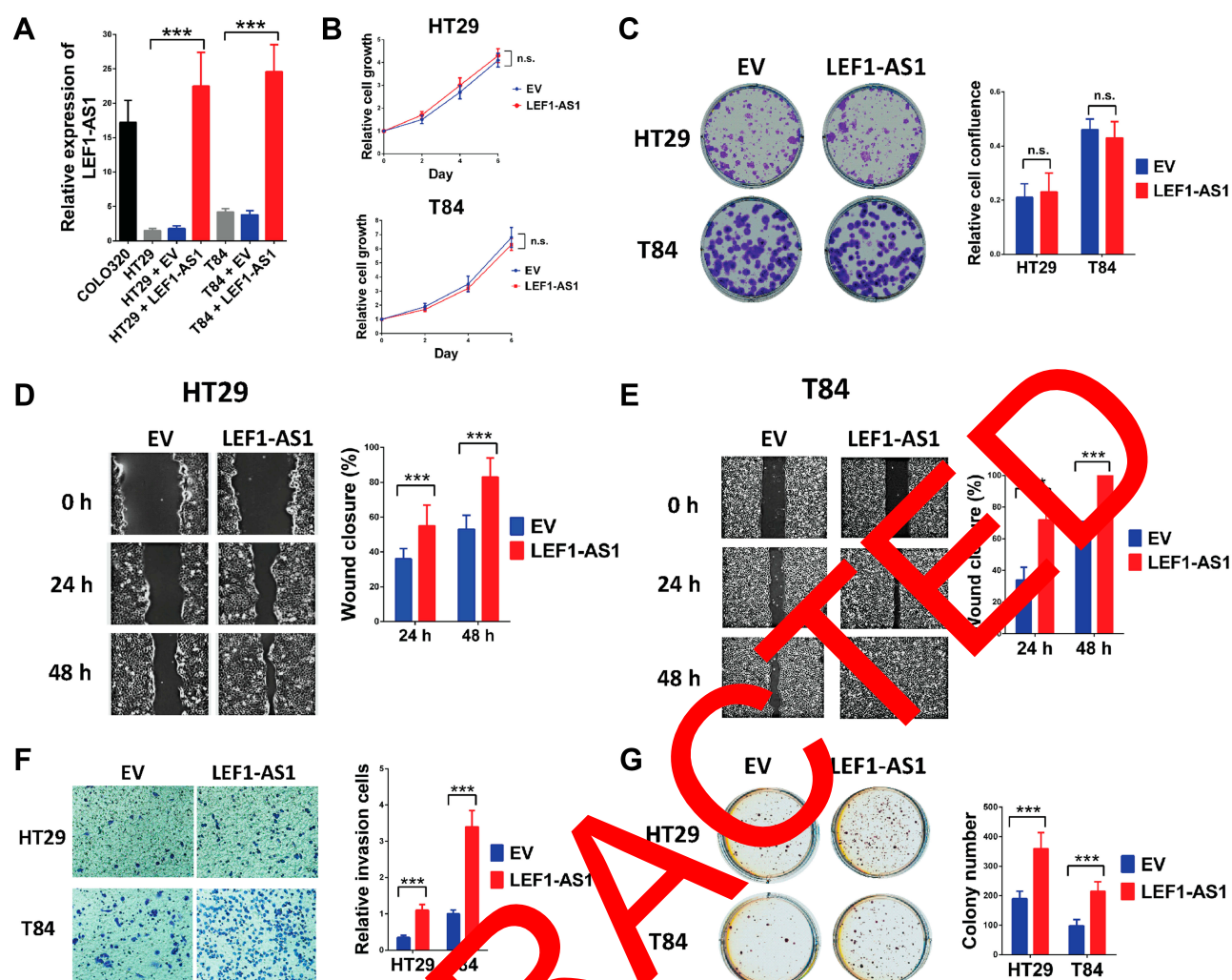


Figure 2 Ectopic expression of LEF1-AS1 increases cell migration and invasion of colon cancer cells. (A) HT29 or T84 cells were transfected with empty vector (EV) or LEF1-AS1 expression vector, then relative expression of LEF1-AS1 was evaluated by qRT-PCR. COLO320 cell was used as internal control. (B) HT29 or T84 cells transfected with EV or LEF1-AS1 expression vector were seeded in 6-well-plate at 500 cells per well, then cell viability assay was conducted every two days. Relative cell growth was calculated by comparing with Day 0. (C) HT29 or T84 cells transfected with EV or LEF1-AS1 expression vector were seeded in 6-well-plate at 1500 cells per well, then cultured for three weeks for colony formation assay. Representative plates and relative cell confluence were shown. (D, E) HT29 or T84 cells transfected with EV or LEF1-AS1 expression vector were used for scratch wound healing assay. Representative images were taken at 0 h, 24 h and 48 h, and the wound closure was calculated. (F) HT29 or T84 cells transfected with EV or LEF1-AS1 expression vector were used for transwell cell migration assay. Representative images at 24 h post-incubation and relative invasion cells were shown. (G) HT29 or T84 cells transfected with EV or LEF1-AS1 expression vector were used for soft agar assay. Representative plates and average colony number were shown. All assays were done in triplicate. *** $P < 0.05$ comparing with EV. n.s., no significance.

