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microRNA-219-5p inhibits epithelial-mesenchymal transition and metastasis of colorectal cancer by targeting lymphoid enhancer-binding factor 1

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Key words

Colorectal cancer, epithelial-mesenchymal transition, LEF1, metastasis, miR-219

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Aberrant expression of microRNAs (miRs) has been shown to play a critical role in the pathogenesis and progression of tumors. microRNA-219-5p (miR-219-5p) has been reported to be abnormally expressed in some types of human tumors. However, the mechanism between miR-219-5p and colorectal cancer (CRC) metastasis remains unclear. In the present study, miR-219-5p was found to be downregulated in CRC tissue compared with matched normal tissue. Through luciferase reporter assay, we demonstrated lymphoid enhancer-binding factor 1 (LEF1) as a direct target of miR-219-5p. Overexpression of miR-219-5p could inhibit motility, migration and invasion of CRC cells, and inhibit epithelial-mesenchymal transition (EMT). Furthermore, silencing LEF1 phenocopied this metastasis-suppressive function. The recovery experiment showed that re-expression of LEF1 rescued this suppressive effect on tumor metastasis and reversed the expression of EMT markers caused by miR-219-5p. Additionally, we demonstrated that miR-219-5p exerted this tumor-suppressive function by blocking activation of the AKT and ERK pathways. Finally, a nude mice experiment showed that miR-219-5p reduced the lung metastasis ability of CRC cells. Taken together, our findings indicate that miR-219-5p inhibits metastasis and EMT of CRC by targeting LEF1 and suppressing the AKT and ERK pathways, which may provide a new antitumor strategy to delay CRC metastasis.

Colorectal cancer (CRC) is a major public health problem and ranks as the fourth leading cause of cancer death worldwide.[1] Approximately 20% of patients have distant metastasis at the time of diagnosis. In addition, 35%–45% of patients with localized disease will experience recurrence within 5 years after surgery. Most of these relapses occur as a result of metastasis, which is the main cause of death in CRC patients.[2] Therefore, it is of great importance to clarify the mechanism of CRC metastasis to find significant biomarkers and develop a novel therapeutic strategy.

miRs are members of a rapidly growing class of small noncoding RNAs 19 to 24 nucleotides in length. They act on target mRNAs in a sequence-specific way to either promote their cleavage and degradation or reduce their translational efficiency, thereby decreasing protein expression.[3] As a result of this regulatory function on gene expression, miRs have been shown to play a critical role in the pathogenesis and progression of tumors.[4] Several studies have indicated that aberrant expression of miRs, such as miR-21, miR-let-7 and miR-24, is associated with proliferation, apoptosis, and invasion of CRC cells by targeting corresponding function proteins.[5] As for miR-219-5p, it has been reported as a tumor suppressor in gastric cancer, glioblastoma and esophageal adenocarcinoma,[6–8] and it also works as a negative regulator of cell proliferation in CRC by targeting platelet-derived growth factor receptor.[9] However, the mechanism between miR-219-5p and metastasis of CRC remains unclear.

LEF1, a member of the high mobility group protein family, can activate the transcription of Wnt target genes, such as *c-myc*, *cyclin D1*, *MMP-1* and *MMP-7*, by combing with β-catenin and forming the β-catenin/TCF/LEF complex, to regulate cell behavior.[10] Kermanshahi *et al.* reported that LEF1 is most commonly expressed in CRC, but is infrequently observed in cancer of the upper gastrointestinal tract and pancreatic adenocarcinoma.[11]. Our previous findings also demonstrated that LEF1 could act as an indicator of poor prognosis in CRC, and LEF1-knockdown reduced viability and invasion but induced apoptosis of colon tumor cells.[12,13] In the present study, we aim to elucidate how LEF1 expression is regulated and the mechanism facilitating its effect on the metastasis of CRC.

EMT is a pivotal process during embryonic development and cancer metastasis, characterized by the loss of adherence junctions and apical–basal polarity and acquisition of

mesenchymal phenotype in epithelial cells.[14,15] The activation of EMT-related transcription factors such as Snail and ZEB leads to downregulation of E-Cadherin and simultaneous upregulation of mesenchymal genes such as N-Cadherin and Vimentin.[16] Several signaling pathways such as the TGF-β and Sonic hedgehog protein pathways participate in this process.[17] A recent study reported that activation of AKT and ERK changes molecular markers of EMT in CRC cells, and another study found that activation of MAPK may promote EMT in breast cancer cells by phosphorylation of Twist1,[18,19] indicating the significance of the AKT/MAPK pathway in EMT.

Herein, we aim to investigate the role of miR-219-5p in CRC metastasis. We identified LEF1 as a direct target of miR-219-5p. Inhibition of LEF1 by miR-219-5p could partly reduce the activation of AKT and ERK signaling pathways, thereby suppressing EMT and metastasis of CRC.

Materials and Methods

Clinical tissue. Twenty paired colorectal cancer tissue and matched normal tissue samples were obtained from patients who underwent surgical resection of colorectal cancer at the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China) from November 2016 to March 2017. None of the patients had received chemotherapy or radiotherapy before surgery. This study was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University. All patients provided written informed consent.

Cell lines and culture conditions. Human colorectal cancer cell lines (Caco-2, HT-29, HCT 116, SW480, SW620 and LoVo) and HEK293T cells were purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM with 10% FBS and 1% penicillin/streptomycin (HyClone, Logan, UT, USA). Human normal colon epithelial cell line FHC was purchased from ATCC (Manassas, VA, USA) and cultured in specific culture medium according to ATCC. TGF-β1 (10 ng/mL; Peprotech, Rocky Hill, CT, USA) was added to culture medium for 72 h to activate the AKT/ERK pathways. All cell lines were grown at 37°C in 5% CO_2.

Western blotting. Total protein was extracted using RIPA lysis buffer supplemented with 1× cocktail and phosphatase inhibitor (Heart Biological, Xi'an, China). After quantitative analysis with bicinchoninic acid (BCA) assay kit (Bioss, Beijing, China), each sample was separated by 10% SDS-PAGE and then transferred onto PVDF membrane (Millipore, Danvers, MA, USA). Incubation with primary antibodies against β-actin, E-Cadherin, N-Cadherin, Vimentin, Snail, MMP-2, MMP-9, Erk1/2, Phospho-Erk1/2 (Thr202/Tyr204), AKT, Phospho-AKT (Ser473) (Cell Signaling Technology, Danvers, MA, USA) was carried out under recommended dilution. After incubation with secondary antibody, chemiluminescence was detected with ECL (Millipore) to analyze the protein levels. Each sample was analyzed three times.

RNA extraction and quantitative RT-PCR (qRT-PCR). Total RNA was purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA synthesis was carried out according to the instruction of PrimeScript RT Master Mix and Mir-X miRNA First-Strand Synthesis Kit (TaKaRa, Dalian, China). Then, cDNA was quantified using SYBR Premix Ex Taq II (TaKaRa) by primers against target genes or miR-specific primers. GAPDH or U6 snRNA served as internal control. Quantification was conducted using the delta-delta C_t method to determine the relative expression of each mRNA or miR. Primer sequences are listed in Table S1. Each sample was carried out in triplicate and repeated three times.

