# Overexpression and knockdown of 5-lipoxygenase regulates the migration and invasion of colorectal cancer cells

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**Abstract.** The overexpression of 5-lipoxygenase (5-LOX) plays a notable role in the development of colorectal cancer (CRC); however, the specific role of 5-LOX in the migration and invasion of CRC cells through the knockdown and overexpression of 5-LOX has not been well studied. Epithelial-mesenchymal transition (EMT) is associated with an invasive phenotype in CRC; however, the relationship between 5-LOX and EMT in CRC cells remains undefined. In the present study, the effects of 5-LOX overexpression and silencing on migration, invasion and EMT in CRC cells was investigated. In addition, the mRNA levels of vascular endothelial growth factor (VEGF) and angiogenin were also analyzed in human umbilical vein endothelial cells (HUVECs) when co-cultured with media acquired from 5-LOX-silenced or 5-LOX-overexpressed CRC cells. Reverse transcription-quantitative PCR and western blotting analyses were used to determine mRNA and protein expression levels, respectively. Transwell and wound healing assays were used to determine the invasion and migration ability of CRC cells. Fluorescent images were captured to demonstrate the lentiviral infection in CRC cells. Analysis revealed that the knockdown of 5-LOX by small interfering RNA significantly inhibited migration, invasion and EMT in HCT116 cells, whereas the overexpression of 5-LOX by recombinant lentiviruses significantly promoted migration, invasion and EMT in RKO cells. Media from 5-LOX-overexpressed RKO cells upregulated the expression of VEGF and angiogenin in HUVECs. These results demonstrated that the overexpression of 5-LOX promoted EMT in CRC cells, which may play a marked role in facilitating cellular migration and invasion. Furthermore, the tumor microenvironment of 5-LOX-overexpressed CRC cells may induce the angiogenesis in HUVECs. The present findings elucidate the role of 5-LOX in the migration and invasion of CRC cells via the knockdown and overexpression of the 5-LOX gene.

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*Key words:* colorectal cancer, 5-lipoxygenase, epithelial-mesenchymal transition, migration, invasion

## Introduction

Colorectal cancer (CRC) has become the third most commonly diagnosed cancer and the second most common cause of cancer-related death globally, as estimated by the International Agency for Research on Cancer (1). In 2022, the National Cancer Center of China recorded 517,100 newly diagnosed cases and 240,000 deaths of CRC (2). Metastasis is a notable cause of CRC-related death (3); although new therapies, such as systemic therapy, extensive surgery and local ablative therapies, have already been utilized for the treatment of metastatic CRC disease and doubled the overall survival for late-stage CRC by up to 3 years, survival rates are still markedly higher for patients with CRC without metastasis (4). Consequently, elucidating the mechanisms underlying the metastasis and invasion of CRC is essential for developing effective therapeutic strategies.

Epithelial-mesenchymal transition (EMT) is a central mechanism for diversifying the cells found in complex tissues and is associated with the formation of the parietal endoderm (5). EMT has been shown to play a marked role in promoting the metastasis of epithelium-derived carcinoma (6,7). The molecular markers for EMT include the increased expression of N-cadherin, vimentin, Snail1, Snail2 (Slug), as well as the reduced expression of E-cadherin. Phenotypic markers for EMT include increased capacity for migration and invasion, as well as resistance to apoptosis (8). In the first step of the invasion-metastasis cascade, CRC cells isolate themselves from one another and nearby normal cells by downregulating E-cadherin. The release of pro-angiogenic factors results in the formation of new blood vessels and the infiltration of tumor cells into the bloodstream (9,10). Snail plays a notable role in the development of CRC and predominantly promotes EMT by suppressing E-cadherin. Furthermore, Snail can induce the

expression of genes related to the mesenchymal phenotype, such as fibronectin and N-cadherin (11). It is reported that the expression levels of Snail in the tumor stroma are associated with distant metastasis and the lower specific survival of patients with CRC (12). Furthermore, the expression of N-cadherin was reported to be upregulated in CRC tissues and negatively associated with the expression of E-cadherin, which was associated with tumor differentiation, metastasis and the reduced survival of patients with CRC (13). Another study demonstrated that the upregulation of vimentin contributed to the progression and poor prognosis of CRC (14).

5-Lipoxygenase (5-LOX) catalyzes the oxygenation of arachidonic acid to generate leukotrienes (15). Furthermore, the overexpression of 5-LOX is known to play a marked role in the development of CRC (16). Clinical studies have shown that the expression levels of 5-LOX are notably upregulated in human colorectal tumors when compared with the adjacent normal mucosa (17,18). Zileuton, a 5-LOX inhibitor, was found to inhibit the growth of colon cancer xenografts in athymic mice (18). Another 5-LOX inhibitor, AA861, notably promotes apoptosis and inhibited the invasion and proliferation of 5-LOX-overexpressed HCA7 cells, while an investigation involving 5-LOX-/- $Apc^{Min/+}$ mice demonstrated a marked reduction in the numbers and sizes of the adenomas, a notable improvement in survival rate and inhibition of the PI3K/AKT pathway via promoting PTEN (19). Bošković *et al* (20) synthesized 13 COX-2 and 5-LOX inhibitors; among these inhibitors, N-hydroxy urea derivatives of indomethacin and diclofenac exhibited a potent antimigratory effect in the SW620 cell line.

Collectively, these existing studies investigated the effects of 5-LOX inhibitors on the proliferation, apoptosis and invasion of CRC cells. However, to the best of our knowledge, no previous study has compared the effects of 5-LOX knockdown and overexpression on the migration and invasion of CRC cells. Furthermore, the specific relationship between 5-LOX and EMT in CRC cells has yet to be elucidated. To elucidate the role of 5-LOX, small interfering RNAs (siRNAs) and recombinant lentiviruses were specifically designed to knock down and overexpress the 5-LOX gene in CRC cells, respectively. Angiogenesis is the beginning of tumor development, which creates necessary conditions for tumor invasion and metastasis (21); vascular endothelial growth factor (VEGF) and angiogenin are two major proangiogenic factors that play marked roles in tumor angiogenesis (22,23). Therefore, the effects of media collected from CRC cells with or without 5-LOX expression on the mRNA levels of proangiogenic factors in human umbilical vein endothelial cells (HUVECs) were also investigated. In the present study, the role of 5-LOX in migration, invasion and EMT of CRC cells was evaluated by overexpression and knockdown of 5-LOX.

## Materials and methods

*Cell lines and culture*. The HCT116, HT29, RKO and SW480 human colorectal cancer cell lines were purchased from Procell Biotechnology Co,. Ltd. Authentication of the aforementioned cell lines was conducted using short tandem repeat profiling. HUVECs were purchased from the Cell Resource Center, Institute of Basic Medical Sciences, CAMS/PUMC. HCT116 and RKO cell lines were cultured with Dulbecco's modified Eagle's medium (Sangon Biotech Co., Ltd.) containing 10% fetal bovine serum (FBS) (PAN-Biotech GmbH) and 1% penicillin/streptomycin (Sangon Biotech Co., Ltd.). HT29 and SW480 cell lines were cultured in RPMI-1640 (Sangon Biotech Co., Ltd.) containing 10% FBS and 1% penicillin/streptomycin. HUVECs were cultured in a HUVEC specific complete medium (Procell Life Science & Technology Co., Ltd.). All cells were maintained in a humidified atmosphere at 37˚C containing 5% $CO_2$.