miR-30d-5p and miR-30e-5p), indicating that LEF1-AS1 might regulate the expression of miR-30-5p (Figure 5A). Moreover, we further validated this in HT29 cells, and found that enforced LEF1-AS1 expression obviously repressed miR-30-5p expression (Figure 5B). These results indicated the expression level of LEF1-AS1 and miR-30-5p were inversely correlated. The putative binding sites for LEF1-AS1 and miR-30-5p predicted by LncBase Predicted V.2 are shown in Figure 5C. In luciferase reporter assay, we found that overexpression of miR-30-5p reduced the luciferase activity of vectors containing wild-type LEF1-AS1, but had no obvious influence on the

luciferase activity of LEF1-AS1 vectors with a 5-bp mutation at the putative binding sites (Figure 5C). Furthermore, in the pull-down assay with biotin-labeled miR-30-5p, LEF1-AS1 was successfully pulled down by wild type miR-30-5p in COLO320 cells, but miR-30-5p with 4-bp mutagenesis at the putative binding sites failed (Figure 5D). These results suggested that LEF1-AS1 might directly interact with and act as a molecular sponge for miR-30-5p family.

In order to explore the potential target genes for miR-30-5p, we used three different databases (starBase V3.0, TargetScan 7.2 and TarBase v.8) to predict the candidate