Wound healing assay. LoVo or SW480 cells were seeded in six-well plates (approximately 4×10^5 cells per well). Three longitudinal scratches were made with sterile 10-μL pipette tips 48 h after transfection. Then, floating cell debris was washed three times with PBS. Subsequently, the cells were cultured in serum-free medium. Typical wound healing images were observed and photographed at 0 h and 30 h under an inverted microscope.

Cell migration and invasion assay. Cell migration and invasion ability was assessed by 24-well transwell chambers (Millipore) in the presence or absence of Matrigel (Corning, Bedford, MA, USA) coating. Forty-eight hours after transfection, the cells were trypsinized and counted. Approximately 1×10^5 cells resuspended in 200-μL serum-free DMEM were seeded into the upper chambers, whereas the bottom chamber was filled with 600-μL 10% FBS medium. Twenty-four hours later, non-migrated/non-invaded cells were wiped off with a cotton bud, and migrated/invaded cells underneath the chamber were fixed with 95% ethyl alcohol and stained with 0.1% crystal violet. The cells were counted and photographed at 200× magnification in five randomly selected fields.

miR, siRNA and expression plasmids. LoVo and SW480 cells were transfected with miR-219-5p mimic, miR-219-5p inhibitor and siRNA targeting LEF1 (si-LEF1) using X-tremeGENE Transfection Reagent (Roche, Indianapolis, IN, USA). miRs, siRNA and their corresponding negative controls were synthesized by GenePharma (Shanghai, China). Sequences are listed in Table S2. For the rescue study, cells were cotransfected with miR-219-5p mimic and LEF1 expression plasmid without the 3′UTR (to avoid downregulation by miR-219-5p). Only the protein-coding region was amplified and inserted into the expression vector pcDNA3.1+. Cells were harvested at 48 h after transfection for RNA analysis and 72 h after transfection for protein analysis as previously described.

Luciferase reporter assay. The wild-type sequence containing the predicted target sites of miR-219-5p in the 3′UTR of *LEF1* mRNA was synthesized by Genewiz (Beijing, China). The mutant sequence was generated with the target sites GACAATCA mutated to TCACCGAC. Both wild-type and mutant sequences were inserted into *Sac*I and *Xho*I restriction sites of the multiple cloning site of pmiRGLO dual-luciferase reporter vector. Then, the pmirGLO-*LEF1*-wt or pmirGLO-*LEF1*-mut vector was cotransfected with miR-219-5p mimic or miR-219-5p negative control into HEK293T cells. Twenty-four hours after transfection, activities of firefly luciferase and *Renilla* luciferase were measured according to the manufacturer's instruction of the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA). The luciferase reporter assay was independently repeated three times.

Animal model. Animal experiment was approved by the Animal Experiment Administration Committee of Xi'an Jiaotong University. Six-week-old male BALB/c nude mice (Xi'an Jiaotong University Health Science Center, Xi'an, China) were used for *in vivo* tumor metastasis studies. LoVo cells were infected with LV-miR-219-5p or LV-miR-control constructed by GeneChem (Shanghai, China) to establish stably overexpressing miR-219-5p CRC cell. Then, LoVo-miR-control or LoVo-miR-219-5p cells (1×10^6 in 100 μL PBS) were injected into the tail vein of each mouse. Mice care was carried out by trained animal care staff. After 5 weeks, mice were killed and lung tissue was obtained for H&E staining.

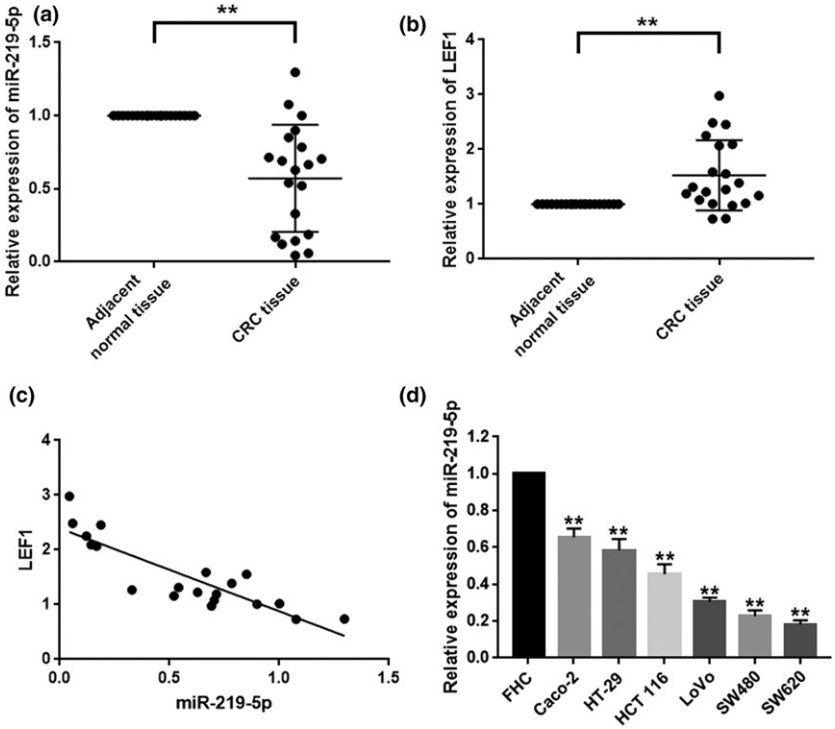

Fig. 1. microRNA-219-5p (miR-219-5p) is downregulated and lymphoid enhancer-binding factor 1 (LEF1) is upregulated in colorectal cancer (CRC) tissue and cell lines. (a,b) Relative expression of miR-219-5p and LEF1 in 20 paired CRC and normal tissue samples. (c) Linear regression analysis between miR-219-5p and LEF1. (d) miR-219-5p expression in six CRC cell lines and the normal colon epithelial cell line FHC. U6 snRNA served as internal control. Data are shown as mean ± SD. $^{**}P < 0.01$.

Table 1. Correlation between clinicopathological features and expression of miR-219-5p and LEF1 in patients with CRC

Characteristics	*n*	Percentage (%)	miR-219-5p relative expression (mean ± SD)	*P*-value	LEF1 mRNA relative expression (mean ± SD)	*P*-value
Clinicopathological features				<0.001**		<0.001**
Adjacent normal tissue	20	100	1		1	
CRC tissue	20	100	0.571 ± 0.367		1.528 ± 0.640	
Age (years)				0.594		0.592
≥55	18	90	0.586 ± 0.383		1.555 ± 0.671	
<55	2	10	0.435 ± 0.150		1.290 ± 0.029	
Gender				0.766		0.733
Male	12	60	0.550 ± 0.397		1.570 ± 0.693	
Female	8	40	0.602 ± 0.342		1.466 ± 0.590	
Pathological stage				0.006**		0.005**
I+II	6	30	0.897 ± 0.247		0.954 ± 0.185	
III+IV	14	70	0.432 ± 0.322		1.774 ± 0.607	
Pathological T stage				0.0098**		0.027*
T1T2	4	20	0.974 ± 0.328		0.912 ± 0.212	
T3T4	16	80	0.471 ± 0.309		1.682 ± 0.619	
Pathological N stage				0.044*		0.029*
N0	7	35	0.793 ± 0.356		1.113 ± 0.453	
N1N2	13	65	0.452 ± 0.326		1.752 ± 0.625	
Pathological M stage				<0.001**		<0.001**
M0	14	70	0.764 ± 0.248		1.160 ± 0.259	
M1	6	30	0.120 ± 0.058		2.388 ± 0.338	

$^{*}P < 0.05$, $^{**}P < 0.01$. CRC, colorectal cancer; LEF1, lymphoid enhancer-binding factor 1; miR-219-5p, microRNA-219-5p.