*siRNA sequence and transfection in vitro*. A total of three different siRNAs (50 $\mu$M) of 5-LOX were synthesized to knock down the expression of 5-LOX in HCT116 cells, and a non-targeting siRNA control (si-NC) was transfected to the cells as a negative control. These sequences were synthesized by Huzhou Hippo Biotechnology Co., Ltd. as follows: si-5-LOX-#1, 5'-CCAAAUGCCACAAGGAUUUTT-3' (sense) and 5'-AAA UCCUUGUGGCAUUUGGCA-3' (antisense); si-5-LOX-#2, 5'-GCAGGAAGACCUGAUGUUUTT-3' (sense); and 5'-AAA CAUCAGGUCUUCCUGCCA- 3' (antisense); si-5-LOX-#3, 5'-CCAUUGCAAUCAACACCAATT-3' (sense); and 5'-UUG GUGUUGAUUGCAAUGGTG-3' (antisense); si-NC, 5'-UUC UCCGAACGUGUCACGUdTdT-3' (sense) and 5'-ACGUGA CACGUUCGGAGAAdTdT-3' (antisense). HCT116 cells in the logarithmic growth phase were seeded into six-well plates at a density of $2x10^5$ per well. Transfection with 40 nM siRNA was performed with Lipofectamine RNAiMAX transfection reagent (cat. no. 13778030; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. Following transfection at 37˚C for 48 h, gene knockdown efficiency was measured by quantitative PCR (qPCR) and western blotting analysis.

*Recombinant lentivirus infection*. Recombinant lentiviruses overexpressing 5-LOX (OE-5-LOX) were constructed by using the GV365 plasmid (*p*Ubi-MCS-3FLAG-*p*CMV-EGFP), with empty vector control lentiviruses (OE-Vector) as control. All lentiviruses were produced by Shanghai GeneChem Co., Ltd.. The lentiviruses were generated by transfecting GV365, pHelper 1.0 and pHelper 2.0 plasmid (Shanghai GeneChem Co., Ltd.) into 293T cells with E-trans transfection reagents (cat. no. REVG007; Shanghai GeneChem Co., Ltd.) at 37˚C for 48 h. The titer of the lentivirus used for infection was $2x10^8$ transducing units/ml. RKO cells in the logarithmic growth phase were seeded into six-well plates at a density of $2x10^5$/well. Lentiviruses were infected into the RKO cells at a multiplicity of infection of 50, supplemented with 5 $\mu$g/ml polybrene (MilliporeSigma) to enhance infection efficiency. After incubation at 37˚C for 72 h, infection efficiency was assessed by fluorescence microscopy. Successful overexpression of the target 5-LOX gene was verified by qPCR and western blotting analysis.

*Incubation of HUVECs*. HCT116 cells ($1x10^6$ cells/well) were transfected with siRNA and RKO cells ($1x10^6$ cells/well) were infected with lentivirus (as aforementioned). Following siRNA transfection (48 h) and lentivirus infection (72 h), the culture media was replaced with fresh media; conditioned media were subsequently collected after 24 h of incubation. HUVECs ($1x10^6$ cells/well) were plated in 6-well plates and incubated

with the media collected from siRNA-transfected HCT116 or lentivirus-infected RKO cells at 37˚C for 24 h. HUVECs were then divided into four groups: si-NC group (transfected with the non-targeting siRNA control) and si-5-LOX-#2 group (transfected with si-5-LOX-#2), OE-Vector group (infected with empty vector control lentiviruses) and OE-5-LOX group (infected with 5-LOX overexpressing lentiviruses). Consequently, qPCR was used to detect the mRNA levels of VEGF and angiogenin in HUVECs. The expression of VEGF protein in HUVECs was determined by western blotting analysis.

*Cell viability assay*. Cell viability was investigated using the cell counting kit (CCK)-8 assay (cat. no. C0037; Beyotime Biotechnology). HUVECs (2,500 cells/well) were cultured with media collected from siRNA-transfected HCT116 cells or lentivirus-infected RKO cells in a 96well culture plate at 37˚C for 24, 48 and 72 h. The absorbance was detected at 450 nm after the cells had been treated with 10% CCK-8 at 37˚C for 2 h. Cell viability was calculated as the ratio of optical density (OD) values of medium-treated cells (24, 48 and 72 h) to those of naive cells (0 h).

*Tumor cell migration and invasion assays*. HCT116 cells transfected with siRNA were divided into si-NC and si-5-LOX#2 groups. RKO cells infected with lentiviruses were divided into OE-Vector group and OE-5-LOX group. HCT116 cells ($8x10^4$ cells/well) and RKO cells ($4x10^4$ cells/well) were suspended in serum-free medium and deposited into the upper Transwell chamber. Upper chambers without Matrigel or Matrigel-coated were used for migration and invasion assays, respectively. For the invasion assay, Matrigel (Corning, Inc.) was diluted in serum-free medium at a ratio of 1:8. A uniform layer was applied to the upper chamber of the Transwell inserts, and the coated inserts were incubated at 37˚C for 1 h to allow polymerization. A medium containing 10% FBS was added to the lower chamber for the two assays. Cells were cultured at 37˚C and 5% $CO_2$ for 24 h and then cleaned with PBS. After discarding the PBS, cells were fixed with methanol (MilliporeSigma) for 15 min at room temperature and then stained with 0.1% crystal violet (MilliporeSigma) for 15 min at room temperature. Stained cells were imaged using a light microscope (x100 magnification), and a total of five random fields per insert were captured.

*Wound healing assay*. Transfected HCT116 cells and infected RKO cells were seeded at a density of $1x10^6$ cells/well in a six-well culture plate attaining 90% confluence, then a wound was created using a 200 $\mu$l pipette tip. Images were captured at three random fields of view by a light microscope (x40 magnification) at 0 and 24 h. Wound area was measured using ImageJ software (version 1.54g; National Institutes of Health). Wound-healing assay results were presented as migration rate (%)=(initial wound area-wound area at 24 h)/initial wound area x100.

*Apoptosis assay*. Transfected HCT116 cells and infected RKO cells were collected and suspended in Binding Buffer at a density of $1x10^6$ cells/ml. HCT116 and RKO cells were stained with Annexin V-FITC and Annexin V-APC Apoptosis Detection kits (cat. no. 88-8005-74/88-8007-74; Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. Apoptosis was examined by flow cytometry (cytoFLEX; Beckman Coulter, Inc.). The apoptotic rate was assessed with CytExpert (V 2.4.0.28) software (Beckman Coulter, Life science).