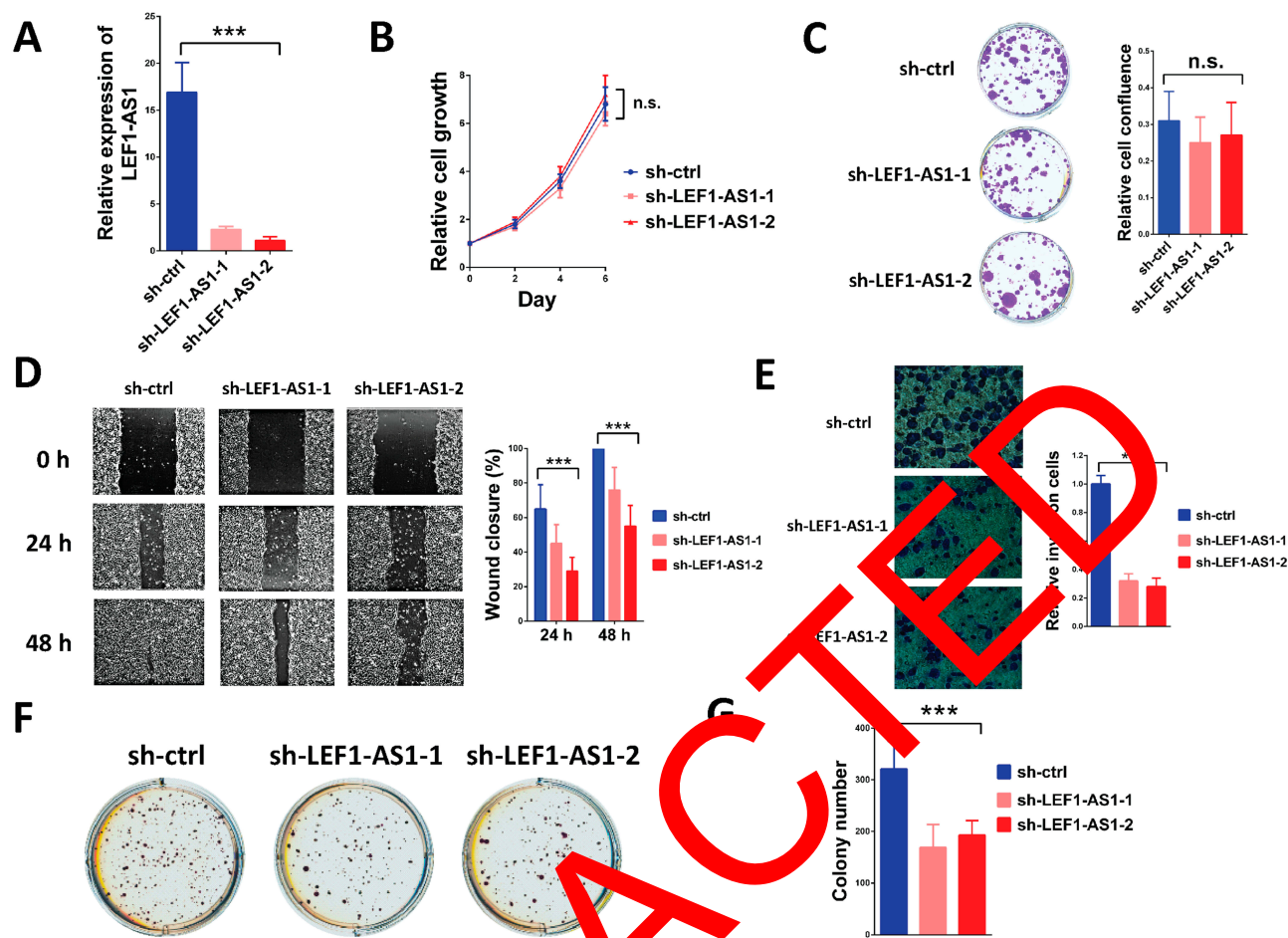


Figure 3 LEF1-AS1 knockdown suppresses cell migration and invasion of colon cancer cells. (A) COLO320 cells were transduced with sh-LEF1-AS1-1, sh-LEF1-AS1-2 or sh-ctrl, then relative expression of LEF1-AS1 was evaluated by qRT-PCR. (B) COLO320 cells transduced with sh-LEF1-AS1-1, sh-LEF1-AS1-2 or sh-ctrl were seeded in 96-well-plate at 5000 cells per well, then cell viability assay was conducted for two days. Relative cell growth was calculated by comparing with Day 0. (C) COLO320 cells transduced with sh-LEF1-AS1-1, sh-LEF1-AS1-2 or sh-ctrl were used for colony formation assay. Represent plates and relative cell confluence were shown. (D) COLO320 cells transduced with sh-LEF1-AS1-1, sh-LEF1-AS1-2 or sh-ctrl were used for scratch wound healing assay. Represent images at 0 h, 24 h and 48 h were shown, and the wound closure was calculated. (E) COLO320 cells transduced with sh-LEF1-AS1-1, sh-LEF1-AS1-2 or sh-ctrl were used for transwell cell invasion assay. Represent images taken 24 h post-incubation and relative invasion cells were shown. (F) COLO320 cells transduced with sh-LEF1-AS1-1, sh-LEF1-AS1-2 or sh-ctrl were used for soft agar assay. Represent plates (F) and average colony number (G) were shown. All assays were done in triplicates. *** $P < 0.05$ comparing with sh-ctrl. n.s., no significance.

genes. We found 2 candidate genes containing the complementary site for the seed region of miR-30-5p, among them *SOX9* was discovered in our study (Figure 6A). Enforced expression of miR-30-5p (miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p and miR-30e-5p) in COLO320 cells significantly suppressed the level of *SOX9* mRNA (Figure 6B). Furthermore, we cloned the 3'UTR sequence of *SOX9* that containing the predicted miR-30-5p binding sites for luciferase reporter assay. In our study, introducing miR-30-5p evidently reduced the luciferase activity of *SOX9* 3'UTR in HEK 293T cells compared with miR-ctrl (Figure 6C). Moreover, a 6-bp mutation of *SOX9* at the seed sequence of the putative binding site for miR-30-5p (Figure 6A) abolished the effect caused by miR-30-5p (Figure 6C). In the pull-down assay with biotin-

labeled miR-30-5p, wild type *SOX9* was successfully pulled down, while *SOX9* with a 6-bp mutation failed (Figure 6D). These results indicated that *SOX9* was a direct target for miR-30-5p. In addition, ectopic expression of LEF1-AS1 in HT29 and T84 cells significantly increased the expression level of *SOX9* mRNA compared with cells transduced with EV (Figure 6E). However, this effect was largely abolished by restoring miR-30-5p (miR-30a-5p or miR-30b-5p) in these cells, indicating that LEF1-AS1 might regulate *SOX9* expression through miR-30-5p (Figure 6E). This was further validated by Western blot (Figure 6F). In our study, overexpressed LEF1-AS1 in HT29 and T84 cells increased the protein expression of *SOX9* compared with EV transduced cells, but this was reversed by restoring miR-30-5p (miR-30a-5p or miR-30b-5p) in these cells