Statistical analysis. Data analysis was carried out using IBM SSPS Statistics Version 22 (IBM Corp., Armonk, NY, USA). Data were reported as mean ± SD and *t*-test was used to determine differences between groups. Differences were considered statistically significant when $P < 0.05$.

Results

miR-219-5p is downregulated and LEF1 is upregulated in CRC tissue and cell lines. By analyzing the expression level of miR-219-5p and LEF1 in 20 paired CRC tissue and corresponding normal tissue samples using qRT-PCR, we found that miR-219-5p was significantly decreased in most of the CRC tissue compared with that in the matched controls (Fig. 1a, $P < 0.001$), whereas LEF1 was increased (Fig. 1b, $P < 0.001$). Paired comparison revealed the same tendency (Fig. S1). Linear regression analysis showed a possible relevance between miR-219-5p and LEF1 in these clinical tissues with $R^2 = 0.755$ (Fig. 1c). Moreover, low miR-219-5p expression was found to be correlated with large tumor size, advanced

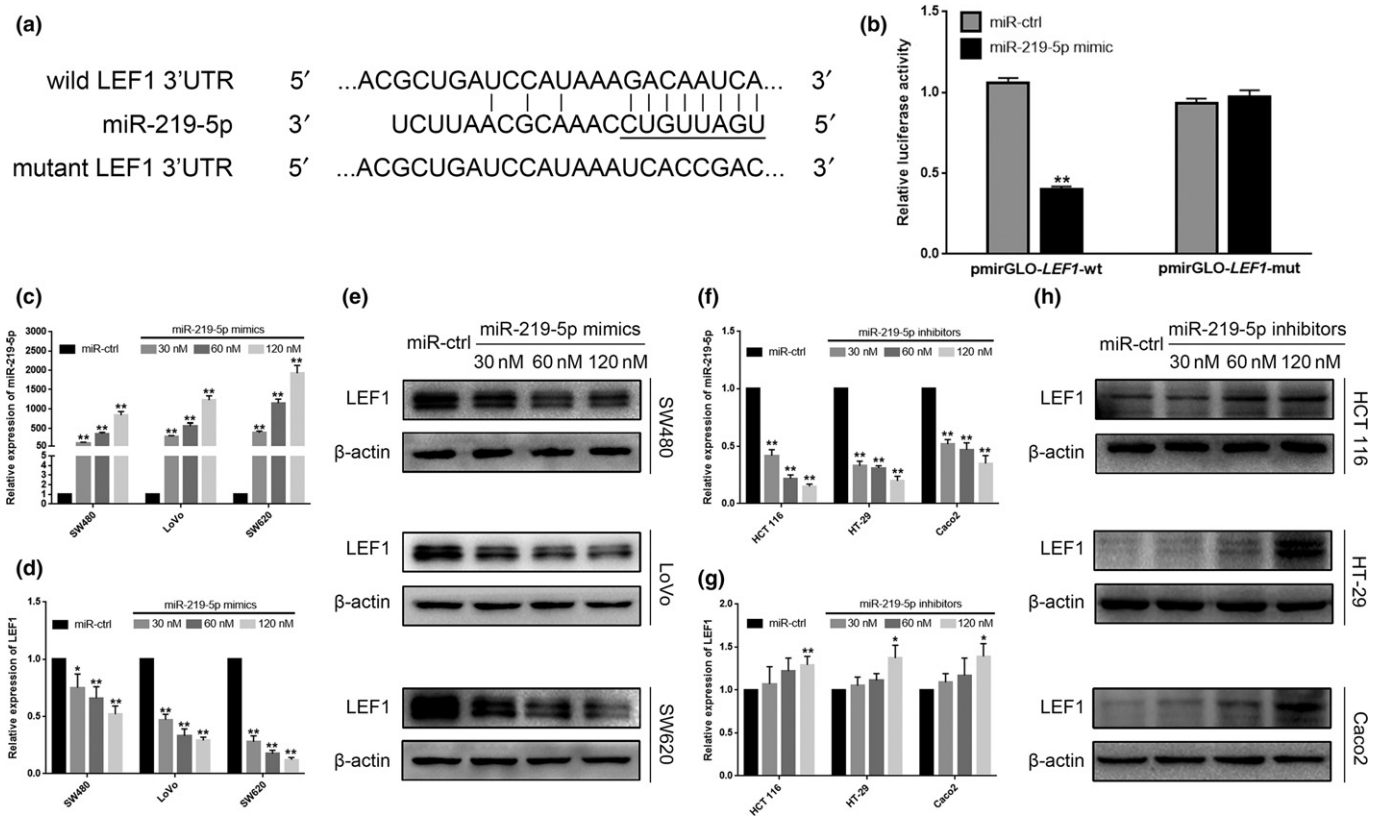

Fig. 2. Lymphoid enhancer-binding factor 1 (LEF1) is a direct target of microRNA-219-5p (miR-219-5p). (a) Sequences of *LEF1* 3′UTR and miR-219-5p according to the prediction of TargetScan. Wild-type and mutated-type binding sequences of *LEF1* 3′UTR are shown. (b) Relative luciferase activity in HEK293T cells transfected with reporter vector containing wild-type binding sequence (pmirGLO-*LEF1*-wt) or mutated-type binding sequence (pmirGLO-*LEF1*-mut) along with miR-219-5p mimic or negative control. (c) Relative miR-219-5p expression in SW480, LoVo and SW620 after transfection with various concentrations of miR-219-5p mimic (30, 60, or 120 nM) or miR-ctrl analyzed by quantitative RT-PCR (qRT-PCR). (d,e) qRT-RCR and Western blotting analysis of LEF1 mRNA and protein levels after cells were transfected with miR-219-5p mimics in a dose-dependent manner. (f) Relative miR-219-5p expression in HCT 116, HT-29 and Caco-2 after transfection with various concentrations of miR-219-5p inhibitor (30, 60, or 120 nM) or miR-ctrl analyzed by qRT-PCR. (g,h) LEF1 mRNA and protein levels after cells were transfected with miR-219-5p inhibitors in a dose-dependent manner. Data are shown as mean ± SD. **P* < 0.05, ***P* < 0.01.