*Total RNA isolation and reverse transcription-qPCR analysis (RT-qPCR)*. Total RNA of HCT116, HT29, RKO, SW480, transfected HCT116, infected RKO and HUVECs was extracted by using RNAprep Pure Cell/Bacteria Kit (cat. no. DP430; Tiangen Biotech Co., Ltd.) in accordance with the manufacturer's instructions. RT was performed with a ReverTra Ace qPCR RT kit (cat. no. FSQ-101; Toyobo Co., Ltd.) in a total volume of 20 $\mu$l. RT was performed as follows: 37˚C for 15 min and 98˚C for 5 min. The qPCR was performed with SYBR Green qPCR Master Mix (cat. no. HY-K0522; MedChemExpress). The reaction protocol was as follows: 5 min initial denaturation at 95˚C, followed by 40 cycles of amplification with 15 sec at 95˚C, 30 sec at 60˚C and 30 sec at 72˚C for each cycle. This was followed by a melt curve analysis to determine the reaction specificity. β-actin was used as the endogenous control. The oligonucleotide primers used were as follows: β-actin, 5'-CATGTACGTTGCTATCCAGGC-3' (forward) and 5'-CTCCTTAATGTCACGCACGAT-3' (reverse); 5-LOX, 5'-ACAAGCCCTTCTACAACGACT-3' (forward) and 5'-AGCTGGATCTCGCCCAGTT-3' (reverse); Angiogenin, 5'-GTTGGTCTTCGTGCTGGGTCTG-3' (forward) and 5'-AGTGCTGGGTCAGGAAGTGTGT-3' (reverse); VEGF, 5'-GGCAGAAGGAGGAGGGCAGAAT-3' (forward) and 5'-GGGCACACAGGATGGCTTGAAG-3' (reverse). The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative RNA expression (24).

*Protein extraction and western blotting (WB)*. HCT116, HT29, RKO, SW480, transfected HCT116 and infected RKO cells were incubated and then centrifuged at 175 x g to collect cells. Total protein was extracted using RIPA buffer (Beyotime Biotechnology). Protein concentration was determined with a BCA protein quantification kit (cat. no. P0009; Beyotime Biotechnology) in accordance with the manufacturer's instructions. Extracted proteins (20 $\mu$g) were then separated by SDS-PAGE in 10-12% acrylamide gel and transferred to a polyvinylidene fluoride membrane (MilliporeSigma). The membrane was subsequently blocked with 5% skimmed milk in TBS containing 0.05% Tween-20 (Beyotime Biotechnology) for 1 h at room temperature. The membrane was then incubated with a primary antibody (1:1,000) overnight at 4˚C. The primary antibodies were 5-LOX (cat. no. 83794-4-RR; Proteintech Group, Inc.), E-cadherin (cat. no. 20874-1-AP; Proteintech Group, inc.), N-cadherin (cat. no. 13116; Cell Signaling Technology, Inc.), Snail (cat. no. 13099-1-AP; Proteintech Group, Inc.), Vimentin (cat. no. 5741; Cell Signaling Technology, Inc.), VEGFA (cat. no. 81323-2-RR; Proteintech Group, Inc.) and GAPDH (cat. no. 81640-5-RR; Proteintech Group, Inc.). The membrane was washed three times in TBST and incubated with diluted secondary antibody (1:10,000) conjugated with horseradish peroxidase solution (cat. no. ab205718; Abcam) for 1 h at room temperature. After washing, the membrane was visualized with ECL substrate (cat. no. SW2050; Beijing Solarbio Science & Technology

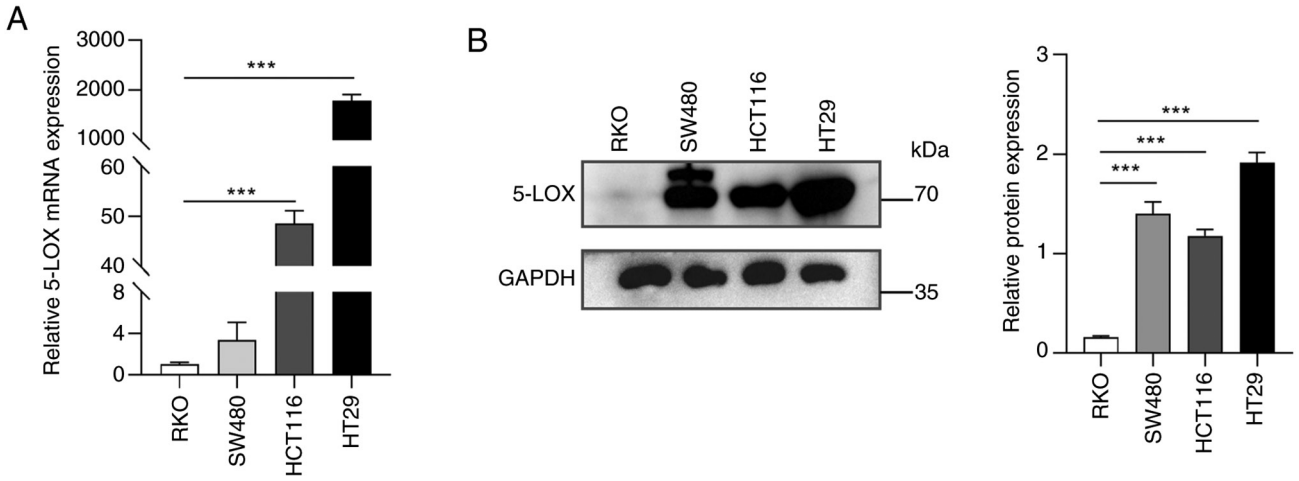


Figure 1. Expression levels of 5-LOX in human colorectal cancer cell lines. (A) Relative mRNA levels of 5-LOX in RKO, SW480, HCT116 and HT29 cell lines were measured by reverse transcription-quantitative polymerase chain reaction. β-actin was used as an internal control. (B) Protein expression levels of 5-LOX in RKO, SW480, HCT116 and HT29 cell lines were determined by western blotting. GAPDH protein was used as an internal control. Values are expressed as the mean ± standard error of the mean (n=3). ***P<0.001. 5-LOX, 5-lipoxygenase.

Co., Ltd.) by using chemiluminescence (AzureC500; Azure Biosystems, Inc.). The gray values of the bands were quantified using ImageJ (version 1.54g; National Institutes of Health), and GAPDH was used as the loading control.

*Enzyme-linked immunosorbent assay (ELISA)*. RKO cells infected with lentiviruses (the OE-Vector and OE-5-LOX groups) were seeded in six-well plates at a density of $5x10^5$ cells/well. After 72 h of incubation at 37˚C, media were replaced with fresh media, and the cells were cultured at 37˚C for another 48 h before supernatant collection. The concentrations of leukotriene $E_4$ ($LTE_4$) and leukotriene $B_4$ ($LTB_4$) in the supernatant were detected using a $LTE_4$ ELISA kit (cat. no. MM-928804O1) and a $LTB_4$ ELISA kit (cat. no. MM-927697O1; both Jiangsu Enzyme Immunoassay Co., Ltd.), in accordance with the manufacturer's instructions. OD values were measured at 450 nm, and sample concentrations were calculated based on the standard curve. Relative expression was calculated as concentration ration of the OE-5-LOX group relative to the OE-Vector group.

*Statistical analysis*. Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism statistical package (version 9.5; Dotmatics). Comparisons between two groups were conducted with Student's t-test. Analysis of more than two groups was performed with one-way ANOVA followed by Dunnett's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Expression of 5-LOX in different CRC cell lines*. To select the prime candidate CRC cell line for further 5-LOX gene knockdown and overexpression, the mRNA and protein expression levels of 5-LOX were analyzed in four CRC cell lines: HCT116, HT29, RKO and SW480. As shown in Fig. 1A, high levels of 5-LOX mRNA were observed in HCT116 and HT29 cells while low levels of 5-LOX mRNA were observed in RKO and SW480 cells. The 5-LOX protein was widely expressed in HCT116, HT29 and SW480 cells other than RKO cells (Fig. 1B). Thus, HCT116 cells, which exhibited high levels of 5-LOX expression, were used for the subsequent gene knockdown investigations. RKO cells, with low levels of 5-LOX expression, were used for gene overexpression investigations.