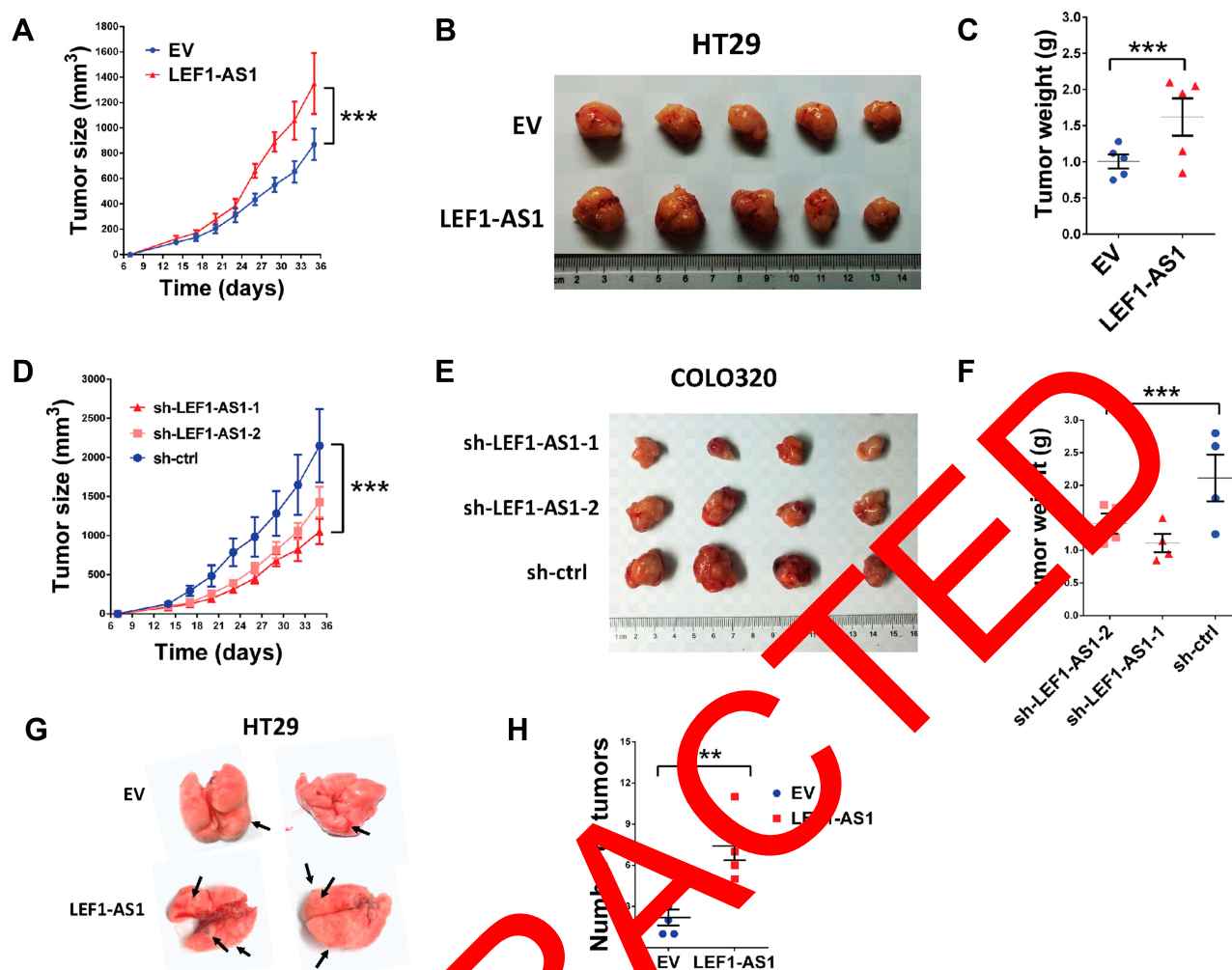


Figure 4 LEF1-AS1 facilitates tumor xenograft growth and lung metastasis of colon cancer cells. (A–C) HT29 cells (2×10^6) transduced with EV or LEF1-AS1 were subcutaneous injected in nude mice ($n = 5$). Tumor growth curves (A), represent images (B) and tumor weight (C) were shown. (D–F) COLO320 cells (2×10^6) transduced with sh-LEF1-AS1-1, sh-LEF1-AS1-2 or sh-ctrl were subcutaneous injected in nude mice ($n = 4$). Tumor growth curves (D), represent images (E) and tumor weight (F) were shown. G–H, HT29 cells (3×10^6) transduced with EV or LEF1-AS1 were injected into the tail vein of nude mice ($n = 5$). Represent images of tumors metastasized to lung were shown (G), and the number of tumors in lung was calculated (H). *** $P < 0.05$ comparing with EV or sh-ctrl.

(Figure 6D). Collectively, our results suggested that LEF1-AS1 mediated SOX9 expression by serving as a molecular sponge for miR-30-5p family.

SOX9 Restoration Abolishes the Effects Caused by LEF1-AS1 Knockdown in COLO320 Cells

To explore whether SOX9 was critical for the effects caused by LEF1-AS1 knockdown, COLO320 cells transduced with sh-LEF1-AS1-1 were restored with SOX9 expression lentiviral vectors, then wound healing assay, transwell cell invasion assay, soft agar assay and tumor xenograft formation assay were conducted. Firstly, we confirmed that LEF1-AS1 expression in COLO320 cells was successfully

knocked down by sh-LEF1-AS1-1 compared with cells transduced with sh-ctrl (Figure 7A). Besides, restoration of SOX9 had little influence on the expression of LEF1-AS1 in sh-LEF1-AS1-1 transduced COLO320 cells (Figure 7A). In wound healing assay, restoring SOX9 reversed the suppressive effect on wound healing caused by LEF1-AS1 knockdown (Figure 7B). In transwell cell invasion assay, LEF1-AS1 knockdown reduced the number of invasion cells but this was partially attenuated by SOX9 restoration (Figure 7C). In addition, LEF1-AS1 knockdown significantly suppressed colony formation of COLO320 cells in soft agar assay, but SOX9 restoration abrogated this effect (Figure 7D). These results indicated that SOX9 restoration abolished the effects caused by LEF1-AS1 knockdown in COLO320 cells in vitro, and this was further