TNM stage, node and distant metastasis ($P < 0.01$, $P < 0.01$, $P < 0.05$ and $P < 0.001$, respectively, Table 1, Table S3), whereas high LEF1 expression was strongly associated with large tumor size, node and distant metastasis and advanced TNM stage ($P < 0.05$, $P < 0.05$, $P < 0.001$ and $P < 0.01$, respectively, Table 1). No significant association between miR-219-5p or LEF1 expression level and age or gender was observed. Parameters of 20 CRC patients are listed in Table 1. Cell lines analysis showed miR-219-5p was decreased in six CRC cell lines compared with the normal colon epithelial cell line FHC (Fig. 1d). These results indicated that miR-219-5p was significantly downregulated and LEF1 was upregulated in CRC tissue and cell lines.

LEF1 is a direct target of miR-219-5p. LEF1 was assumed to be a direct target of miR-219-5p, as it was simultaneously predicted by all three miR target gene prediction software packages (TargetScan, miRDB, DIANA). In addition, the potential binding sequence in *LEF1* 3′UTR was identified (Fig. 2a). To validate that LEF1 is a direct target of miR-219-5p, a dual-luciferase reporter assay was carried out. The reporter vector containing wild-type binding sequence (pmirGLO-*LEF1*-wt) or mutated-type binding sequence (pmirGLO-*LEF1*-mut) was transfected into HEK293T cells along with miR-219-5p mimic or miR-219-5p negative control. Results showed that cotransfection of pmirGLO-*LEF1*-wt and miR-219-5p mimic led to a significant decrease (approximately 60%) in luciferase activity compared with the control group, whereas cotransfection of pmirGLO-*LEF1*-mut and miR-219-5p mimic had no effect on luciferase activity (Fig. 2b).

To further confirm the functional significance of miR-219-5p on LEF1, we overexpressed or downregulated miR-219-5p in multiple CRC cell lines. Results showed that forced transfection of miR-219-5p mimic into LEF1 high-expression cell lines SW620, SW480 and LoVo led to a sharp increase in miR-219-5p level (Fig. 2c), meanwhile it caused a significant decrease in LEF1 expression both at mRNA and protein levels in a dose-dependent manner (Fig. 2d,e). Conversely, an obvious increase in LEF1 expression was observed in LEF1 low-expression cell lines Caco-2, HT-29 and HCT 116 when transfected with miR-219-5p inhibitor in a dose-dependent manner (Fig. 2f–h).

These results suggest that LEF1 is a direct target of miR-219-5p. In addition, through promoting degradation of LEF1 mRNA, miR-219-5p can reduce LEF1 expression both at mRNA and protein levels.

miR-219-5p inhibits motility and invasion of CRC cells by suppressing LEF1. SW480 and LoVo cells were chosen for further study because they have low expression of miR-219-5p.

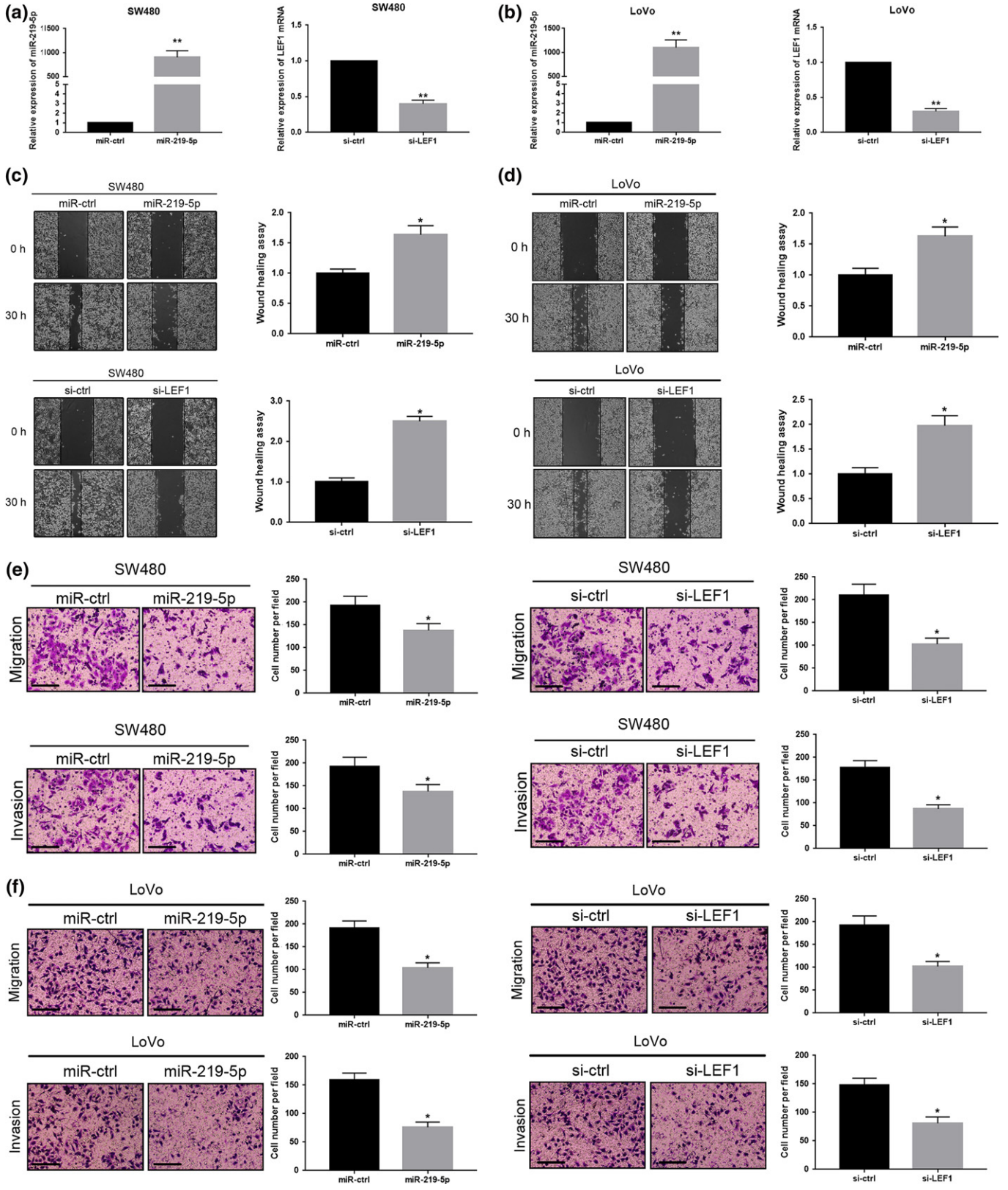

Fig. 3. microRNA-219-5p (miR-219-5p) inhibits motility and invasion of colorectal cancer (CRC) cells. (a,b) Expression of miR-219-5p after transfection of miR-219-5p mimic (120 nM, left panel) and expression of lymphoid enhancer-binding factor 1 (LEF1) after transfection of siRNA targeting lymphoid enhancer-binding factor 1 (si-LEF1) (right panel) into (a) SW480 and (b) LoVo cells determined by quantitative RT-PCR (qRT-PCR). (c,d) Left panel, wound healing assay in (c) SW480 and (d) LoVo cells treated with miR-219-5p mimic or si-LEF1 and the corresponding negative control. Right panel, quantitative results of wound healing assay shown on the left. (e,f) Left panel, migration and invasion of cells determined by transwell assay in (e) SW480 and (f) LoVo cells treated with miR-219-5p mimic or si-LEF1 and the corresponding negative control. Scale bar, 100 μm. Right panel, quantitative results of transwell assay shown on the left. Data are shown as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$.