*Inhibitory effects of siRNAs designed to target 5-LOX*. The mRNA levels of 5-LOX in HCT116 cells were determined by RT-qPCR following treatments with siRNA for 48h. As shown in Fig. 2A, three different siRNAs inhibited the 5-LOX mRNA levels up to 72% compared with the si-NC group. Of these three siRNAs, si-5-LOX-#2 inhibited the expression of 5-LOX protein more effectively than the other siRNAs (Fig. 2B). Therefore, the following investigations were conducted by using si-5-LOX-#2.

*Overexpression of 5-LOX in RKO cells*. Recombinant lentiviruses expressing 5-LOX were used to infect RKO cells. An embedded green fluorescent protein-tag was used to visualize viral infection. As shown in Fig. 3A, RKO cells were successfully infected with the recombinant lentiviruses after 72 h of incubation. The mRNA level of 5-LOX in the OE-5-LOX group was significantly higher than that in the OE-Vector group (Fig. 3B). As shown in Fig. 3C, the expression of 5-LOX protein in the OE-Vector group remains at the basal level, consistent with the inherently low endogenous 5-LOX expression in RKO cells. However, the expression of 5-LOX protein in the OE-5-LOX group was significantly higher than that in OE-Vector group. 5-LOX is a key enzyme in leukotriene biosynthesis (25); as shown in Fig. S1, the overexpression of 5-LOX significantly increased levels of $LTB_4$ and $LTE_4$ in RKO cells.

*Knockdown and overexpression of 5-LOX impacts the migration and invasion of CRC cells*. Transwell migration (Fig. 4A and B) and wound healing assays (Fig. 4I) revealed

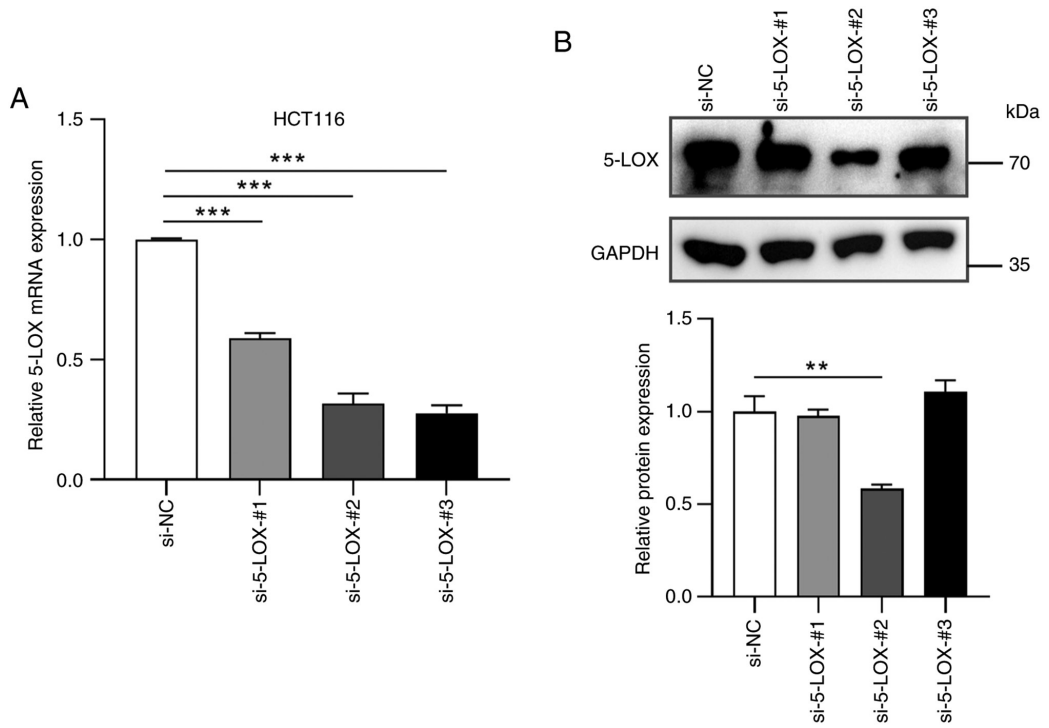


Figure 2. Knockdown efficiency of HCT116 cells transfected by siRNAs. (A) Relative mRNA levels of 5-LOX in HCT116 cells with three different siRNAs treatments were measured by reverse transcription-quantitative PCR. β-actin was used as an internal control. (B) Relative protein expression of 5-LOX in HCT116 cells with siRNA transfection were determined by western blotting. GAPDH protein was used as an internal control. Values are expressed as the mean ± standard error of the mean (n=3). $^{**}$P<0.01, $^{***}$P<0.001. 5-LOX, 5-lipoxygenase; siRNA, small interfering RNA; NC, negative control.