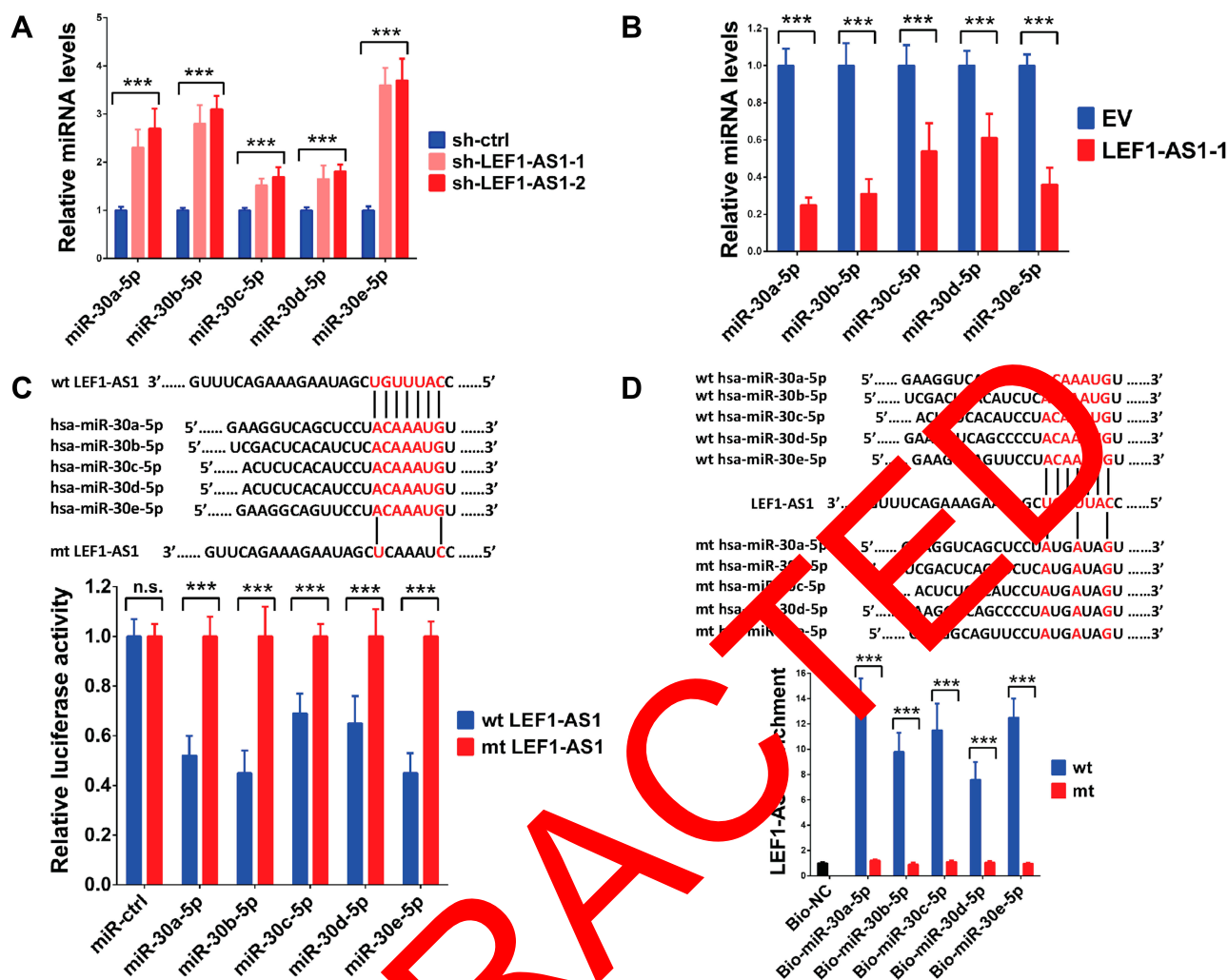


Figure 5 LEF1-AS1 acts as a molecular sponge for miR-30-5p in colon cancer cells. **(A)** COLO320 cells were transduced with sh-LEF1-AS1-1, sh-LEF1-AS1-2 or sh-ctrl, then relative expression of miR-30-5p family (miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p and miR-30e-5p) was evaluated by qRT-PCR. **(B)** HT29 cells were transduced with empty vector (EV) or LEF1-AS1 expression vector, then relative expression of the miR-30-5p family was evaluated by qRT-PCR. **(C)** HEK293T cells transduced with wild-type LEF1-AS1 (wt LEF1-AS1) or mutant LEF1-AS1 (mt LEF1-AS1) expression pMIR-REPORT vector were used for luciferase reporter assay. Cells were co-transfected with miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p or miR-ctrl expression vector as indicated. The putative binding sites of miR-30-5p family for wt LEF1-AS1 or mt LEF1-AS1 were shown. **(D)** the putative binding sites of LEF1-AS1 for wt or mt miR-30-5p were shown. Relative expression of LEF1-AS1 in the bound RNAs pulled down by biotinylated wild-type (wt) miR-30-5p, mutant (mt) miR-30-5p and negative control (NC) were evaluated by qRT-PCR. All assays were done in triplicates. *** $p < 0.05$ comparing with sh-ctrl, EV or mt LEF1-AS1 or mt. n.s., no significance.

validated in mouse tumor xenograft model in vivo. In our study, tumor growth of COLO320 cells was obviously repressed by transducing with sh-LEF1-AS1-1, while cells introducing with sh-LEF1-AS1-1 and SOX9 together showed no great difference in tumor growth compared with cells transduced with sh-ctrl and EV, indicating that SOX9 restoration abolished the effects of LEF1-AS1 knockdown in vivo (Figure 7E–G). Overall, our results suggested that the inhibitory effects of LEF1-AS1 knockdown on cell migration, invasion, anchorage-independent growth and tumor xenograft growth of COLO320 cells were at least partially due to SOX9 inhibition.

Discussion

Human transcriptome sequencing identifies a great number of dysregulated lncRNAs in colon cancer, some of which play essential roles in tumorigenesis and progression.²⁶ For example, lncRNA H19 was found to be overexpressed in colon cancer tissues and cell lines, and H19 knockdown inhibited migration and invasion of colon cancer cells through miR-138/HMGA1 axis.²⁷ lncRNA CRNDE was upregulated in colon cancer tissues, and CRNDE overexpression promoted cell proliferation and chemoresistance of colon cancer cells via miR-181a-5p mediated regulation of Wnt/ β -catenin signaling.²⁸ lncRNA Linc00659 was significantly increased