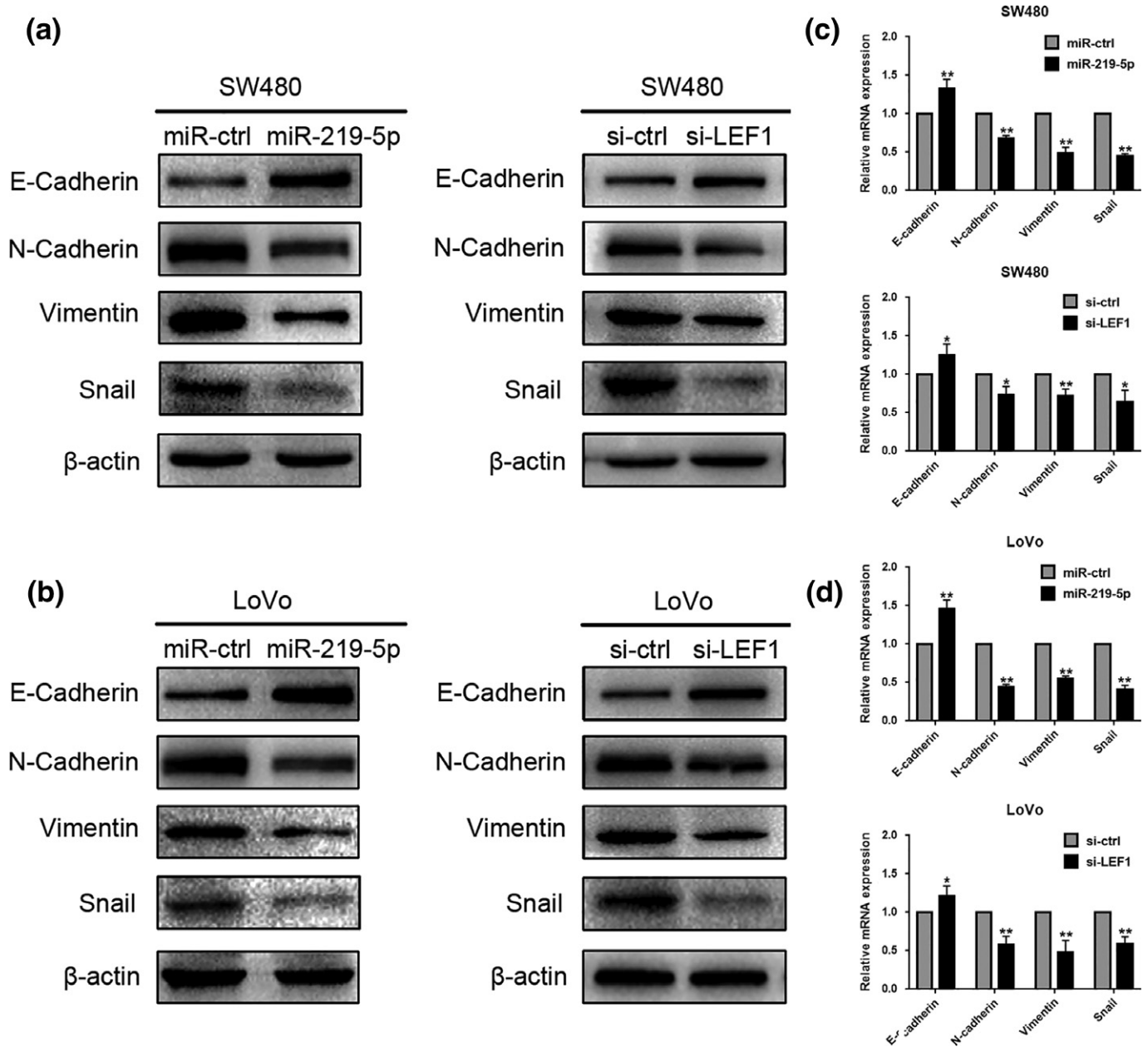

Fig. 4. microRNA-219-5p (miR-219-5p) suppresses epithelial-mesenchymal transition (EMT) in colorectal cancer (CRC) cells. (a,b) Protein and mRNA levels of EMT markers E-Cadherin, N-Cadherin, Vimentin and Snail in SW480 cells transfected with miR-219-5p or siRNA targeting lymphoid enhancer-binding factor 1 (si-LEF1) determined by Western blotting and quantitative RT-PCR (qRT-PCR). (c,d) Protein and mRNA levels of EMT markers in LoVo cells transfected with miR-219-5p or si-LEF1. Data are shown as mean ± SD. $*P < 0.05$, $**P < 0.01$.

Through transfection with miR-219-5p mimic, there was a robust increase in miR-219-5p mRNA level (Fig. 3a,b). Moreover, to validate whether the molecular biological function of miR-219-5p was mediated by suppression of LEF1, we used a siRNA targeting LEF1, which induced a decrease in LEF1 mRNA by 70% (Fig. 3a,b). First, we detected cell motility. Results of wound healing assay suggested that miR-219-5p inhibited motility of SW480 and LoVo cells (Fig. 3c,d). Similarly, cell motility was inhibited when transfected with si-LEF1 into SW480 and LoVo cells (Fig. 3c,d). Next, transwell assay was carried out to evaluate migration and invasion ability. Results showed that the number of migrated and invaded cells was significantly decreased after transfection of miR-219-5p or si-LEF1 compared with that of the control group (Fig. 3e,f). Taken together, these findings demonstrate that miR-219-5p inhibits motility, migration and invasion of CRC cell lines, and that silencing LEF1 can replicate these effects of miR-219-5p.

miR-219-5p suppresses EMT in CRC cells. To explore the effect of miR-219-5p on EMT in CRC cell lines, we monitored changes in EMT markers E-Cadherin, N-Cadherin, Vimentin and Snail. qRT-PCR and Western blotting results all showed that the expression of E-Cadherin was higher in miR-219-5p mimic-transfected and si-LEF1-transfected groups compared