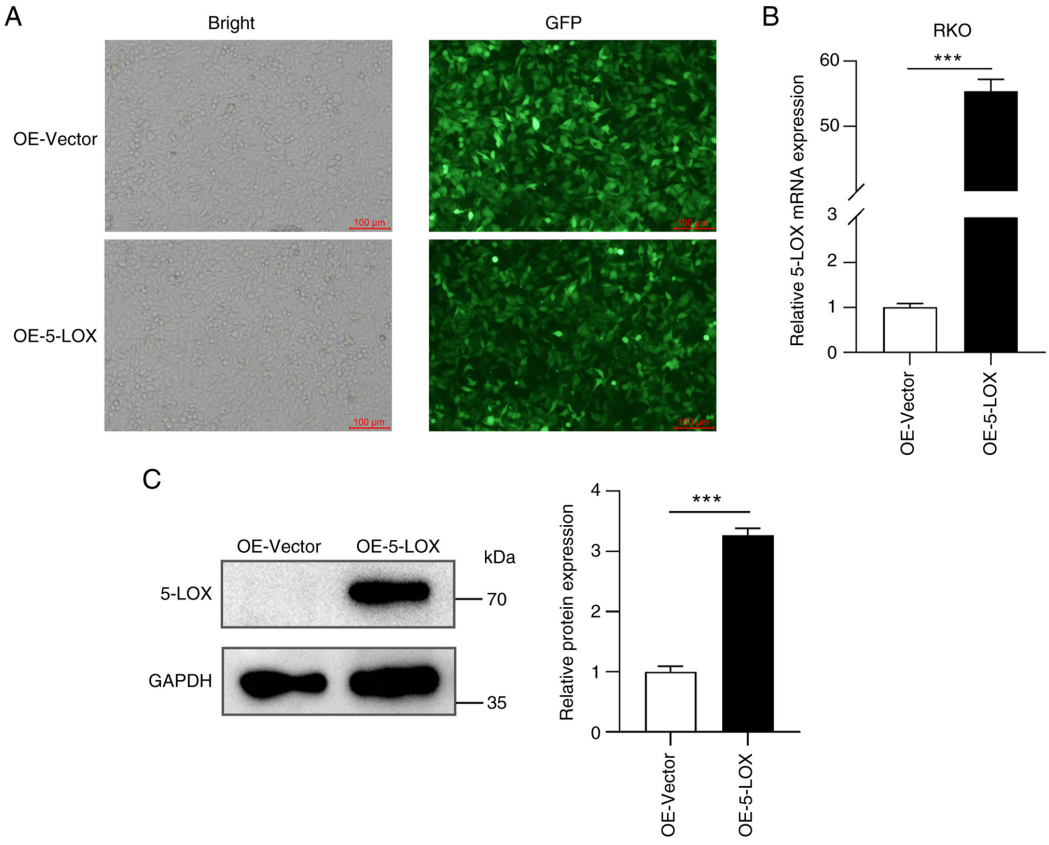


Figure 3. Efficiency of lentiviral infection on 5-LOX OE in RKO cells. (A) Bright field and fluorescence images following the lentiviral infection of RKO cells in OE-Vector and OE-5-LOX groups. (B) Relative mRNA expression of 5-LOX in RKO cells with two treatments (OE-Vector and OE-5-LOX) was determined by reverse transcription-quantitative PCR. (C) Relative protein expression of 5-LOX in RKO cells with two treatments (OE-Vector and OE-5-LOX) was determined by western blotting. Values are expressed as the mean ± standard error of the mean (n=3). $^{***}$P<0.001. OE-Vector, cells infected with empty vector control lentivirus; OE-5-LOX, cells infected with 5-LOX recombinant lentivirus; GFP, green fluorescent protein; 5-LOX, 5-lipoxygenase.

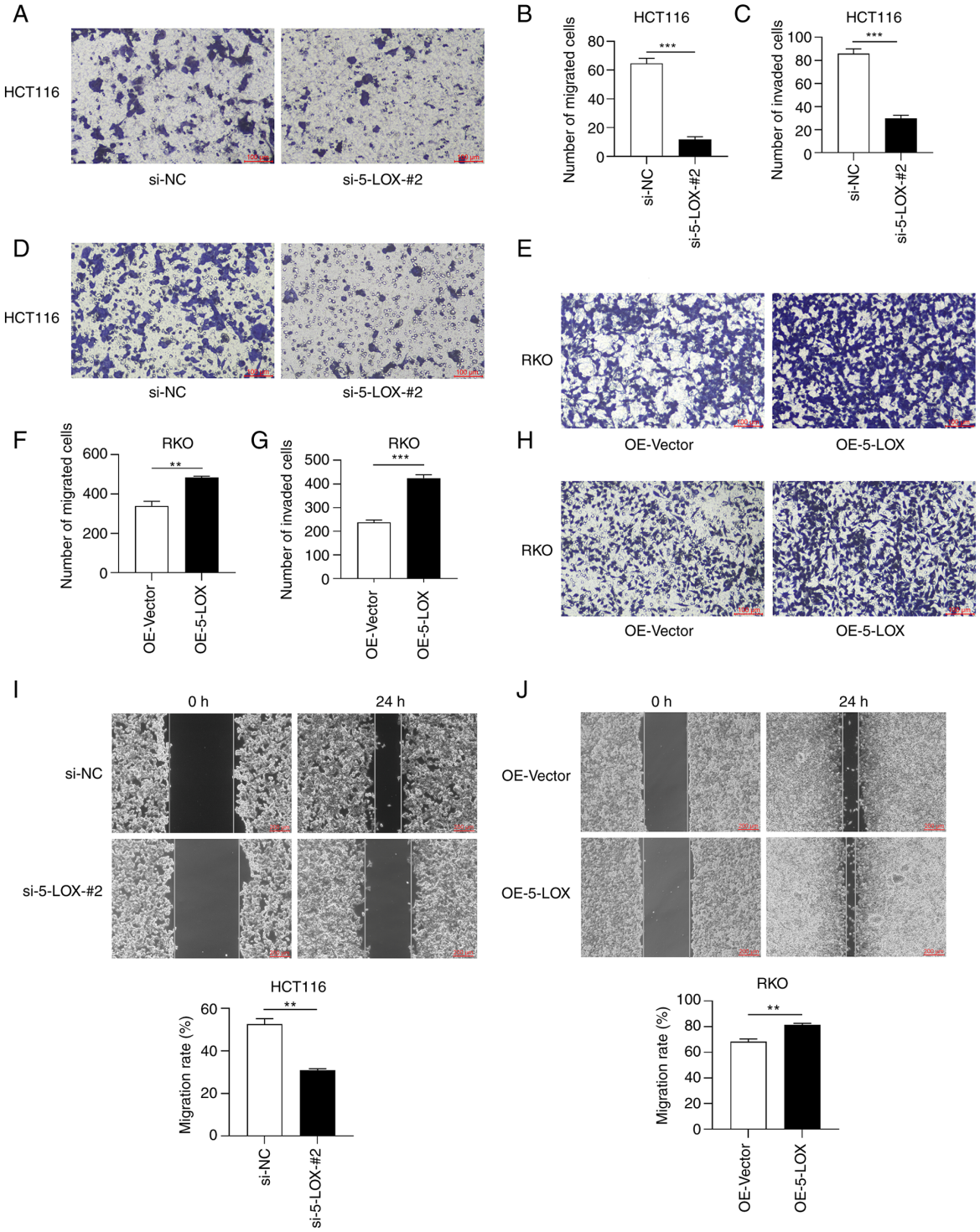


Figure 4. Migratory and invasive abilities of 5-LOX-silenced HCT116 cells and 5-LOX-OE RKO cells. HCT-116 cells transfected with siRNAs were divided into si-NC and si-5-LOX-#2 groups. RKO cells infected with lentivirus were divided into OE-Vector and OE-5-LOX groups. (A) Representative migrated HCT116 cells stained with crystal violet on the lower side of the membrane and (B) quantified numbers of migrated HCT116 cells in the Transwell migration assay. (C) Number and (D) representative images of invasive HCT116 cells in the Transwell invasion assay. (E) Representative images and (F) quantified numbers of migrated RKO cells in the Transwell migration assay. (G) Number of (H) invasive RKO cells in the Transwell invasion assay. The migration rate of (I) HCT-116 cells and (J) RKO cells was determined by woundhealing assay. Values are expressed as the mean ± standard error of the mean (n=3). $^{**}$P<0.01, $^{***}$P<0.001. OE-Vector, cells infected with empty vector control lentivirus; OE-5-LOX, cells infected with 5-LOX recombinant lentivirus; siRNA, small interfering RNA; 5-LOX, 5-lipoxygenase; NC, negative control.

that the migration ability of HCT116 cells in the si-5-LOX-#2 group was significantly reduced compared with the si-NC group. Transwell invasion assay revealed that the si-5-LOX-#2 group had a significantly lower number of invaded cells than the si-NC group (Fig. 4C and D). However, the migration ability of RKO cells in the OE-5-LOX group was significantly higher than that in the OE-Vector group which was determined by Transwell migration (Fig. 4E and F) and wound healing