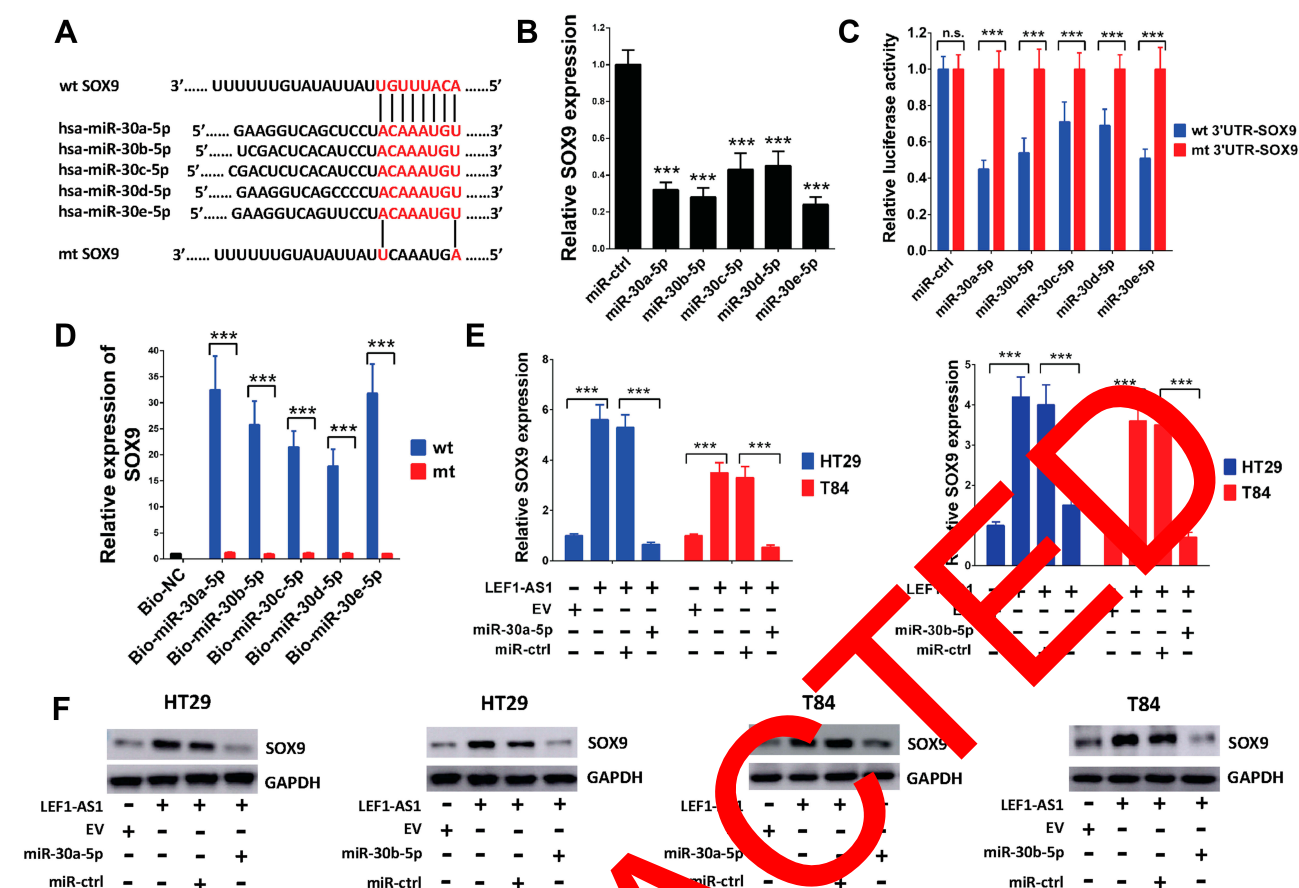


Figure 6 LEF1-AS1 mediates SOX9 expression through miR-30-5p in colon cancer cells. (A) The putative binding sites of miR-30-5p family with wt SOX9 or mt SOX9 were shown. (B) COLO320 cells were transduced with miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p or miR-ctrl, then relative expression of SOX9 was evaluated by qRT-PCR. (C) HEK293T cells transduced with wt SOX9 or mt SOX9 expression pMIR-REPORT vector were used for luciferase reporter assay. Cells were co-transfected with miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p or miR-ctrl expression vector as indicated. (D) Relative expression of SOX9 in the bound RNAs pulled down by biotinylated wild-type (wt) miR-30-5p, mutant (mt) miR-30-5p and negative control (NC) were evaluated by qRT-PCR. (E, F) HT29 and T84 cells were transduced with LEF1-AS1, EV, miR-30a-5p, miR-30b-5p or miR-ctrl as indicated, then SOX9 expression was evaluated by qRT-PCR (E) or Western blot (F). All assays were done in triplicates. *** $P < 0.05$ comparing with miR-ctrl or EV.

and positively correlated with poor survival in colon cancer patients, and Linc00659 knockdown impaired cell cycle progression and suppressed PI3K/AKT signaling.²⁹ Besides the well-characterized lncRNAs, it is also important to elucidate the biological functions of some newly found and dysregulated lncRNAs in colon cancer. Among them, lncRNA LEF1-AS1 was reported to be upregulated and correlated with lymph node metastasis and poor survival in colon cancer patients, but the exact role of LEF1-AS1 was uncertain.^{13,14} In our study, we have demonstrated this, and furthermore, we found that enforced LEF1-AS1 expression in colon cancer cells promoted migration, invasion and anchorage-independent growth in vitro and tumor xenograft growth and lung metastasis in vivo. Instead, LEF1-AS1 knockdown suppressed migration, invasion, anchorage-independent growth and tumor xenograft formation. In addition, LEF1-AS1 acted as a molecular sponge for miR-30-5p, and further regulated SOX9 expression.

Restored SOX9 expression in colon cancer cells abolished the inhibitory effects caused by LEF1-AS1 knockdown. Our results revealed the potential role of LEF1-AS1 in regulating migration, invasion and metastasis of colon cancer cells through miR-30-5p/SOX9 axis.