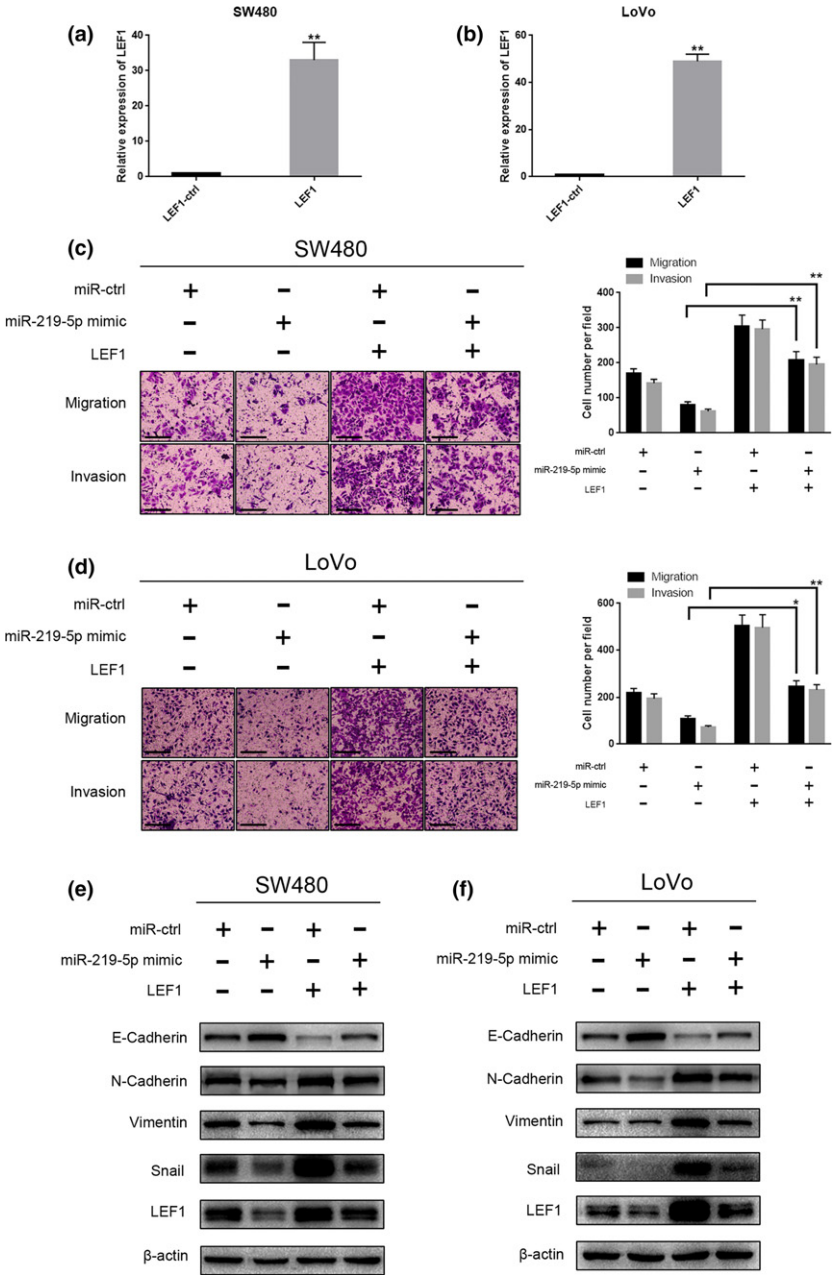

Fig. 5. Lymphoid enhancer-binding factor 1 (LEF1) rescues the inhibitory effect of microRNA-219-5p (miR-219-5p) on metastasis and epithelial-mesenchymal transition (EMT) of colorectal cancer (CRC) cells. (a,b) Quantitative RT-PCR (qRT-PCR) analysis of LEF1 mRNA expression after transfection of LEF1 expression vector into (a) SW480 and (b) LoVo cells. (c,d) Left panel, rescue of the migration and invasion ability of (c) SW480 and (d) LoVo cells by exogenous expression of LEF1. Scale bar, 100 μm. Right panel, quantitative results of transwell assay on the left. (e,f) Western blotting analysis of protein levels of LEF1 and EMT markers after transfection of miR-219-5p mimic or LEF1 expression vector alone or together into (e) SW480 and (f) LoVo cells. Data are shown as mean ± SD. $*P < 0.05$, $**P < 0.01$.

with that in their corresponding controls, whereas the level of N-Cadherin, Vimentin and Snail was lower in CRC cells transfected with miR-219-5p or si-LEF1 (Fig. 4). Activation of the EMT transcription factor Snail and downregulation of the epithelial marker E-Cadherin along with the upregulating of mesenchymal markers N-Cadherin and Vimentin indicate the progress of EMT. Therefore, the above findings suggest that ectopic expression of miR-219-5p can suppress EMT, which is in accordance with its suppressive function on migration and invasion of CRC cells. Moreover, the loss of LEF1 phenocopied this inhibitory function.

LEF1 rescues the inhibitory effect of miR-219-5p on metastasis and EMT of CRC cells. To further demonstrate whether LEF1 could mediate the function of miR-219-5p in suppressing metastasis and EMT of CRC cells, a rescue experiment was conducted. First, SW480 and LoVo cells were transfected with LEF1-expressing vector. qRT-PCR results showed that the LEF1-expressing vector could notably increase LEF1 expression at the mRNA level (Fig. 5a,b). Transwell assay also validated its ability on promoting migration and invasion (Fig. 5c,d). Then, SW480 and LoVo cells were transfected with miR-219-5p only or cotransfected with miR-219-5p and LEF1-expressing vector. Interestingly, transwell assay revealed that the inhibitory effect of miR-219-5p on migration and invasion of CRC cells could be significantly rescued by re-expressing exogenous LEF1 (Fig. 5c,d). Consistently, expression of EMT markers was also recovered by overexpression of LEF1. Western blotting analysis showed that exogenous expression of LEF1 inhibited expression of E-Cadherin but restored expression of N-Cadherin, Vimentin and Snail (Fig. 5e,f). Taken together, our present results demonstrate that miR-219-5p exerts its inhibitory effect on

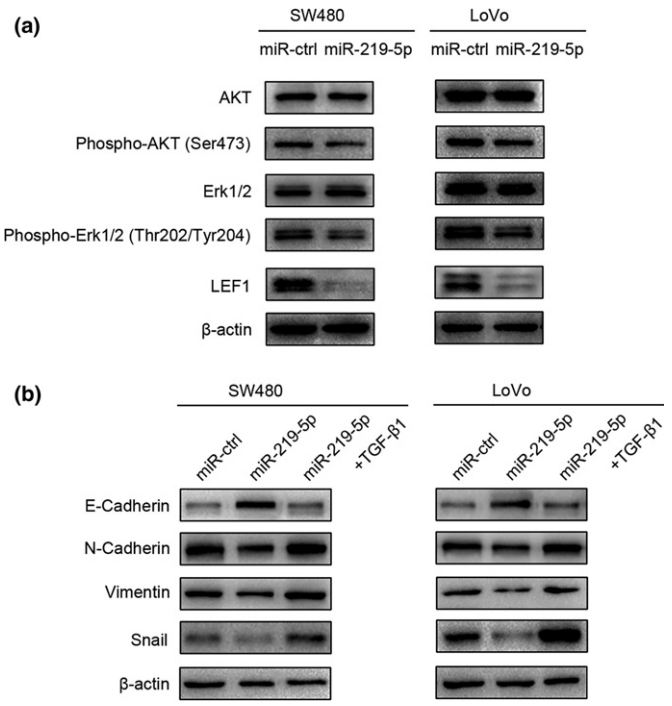

Fig. 6. microRNA-219-5p (miR-219-5p) suppresses epithelial-mesenchymal transition (EMT) by reducing activation of AKT/ERK pathways. (a) Western blotting analysis of lymphoid enhancer-binding factor 1 (LEF1), AKT, p-AKT, ERK, p-ERK in SW480 and LoVo cells after transfection of miR-219-5p or miR-ctrl. (B) Protein levels of EMT markers in SW480 and LoVo cells transfected with miR-ctrl or miR-219-5p mimic, or transfected with miR-219-5p mimic and incubated with 10 ng/mL transforming growth factor beta 1 (TGF-β1) for 72 h.