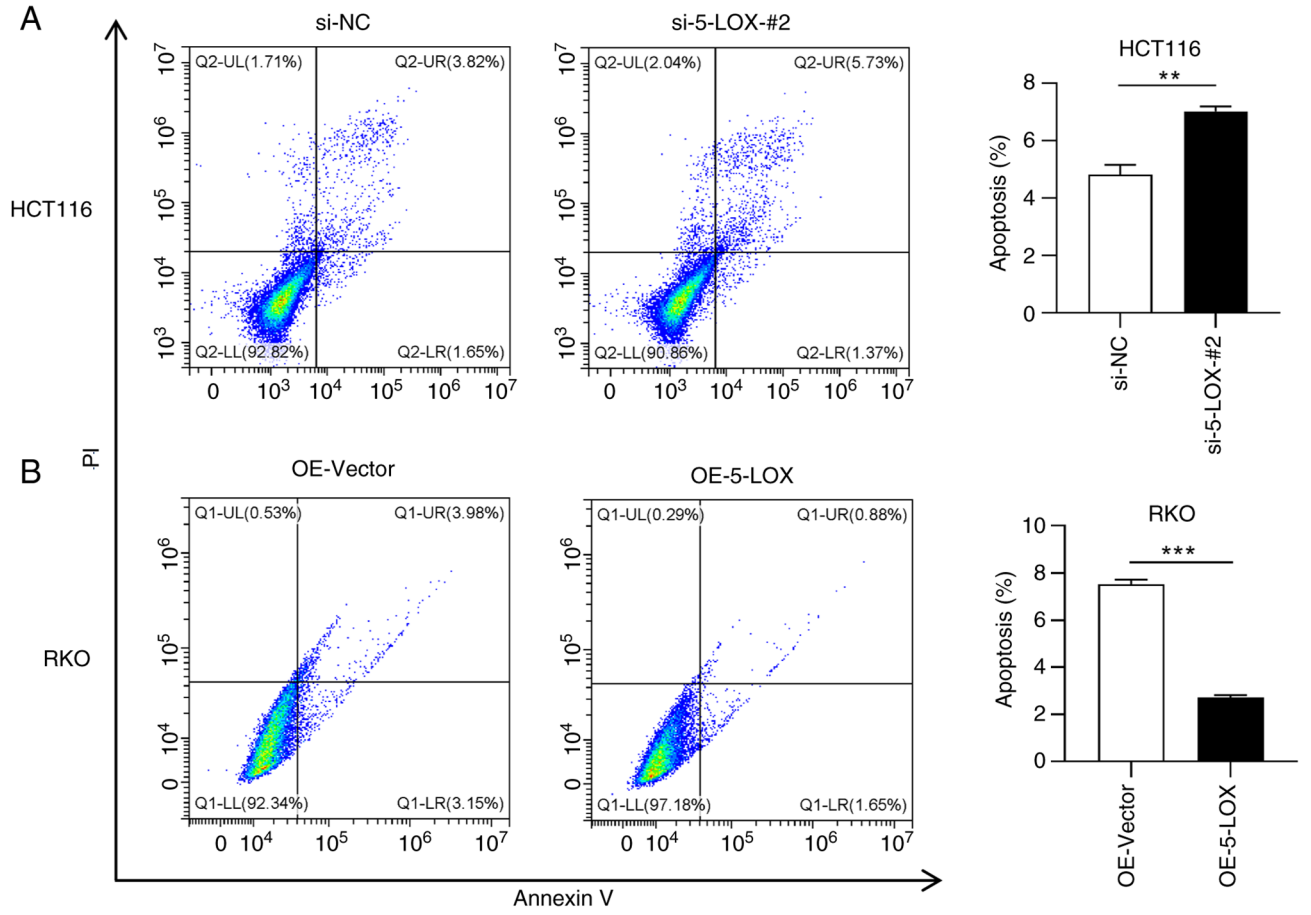


Figure 5. Apoptosis rate regulated by 5-LOX knockdown and overexpression in CRC cells. The apoptotic rates of (A) siRNA-silenced HCT116 cells (si-NC and si-5-LOX-#2 groups) and (B) lentivirus-overexpressed RKO cells (OE-Vector and OE-5-LOX groups) were determined by flow cytometry. $^{**}$P<0.01, $^{***}$P<0.001. OE-Vector, cells infected with empty vector control lentivirus; OE-5-LOX, cells infected with 5-LOX recombinant lentivirus; siRNA, small interfering RNA; CRC, colorectal cancer; 5-LOX, 5-lipoxygenase; NC, negative control.

assays (Fig. 4J). RKO cells overexpressing 5-LOX (the OE-5-LOX group) exhibited significantly higher invasion (Fig. 4G and H) ability compared with the cells infected with the control-lentiviruses (the OE-Vector group).

*Knockdown and overexpression of 5-LOX affects the apoptosis of CRC cells*. As shown in Fig. 5A, the apoptotic rate of 5-LOX-silenced HCT116 cells (the si-5-LOX-#2 group) was significantly increased compared with the cells transfected with control siRNA (the si-NC group). Alternatively, RKO cells overexpressing 5-LOX (the OE-5-LOX group) exhibited significantly lower apoptotic rate in comparison to the OE-Vector group (Fig. 5B).

*Knockdown and overexpression of 5-LOX regulates EMT*. To evaluate the relationship between 5-LOX and EMT in CRC cells, the expression of molecular markers for EMT were analyzed by WB in 5-LOX-silenced CRC cells, 5-LOX-overexpressed CRC cells and their corresponding control cells. WB analyses revealed that 5-LOX knockdown significantly increased the expression of E-cadherin and reduced the expression of N-cadherin, Snail and Vimentin in HCT116 cells (Fig. 6A). However, the overexpression of 5-LOX significantly downregulated the expression of E-cadherin but upregulated the expression of N-cadherin, Snail and Vimentin in RKO cells (Fig. 6B).

*Effects of culture media from CRC cells on HUVECs*. To elucidate whether the products secreted by 5-LOX-silenced and 5-LOX-overexpressed CRC cells affect the angiogenesis of HUVECs, HUVECs were treated with media from 5-LOX-silenced HCT116 cells, 5-LOX-overexpressed RKO cells and their corresponding control cells *in vitro*. The CCK-8 assay showed that cell viability of HUVECs was significantly suppressed by treating with culture medium from the si-5-LOX-#2 group (Fig. 7A), while cell viability of HUVECs was significantly promoted by treating with culture medium from the OE-5-LOX group (Fig. 7B). The mRNA levels of VEGF and angiogenin were analyzed by RT-qPCR; as shown in Fig. 7C, the mRNA levels of VEGF and angiogenin were significantly lower in HUVECs which had been treated with culture medium from the si-5-LOX-#2 group than in HUVECs which had been treated with culture medium from the si-NC group. However, the mRNA levels of VEGF and angiogenin were significantly increased when HUVECs were treated with culture medium from the OE-5-LOX group rather than with culture medium from the OE-Vector group (Fig. 7D). The expression of VEGF protein was significantly decreased in HUVECs treated with conditioned media from 5-LOX silenced HCT116 cells but significantly increased in HUVECs treated with conditioned media from 5-LOX overexpressed RKO cells, compared with their respective control groups (Fig. S2).