Accumulated evidences demonstrate that LEF1-AS1 is enrolled in the tumorigenesis and progression of a variety of cancers. In glioblastoma, LEF1-AS1 was upregulated and correlated with poor overall survival, while knockdown of LEF1-AS1 suppressed proliferation and invasion and promoted apoptosis of glioblastoma cells through ERK and AKT/mTOR pathway.⁹ In oral squamous cell carcinoma, LEF1-AS1 silence inhibited cell proliferation, migration, G0/G1 cell cycle progression and tumor xenograft growth by directly interacting with LATS1 and subsequently suppressing Hippo signaling pathway.¹⁰ LEF1-AS1 was also markedly upregulated in non-small-cell lung cancer

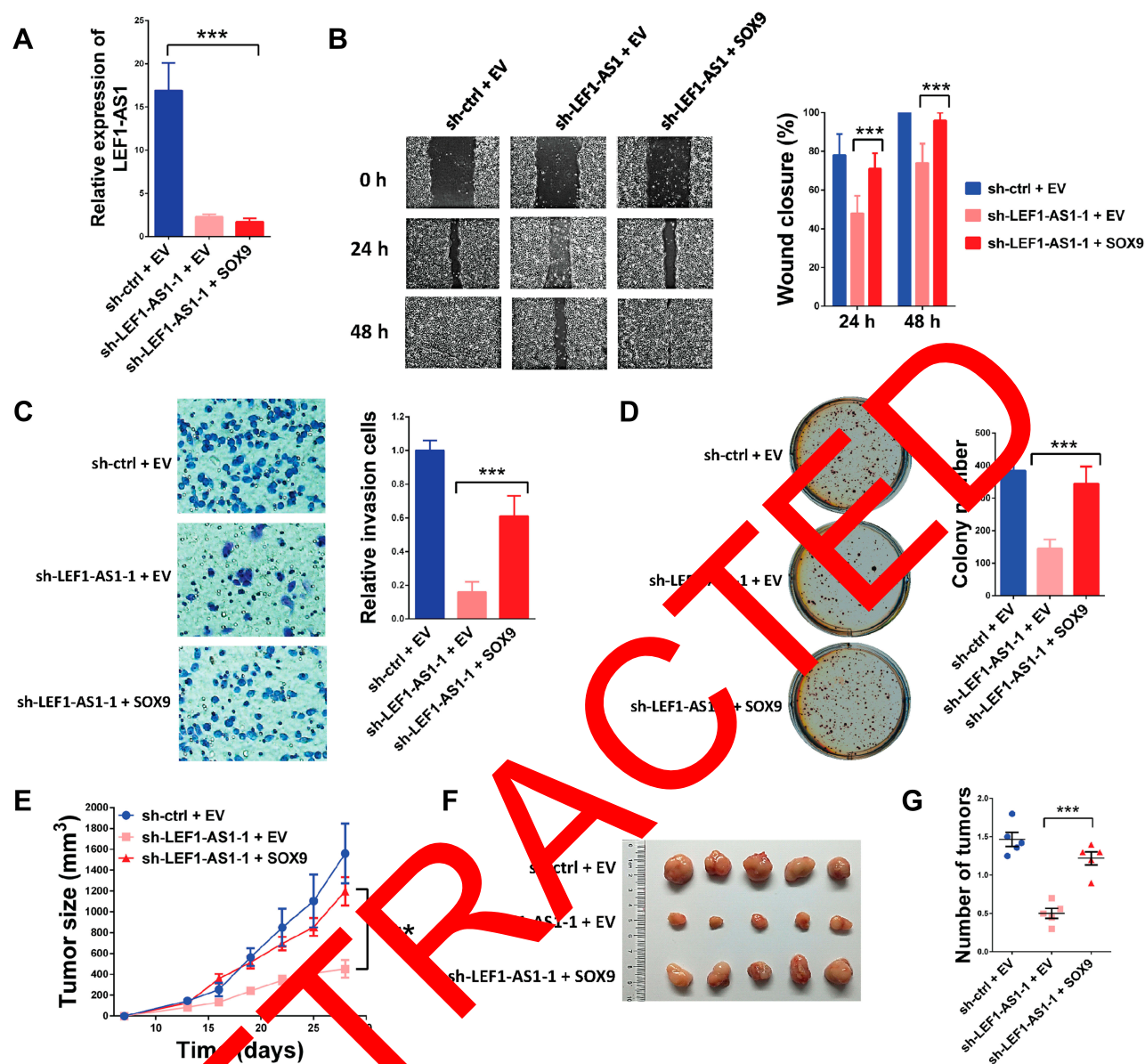


Figure 7 SOX9 restoration abolishes the effects caused by LEF1-AS1 knockdown in COLO320 cells. **(A)** COLO320 cells were transduced with sh-LEF1-AS1-1, sh-ctrl, SOX9 or EV as indicated, then cells were used for qRT-PCR. **(B)** COLO320 cells were transduced with sh-LEF1-AS1-1, sh-ctrl, SOX9 or EV as indicated, then cells were used for scratch wound healing assay. Represent images at 0 h, 24 h and 48 h were shown, and the wound closure was calculated. **(C)** COLO320 cells were transduced with sh-LEF1-AS1-1, sh-ctrl, SOX9 or EV as indicated, then cells were used for scratch wound healing assay. Represent images taken 24 h post-incubation and relative invasion cells were shown. **(D)** COLO320 cells were transduced with sh-LEF1-AS1-1, sh-ctrl, SOX9 or EV as indicated, then cells were used for soft agar assay. Represent images and average colony number were shown. **(E-G)** COLO320 cells (2×10^6) were transduced with sh-LEF1-AS1-1, sh-ctrl, SOX9 or EV as indicated, then cells were subcutaneously injected in nude mice ($n = 5$). Tumor growth curves **(E)**, represent images **(F)** and tumor weight **(G)** were shown. All assays were done in triplicates. *** $p < 0.001$.