migration, invasion and EMT of CRC cells by targeting and suppressing LEF1.

miR-219-5p suppresses EMT by reducing activation of AKT/ERK pathways. To clarify the specific molecular mechanisms under miR-219-5p suppressing EMT, we assessed the activation of AKT and ERK pathways as they were previously reported to participate in EMT and associated with the Wnt/β-catenin/LEF1 pathway. After overexpression of miR-219-5p, a decrease in phosphorylation of AKT and ERK was observed, whereas levels of total AKT and ERK remained unchanged (Fig. 6a). In addition, through reactivation of the AKT/ERK pathways with TGF-β1, expression of EMT markers was reversed compared with that in miR-219-5p overexpressed cells. As shown in Figure 6b, in response to stimulation with 10 ng/mL TGF-β1 for 72 h in miR-219-5p overexpressed cells, E-Cadherin decreased, whereas N-Cadherin, Vimentin and Snail increased. This indicates that with activation of the AKT/ERK pathways, EMT was induced again. These results demonstrate that miR-219-5p suppresses EMT by reducing activation of the AKT/ERK pathways.

miR-219-5p inhibits lung metastasis of CRC cells *in vivo*. The results above demonstrate the critical metastasis suppressive function of miR-219-5p *in vitro*. Then, a lung metastasis experiment on nude mice was conducted to further validate this conclusion. LoVo cells stably overexpressing miR-219-5p were established by infection with LV-miR-219-5p. qRT-PCR results showed an eight-fold increase of miR-219-5p level in LV-miR-219-5p-infected cells compared with that in the controls (Fig. 7a). Then, LoVo-miR-219-5p or LoVo-miR-control cells were injected into the tail vein of nude mice. Five weeks after injection, incidence of lung metastases in mice injected with LoVo-miR-219-5p cells was significantly lower than that in LoVo-miR-control cell-injected mice (2/6 *vs* 6/6, Fig. 7b). Lung metastatic nodules were confirmed by H&E staining (Fig. 7c). This *in vivo* result suggests that miR-219-5p functions as a suppressor of CRC metastasis, which is in accordance with the results obtained *in vitro*.

Discussion

Distant metastasis is the major cause of CRC-related mortality. An increasing number of studies have reported the significant role of miRs in the metastatic cascade of CRC, including angiogenesis, intravasation, circulation, extravasation and metastatic colonization.[20,21] Moreover, miR-based therapies have lately been emphasized. Investigation of the use of miRs as drugs or drug targets against tumors is under way.[22] There have been various small molecule inhibitors that can inhibit the function of miR-21.[23] In addition, RNAi strategies including miRNAs have been used as genetic tools and are one of the most promising therapies. Pre-miR-34a or pre-miR-199a transfection was shown to reduce the number of liver metastases in nude mice.[24] These results indicate miRs are potential and promising strategies to inhibition and treatment of CRC metastasis.

Accumulating evidence has demonstrated that a series of tumors, including gastric cancer, glioblastoma and pancreatic cancer, has a reduced level of miR-219-5p, indicating the tumor-suppressive effect of this miR. Overexpression of miR-219-5p can decrease the proliferation and invasion of gastric cancer cells by targeting the liver receptor homolog-1, meanwhile repressing activation of the Wnt/β-catenin signaling pathway.[6] In addition, another group demonstrated that miR-219-1-3p can negatively regulate Mucin-4 expression, thereby decreasing the proliferation of pancreatic cancer cells associated with a decrease in activation of the AKT and ERK pathways, which is partly consistent with our findings in the CRC model.[25] Moreover, Rao *et al.* reported that miR-219-5p could inhibit MAPK and PI3K pathways in glioma cells because of its ability to suppress epidermal growth factor receptor (EGFR).[26] However, we did not detect any change in EGFR expression level. A likely reason for this discrepancy is the difference of cell lines.

LEF1 is an oncoprotein that also serves as a prognostic marker in several human malignancies. As a transcription factor of the Wnt/β-catenin pathway, dysregulation of LEF1 transcriptional activity plays a critical role in oncogenesis.[10] In order to further understand whether miR-219-5p can affect LEF1 transcriptional activity, a Top/Fop flash assay was conducted, which showed a two-fold decrease in luciferase activities in the miR-219-5p transfected group (Fig. S2a). Also, this decrease did not depend on β-catenin as the immunofluorescence experiment showed subcellular localization of β-catenin had no obvious change (Fig. S2b). These results support that miR-219-5p can decrease the transcriptional activity of LEF1 by downregulating LEF1 expression.

In the present study, we demonstrated that miR-219-5p bound to the 3′UTR of *LEF1*, leading to degradation and translational repression of LEF1 mRNA, thus inhibiting EMT-associated migration and invasion through reducing the activation of AKT and ERK pathways.

EMT is an important process during development and oncogenesis and mediated by several important pathways. As the