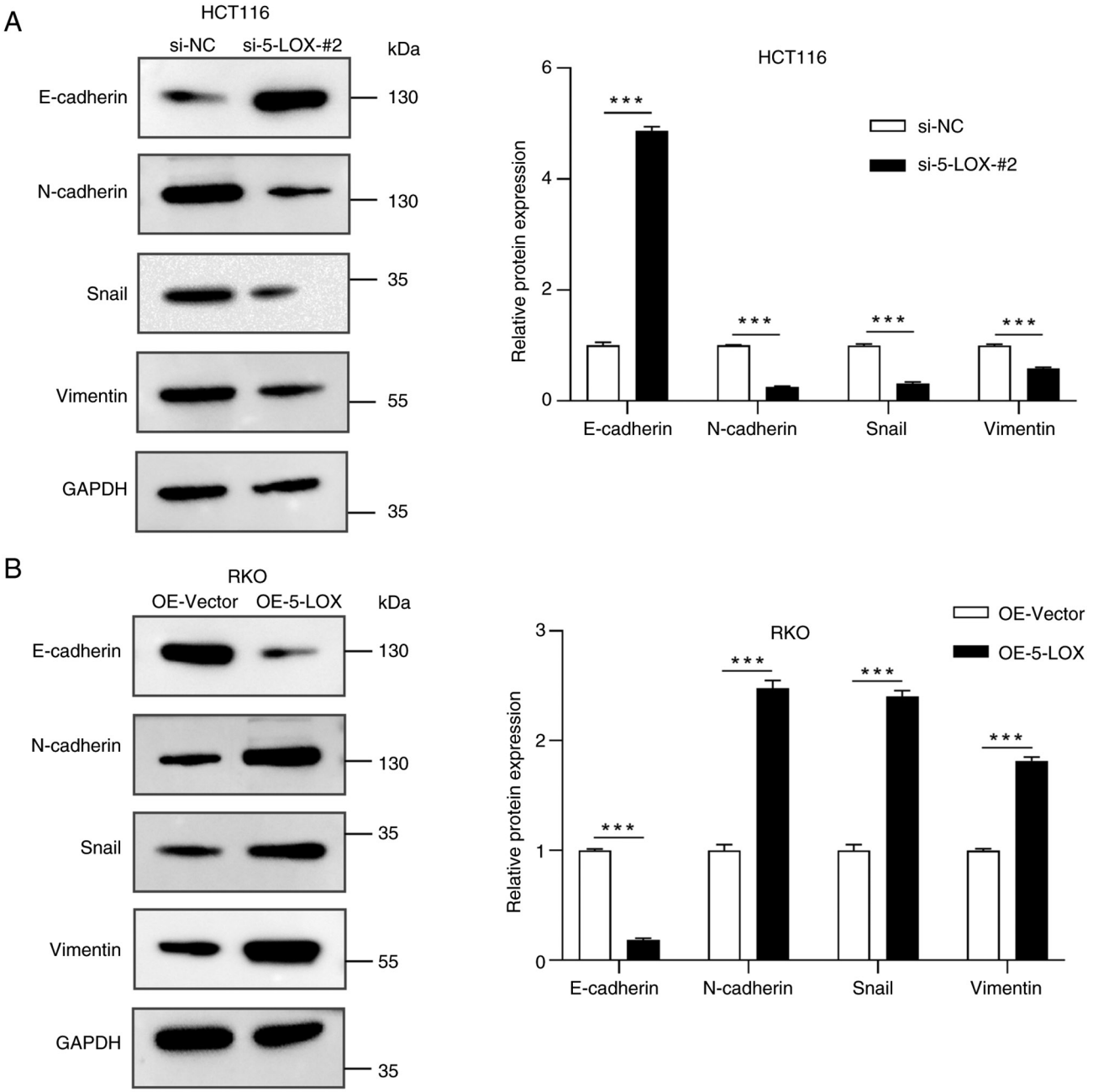


Figure 6. Levels of EMT-associated proteins regulated by 5-LOX knockdown and overexpression in CRC cells. (A) The levels of EMT-associated proteins (E-cadherin, N-cadherin, Snail and Vimentin) were determined by WB in siRNA-silenced HCT116 cells (si-NC and si-5-LOX-#2 groups). (B) The levels of EMT-associated proteins were determined by WB in lentivirus-overexpressed RKO cells (OE-Vector and OE-5-LOX groups). Values are expressed as the mean ± standard error of the mean (n=3). ***P<0.001. OE-Vector, cells infected with empty vector control lentivirus; OE-5-LOX, cells infected with 5-LOX recombinant lentivirus; siRNA, small interfering RNA; CRC, colorectal cancer; 5-LOX, 5-lipoxygenase; NC, negative control; WB, western blot; EMT, epithelial-mesenchymal transition.

## Discussion

5-LOX is upregulated in CRC and plays a notable role in the development of other forms of cancer, including pancreatic cancer (26), prostate adenocarcinoma (27) and esophageal adenocarcinoma (28). The overexpression of 5-LOX gene which increase mitogenesis, mutagenesis, angiogenesis, cell survival, immunosuppression and metastasis in the inflammogenesis of CRC (29). In the present study, the role of 5-LOX in CRC was demonstrated by both silencing and overexpressing 5-LOX in CRC cells.

RNA interference is the process of sequence-specific, post-transcriptional gene silencing in animals and plants. siRNA-mediated gene silencing is an excellent tool for studying gene function in mammalian cells and may eventually be used for gene-specific therapeutics (30). The results of the present study demonstrated that 5-LOX was widely expressed in both HT29 and HCT116 CRC cell lines. Of the three siRNAs tested in the present study, si-5-LOX-#2 exhibited notable ability to inhibit both the mRNA and protein expression of 5-LOX in HCT116 cells. After treating HT29 with si-5-LOX-#2, the same inhibitory effect was observed, but the treated HT29