specimens and, and enforced expression of LEF1-AS1 enhanced proliferation and migration of lung cancer cells through miR-489/SOX4 axis.¹¹ In prostate cancer, LEF1-AS1 knockdown repressed proliferation, invasion, migration, epithelial-mesenchymal transition and tumor xenograft formation of prostate cancer cells by acting as a molecular sponge for miR-330-5p.¹² These results indicate that LEF1-AS1 is overexpressed and may act like oncogene in cancers.

Indeed, we found that LEF1-AS1 was upregulated in colon cancer and overexpression of LEF1-AS1 promoted cell migration, invasion, anchorage-independent growth and tumor xenograft growth, suggesting that LEF1-AS1 behaved like oncogene in colon cancer, too.

LncRNAs have many different biological functions, one of which is act as competing endogenous RNAs (ceRNAs) or natural microRNA sponges, thus regulating

microRNA expression level and its downstream targeting protein-coding genes.^{30,31} For example, a muscle-specific lncRNA linc-MD1 mediated muscle differentiation by acting as a molecular sponge for miR-133 and subsequently regulating the expression of transcription factors MAML1 and MEF2C to activate muscle-specific gene expression.³² In hepatocellular carcinoma, a novel lncRNA MCM3AP-AS1 enhanced the growth of hepatocellular carcinoma cells by directly interacting with miR-194-5p and further promoted the expression of FOXA1.²⁴ In our study, we found that LEF1-AS1 was directly interacted and inversely correlated with miR-30-5p in colon cancer cells, suggesting that LEF1-AS1 acted as ceRNA or molecular sponges for miR-30-5p. Furthermore, a variety of studies have indicated that miR-30-5p functioned as a tumor suppressor in cancers. In myeloma cells, downregulation of miR-30-5p promoted BCL9 expression and further activated the oncogenic Wnt signaling pathway to facilitate cell proliferation, migration and formation of cancer stem cells.³³ Enforced expression of miR-30 suppressed migration, invasion and epithelial-mesenchymal transition of prostate cancer cells by targeting oncogenic TMPRSS2-ERG fusion gene.³⁴ In head and neck squamous cell carcinoma (HNSCC), all 5 members of miR-30-5p family were downregulated, and restoration of miR-30a-5p inhibited cell proliferation, migration, invasion and tumor xenograft growth.³⁵ In our study, as miR-30-5p is prone to acting like tumor suppressor, this may partially explain the oncogenic function of LEF1-AS1 by suppressing miR-30-5p in colon cancer.

Whether *SOX9* is an oncogene or tumor suppressor in colon cancer is controversial because both oncogenic and tumor-suppressing functions of this protein have been described.³⁶⁻³⁹ Evidence for *SOX9* tumor suppressor activity included that *SOX9* overexpression suppressed cell proliferation of human colon cancer cells while *SOX9* knockdown promoted cell proliferation of HT29 cell, and knocked out *SOX9* in the intestine epithelium of APC^{Min/+} mouse increased tumor burden.⁴⁰ In addition, most of *SOX9* mutations in colon cancer were frameshift or non-sense mutations, which was seen in classical tumor suppressors.⁴¹ However, study of the truncating mutations of *SOX9* indicating that these mutations were likely oncogenic and overexpressed in colon cancer.⁴² Besides, data across TCGA showed that *SOX9* was mutated and amplified in multiple cancers and whole gene deletion was rarely detected, a pattern typically seen in oncogenes.⁴³

Moreover, a variety of studies demonstrated that *SOX9* was overexpressed in colon cancer tissues and cell lines compared with healthy colon epithelia, suggesting that *SOX9* was inclined to be oncogenic in colon cancer.^{36,37,42,44,45} In our study, we found that *SOX9* was a downstream target for miR-30-5p, and LEF1-AS1 overexpression increased the expression level of *SOX9* in colon cancer cells. Moreover, restoration of *SOX9* attenuated the effects caused by LEF1-AS1 knockdown. These results indicated that the oncogenic property of LEF1-AS1 in colon cancer might partially due to regulating *SOX9* expression through miR-30-5p.

In conclusion, our study indicated that LEF1-AS1 was upregulated and correlated with unfavorable prognosis in colon cancer patients. Enforced expression of LEF1-AS1 promoted migration, invasion, and anchorage-independent growth, tumor xenograft formation and lung metastasis of colon cancer cells, while knockdown of LEF1-AS1 suppressed cell migration, invasion, anchorage-independent growth and tumor xenograft formation. LEF1-AS1 mediated *SOX9* expression by acting as a molecular sponge for miR-30-5p, and restoration of *SOX9* abrogated the effects caused by LEF1-AS1 knockdown. Our results suggested that LEF1-AS1 was oncogenic and could be a potential prognosis biomarker for colon cancer.

Data Sharing Statement

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

Ethics and Consent Statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. All procedures performed in studies involving animals were in accordance with the ethical standards of the ethics committee of the Cancer Hospital of China Medical University.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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