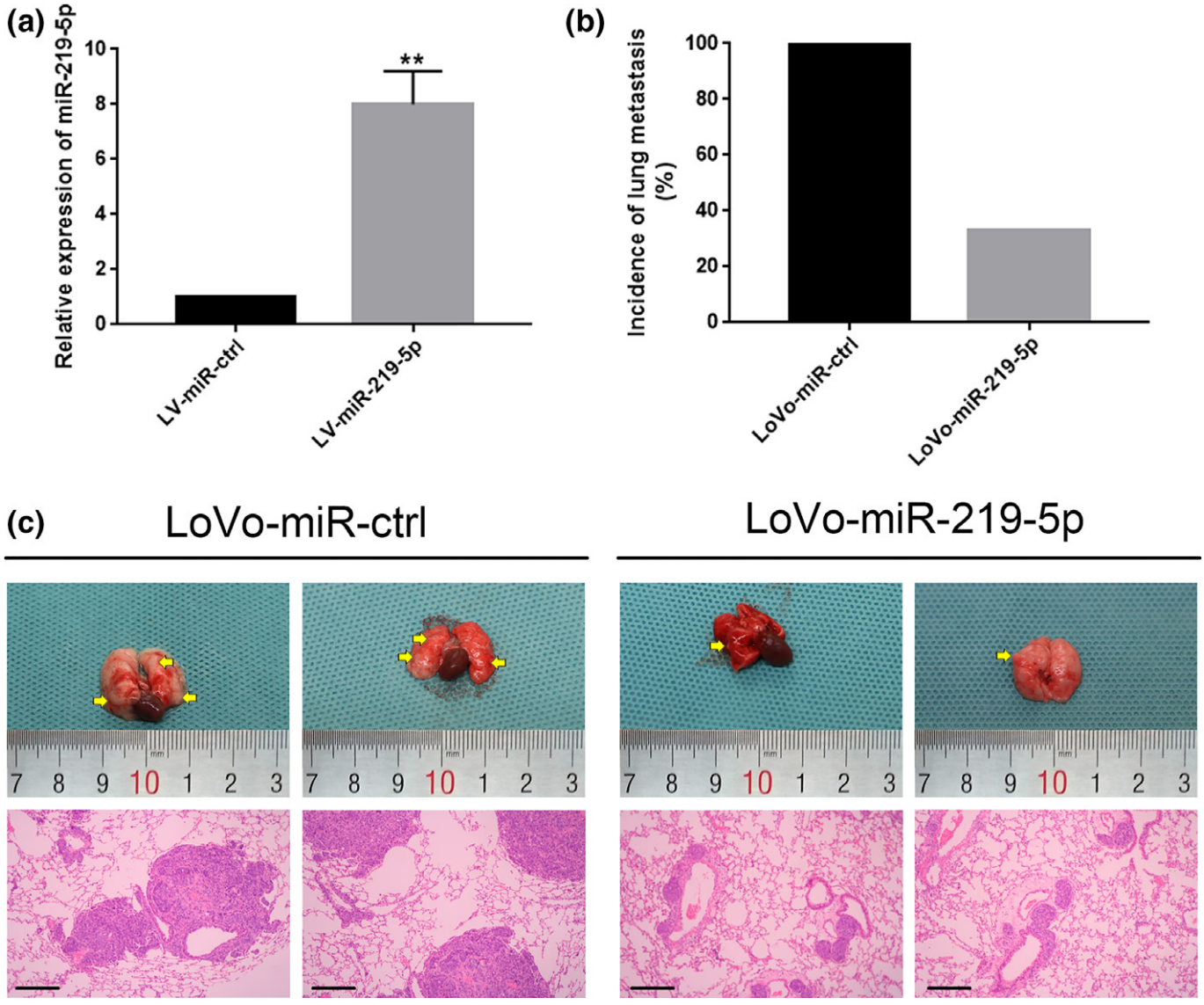

Fig. 7. microRNA-219-5p (miR-219-5p) inhibits lung metastasis of colorectal cancer (CRC) cells *in vivo*. (a) Quantitative RT-PCR (qRT-PCR) analysis of miR-219-5p expression level in LoVo cells infected with LV-miR-219-5p or LV-miR-ctrl. (b) Incidence of lung metastasis. (c) Representative images of lung metastasis in nude mice injected with LoVo-miR-219-5p or LoVo-miR-ctrl cells (upper panel). H&E stains of lung tissues (lower panel). Scale bar, 200 μm. Data are shown as mean ± SD. $^{**}P < 0.01$.

AKT/ERK pathways widely participate in tumor proliferation, angiogenesis and metastasis, we decided to investigate the change of AKT/ERK pathways in the EMT process.[27–29] In the present study, after overexpressing miR-219-5p, a decrease in the phosphorylation of AKT and ERK was observed. Furthermore, when reactivating the AKT/ERK pathways with TGF-β1, expression of EMT markers was reversed, suggesting miR-219-5p inhibited EMT in CRC by reducing the activation of AKT and ERK pathways.

Studies have reported that the connection between Wnt/β-catenin/LEF1 and AKT/ERK pathways may be mediated by metalloproteinases, which have proteolytic properties and therefore can activate cell surface molecules through proteolytic cleavage or "shedding", including EGFR ligands such as TGF-α, adhesion molecules such as integrin β1 and proinflammatory cytokines such as TNF-α etc. Specific MMP, activated by LEF1, can cleave the ectodomains of various ligands, leading to an increase of soluble ligands and binding with their receptors, thus activating cellular signaling including AKT/ERK pathways. The EGFR ligands such as TGF-α may activate downstream AKT/ERK pathways by activating EGFR.[30,31] Integrin β1 driven Src-Akt hyperactivation can trigger EGFR ligand-independent signaling.[32,33] Besides, TGF-β can activate downstream PI3K/AKT and p-ERK by phosphorylating FAK.[34,35] Conversely, when the activity of MMP is inhibited, this MMP-induced cleavage of the ligands is blocked, resulting in downregulation of the activity of AKT/ERK pathways. We have measured the expression level of MMP-2 and MMP-9 after transfection of miR-219-5p. Results showed that the level of MMP-2 and MMP-9 decreased after overexpressing miR-219-5p (Fig. S3b). Furthermore, the level of TGF-α and TGF-β in miR-219-5p overexpressed cell supernatant was measured by an ELISA assay, which showed a ~two-fold decrease compared with the controls (Fig. S3a).

However, the specific mechanism of miR-219-5p regulating the AKT/ERK pathways needs further exploration.

Meanwhile, further analysis on the association between miR-219-5p expression level and recurrent or overall survival is needed to clarify whether miR-219-5p can be used as a prognostic biomarker. Moreover, we focused on the function of miR-219-5p only, but the mechanism of its downregulation in CRC progression remains unclear. Further studies are required to fully understand the complex mechanism behind miR-219-5p expression and maturation.

Briefly, we determined miR-219-5p as a tumor suppressor that can block the expression of LEF1, thereby inhibiting EMT in CRC by reducing the activation of AKT and ERK signaling pathways. Our elucidation of the roles of miR-219-5p in CRC will enable the discovery of specific therapeutic approaches for CRC patients. Nevertheless, the importance of miR-219-5p in inhibition of EMT and, presumably, suppression of the early phases of metastasis need further exploration in the clinical setting for human colorectal cancer.

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Disclosure Statement

Authors declare no conflicts of interest for this article.

Abbreviations

CRC	colorectal cancer
EMT	epithelial-mesenchymal transition
LEF1	lymphoid enhancer-binding factor 1
microRNA	miR
miR-219-5p	microRNA-219-5p
si-LEF1	siRNA targeting lymphoid enhancer-binding factor 1
TGF-β	transforming growth factor beta

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Paired comparison of miR-219-5p or LEF1 expression level in 20 paired CRC and normal tissue samples. Paired comparison of miR-219-5p (a) or LEF1 (b) expression level in 20 paired clinical tissue samples.

Fig. S2. miR-219-5p downregulates the transcriptional activity of LEF1. (a) Luciferase activity in SW480 cells transfected with miR-219-5p or miR-ctrl along with either TOPflash or FOPflash. (b) Immunofluorescence of β-catenin in SW480 cells after transfection with miR-219-5p or miR-ctrl.

Fig. S3. Change of MMPs and TGF-β, TGF-α in SW480 and LoVo cells after transfection with miR-219-5p or miR-control. (a) ELISA assay of TGF-α and TGF-β in SW480 and LoVo cells supernatant after transfection of miR-219-5p or miR-ctrl. (b) Western blotting analysis of LEF1 and MMP-2, MMP-9.

Table S1. Primer sequences used in qRT-PCR.

Table S2. Sequences of miR and siRNA.

Table S3. Characteristics of patients with CRC.