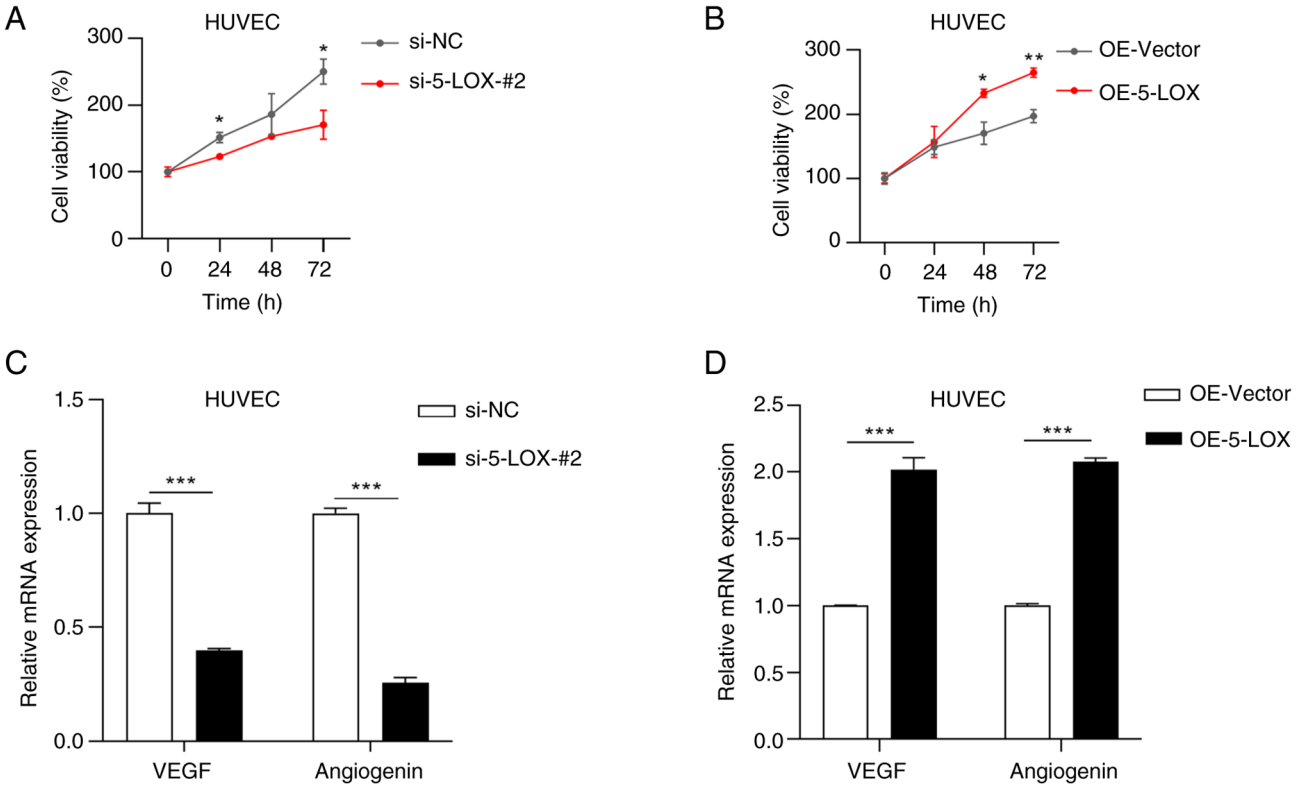


Figure 7. Cell viability and mRNA expression of VEGF and angiogenin in HUVECs. The cell viability of HUVECs incubated with collected media from (A) siRNA-silenced HCT116 cells (si-NC and si-5-LOX-#2 groups) and (B) lentivirus-overexpressed RKO cells (OE-Vector and OE-5-LOX groups) for 24, 48 and 72 h. mRNA expression of VEGF and angiogenin in HUVECs were quantified by RT-qPCR after 24 h incubation with collected media from (C) siRNA-silenced HCT116 cells and (D) lentivirus-overexpressed RKO cells. Values are expressed as the mean ± standard error of the mean (n=3). $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001. OE-Vector, cells infected with empty vector control lentivirus; OE-5-LOX, cells infected with 5-LOX recombinant lentivirus; siRNA, small interfering RNA; 5-LOX, 5-lipoxygenase; NC, negative control; HUVEC, human umbilical vein endothelial cell.

cells did not exhibit any migration or invasion abilities (data not shown). Therefore, the HCT116 cell line was considered as the best candidate for the 5-LOX knockdown investigations. A previous study reported that the overexpression of 5-LOX by lentivirus increased the invasion and migration abilities of PANC-1 cells (31). However, additional studies regarding the utilization of 5-LOX lentivirus infection and the overexpression effects of 5-LOX in CRC cells are needed. RKO cells with low mRNA and protein expression of 5-LOX were selected for lentiviral infection experiments. 5-LOX lentiviral infection significantly increased the expression of 5-LOX, as determined by WB and RT-qPCR. The 5-LOX catalyzes arachidonic acid to produce $LTB_4$, $LTD_4$ and $LTE_4$ (32), and in the present study, the OE-5-LOX group exhibited significantly higher levels of $LTB_4$ and $LTE_4$, which indicating the increased 5-LOX enzymatic activity in RKO cells following 5-LOX lentiviral infection.

CRC is a highly metastatic malignancy; therefore, the impacts of 5-LOX knockdown and overexpression on the migration and invasion of CRC cells were investigated. Downregulation of 5-LOX expression in HCT116 cells significantly decreased migration and invasion capacities *in vitro*. However, the overexpression of 5-LOX in RKO cells significantly upregulated their migration and invasion capacities *in vitro*. In CRC, EMT is a widely-known biological process that plays a marked role in tumor progression, invasion and metastasis. Markers of EMT (E-cadherin, N-cadherin, Vimentin and Snail) all acts as key indicators of invasion and metastasis in CRC (33). In the present study, it was demonstrated that the downregulation of 5-LOX expression in HCT116 cells inhibited the expression of N-cadherin, Vimentin, Snail; however, improved the expression of E-cadherin. By contrast, high levels of N-cadherin, Vimentin, Snail along with low level of E-cadherin were detected in RKO cells with overexpression of 5-LOX. Collectively, these results suggest that EMT may contribute to the 5-LOX-mediated invasion and migration of CRC cells. Moreover, the silencing of 5-LOX significantly increased the apoptotic rate in HCT116 cells, whereas the overexpression of 5-LOX significantly decreased the apoptotic rate in RKO cells. However, the apoptotic rates were relatively low, indicating a limited magnitude of apoptosis induced by modulating 5-LOX expression. Although siRNA-mediated 5-LOX knockdown and lentiviral 5-LOX overexpression were used as complementary strategies in the present study, re-overexpression of 5-LOX after knockdown requires further validation in future work.

It was reported that F3, an inhibitor of 5-LOX, effectively suppressed angiogenesis in endothelial cells (34). Angiogenesis *in vivo* usually occurs in a complex microenvironment in which a variety of cells interact together. Endothelial cells in the tumor microenvironment serve a notable role in the development and progression of cancer by regulating angiogenesis (35). Previous research demonstrated that hypoxia (36) or tumor microenvironment stimuli (37) could increase the VEGF expression in

HUVECs. Downregulation of angiogenin in HUVECs caused a reduction in cell proliferation and angiogenesis (38). To understand the effect of the microenvironment produced by CRC cells with or without the expression of 5-LOX on HUVECs, HUVECs *in vitro* were treated with culture media from CRC cells to simulate the physiological environment *in vivo*. Conditioned media was collected from 5-LOX-silenced CRC cells, 5-LOX-overexpressed CRC cells and their corresponding control cells and then incubated media with HUVECs. The cell viability and mRNA levels of VEGF and angiogenin were upregulated in HUVECs that had been incubated with culture medium collected from 5-LOX-overexpressed CRC cells but downregulated in HUVECs that had been incubated with culture medium collected from 5-LOX-silenced CRC cells. Consistent results were also observed for VEGF protein expression in HUVECs. Conditioned medium collected from CRC cells with 5-LOX overexpression stimulated the expression of proangiogenic factors in HUVECs, indicating that the tumor microenvironment in CRC cells overexpressing 5-LOX may be angiogenically active. Further studies are needed to determine whether 5-LOX can influence the tube formation of HUVECs *in vitro*.

Taken together, the present study demonstrated that the overexpression of 5-LOX in CRC cells promoted the migration and invasion of CRC cells, whereas the silencing of 5-LOX inhibited the migration and invasion of CRC cells. In addition, the knockdown but not overexpression of 5-LOX in CRC cells inhibited EMT, which consisted with the results of migration and invasion. Furthermore, the mRNA levels of proangiogenic factors in HUVECs were upregulated upon treatment with conditioned medium from 5-LOX-overexpressed CRC cells but not medium from CRC cells in which 5-LOX had been silenced. Collectively, the present findings suggest that 5-LOX plays a notable role in promoting migration, invasion and EMT in CRC cells, and that the tumor microenvironment of 5-LOX-overexpressed CRC cells may contribute to the angiogenesis in HUVECs. Thus, 5-LOX represents a potential target for the migration and invasion of CRC. Further investigation will focus on establishing CRC xenograft models in nude mice with 5-LOX-knockout and 5-LOX-overexpressing cell lines, to verify the biological function of 5-LOX in CRC *in vivo*.

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**Availability of data and materials**

The data generated in the present study may be requested from the corresponding author.

**Authors' contributions**

XuL, JH and XW designed and conceived the study. XuL, JH, JZ and TS performed the experiments. XiL, CB and XG analyzed data. XuL, TS and XW confirm the authenticity of all the raw data. XuL wrote the manuscript. All authors read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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