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ORIGINAL RESEARCH

MicroRNA-145 Inhibits Cell Migration and Invasion in Colorectal Cancer by Targeting TWIST

Xuning Shen[1]
Honggang Jiang[1]
Zhiheng Chen[1]
Bohao Lu[1]
Yi Zhu [ID][1]
Jiayan Mao[2]
Kequn Chai[2,3]
Wei Chen[2,3]

[1]Department of Gastroenterological Surgery, Affiliated Hospital of Jiaxing University, Jiaxing, Zhejiang, People's Republic of China; [2]Cancer Institute of Integrated Traditional Chinese and Western Medicine, Key Laboratory of Cancer Prevention and Therapy Combining Traditional Chinese and Western Medicine, Zhejiang Academy of Traditional Chinese Medicine, Hangzhou, Zhejiang 310012, People's Republic of China; [3]Department of Medical Oncology, Tongde Hospital of Zhejiang Province, Hangzhou, Zhejiang 310012, People's Republic of China

Correspondence: Wei Chen; Kequn Chai
Cancer Institute of Integrated Traditional Chinese and Western Medicine, Key Laboratory of Cancer Prevention and Therapy Combining Traditional Chinese and Western Medicine, Zhejiang Academy of Traditional Chinese Medicine, Hangzhou, Zhejiang 310012, People's Republic of China
Tel +86-571-89972114
Fax +86 571-88853199
Email wei_chen@zju.edu.cn;
ckq3301@aliyun.com

Introduction: MicroRNAs function as oncogenes or tumor suppressors in the development of various human cancers. We investigated the effect of microRNA-145 (miR-145) on colorectal cancer (CRC) cell invasion and migration.

Methods: The levels of miR-145 in CRC cells were examined by quantitative PCR; Western blotting was used to detect TWIST1 (twist family bHLH transcription factor 1) protein and the epithelial–mesenchymal transition (EMT)-related proteins (E-cadherin, vimentin). Then, we transfected miR-145 mimics or inhibitor into CRC cells and used the wound healing and Transwell invasion assays to investigate their migration and invasive capability, respectively.

Results: The miR-145 mimics suppressed CRC cell invasion and migration significantly; in contrast, miR-145 downregulation had the opposite effect. Furthermore, miR-145 regulated TWIST1 levels negatively at transcriptional level. TWIST1 knockdown significantly inhibited the CRC cell migration ability and the number of CRC cells that crossed the Transwell membrane. There was no significant difference in terms of migration and invasive capability after the cells had been transfected with miR-145 mimics or inhibitor plus TWIST1 small interfering RNA (siRNA) as compared to the TWIST1 siRNA–only group. Furthermore, we demonstrate that the inhibition of miR-145 could enhance the capability for lung metastasis in vivo.

Conclusion: Taken together, these findings indicate that miR-145 acts as a new tumor suppressor by regulating TWIST1 and plays a vital role in the invasive and migration ability of CRC cells.

Keywords: MicroRNA-145, cell migration and invasion, colorectal cancer, TWIST, EMT

Introduction

Colorectal cancer (CRC) is the third most common malignant tumor in the world, with high tumor migration and invasion ability.[1,2] In CRC, the development of normal epithelial cells into malignant tumors is considered to be a multi-stage process involving genetic alterations, leading to oncogene activation and tumor suppressor gene inactivation. Although migration and invasion are considered the deadliest features of solid tumors, the potential molecular mechanisms are rarely known. Therefore, it is very important to identify novel molecules with different expression in CRC cells, and it may aid the obtainment of insight into the involved mechanisms.

MicroRNAs (miRNAs) are small noncoding RNAs that are 18–22 nucleotides in length and that regulate gene expression negatively, affecting either mRNA cleavage or the translational inhibition of genes at post-transcriptional level.[3] They play vital effects in tumorigenesis and metastasis, regulating gene expression

through knockdown of mRNA translation, and also play oncogenic or tumor-suppressive roles according to the regulatory targets.[4] Recent reports have indicated that miRNAs play important roles in the occurrence and development of different human cancers.[5–7] miRNA-145 (miR-145), a tumor-suppressive miRNA, is downregulated in several malignancies such as breast cancer, lung cancer, bladder cancer, ovarian cancer, colon cancer, and prostate cancer.[8–13] miR-145 was first found to be downregulated in CRC in 2003, and it is considered a tumor suppressor gene.[14] Furthermore, miR-145 can regulate the expression response of a specific set of certain genes to tumorigenesis and metastasis by participating in CRC.[14,15] However, the potential molecular mechanism of miR-145 in the progression of CRC is unclear.

TWIST1 (twist family bHLH transcription factor 1), a highly conserved basic helix-loop-helix (bHLH) transcription factor, is represented by a basic DNA-binding region targeting the consensus E-box sequence 5′-CANNTG-3′ and a HLH area.[16] Epithelial–mesenchymal transition (EMT) is a new cellular process that is very important for the development of metastatic disease. TWIST1 is considered an inducer of EMT and a basic regulator of cancer metastasis.[17,18] TWIST1 promotes cancer metastasis by promoting EMT.[19,20] Based on its function as a cell adhesion molecule, the deletion of E-cadherin is considered a prerequisite for EMT to facilitate cancer cell proliferation and metastasis.[19] Therefore, regulating the expression of TWIST1 might be a potential target for suppressing CRC cell invasion and migration.

In our present study, we investigated miR-145 and determined its effect on CRC cell invasion and migration. We prove that TWIST1 expression correlated negatively with miR-145 expression in CRC. We also observed that miR-145 overexpression inhibited the cell invasive and migration ability by regulating TWIST1 in CRC cells.

Materials and Methods
Cell Culture
Human CRC cells (LOVO, HCT116, HT29) were obtained from American Type Culture Collection (Manassas, VA, USA). LOVO and HT29 cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA). The HCT116 cells were cultured in McCoy's 5A medium (Gibco) supplemented with 10% FBS and 1% penicillin/

streptomycin (Sigma). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

RNA Oligoribonucleotides and Transfection
miR-145 mimics, inhibitor, and negative control were synthesized by GenePharma (Shanghai, China). TWIST1 small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect the CRC cells according to the manufacturer's protocol. The oligoribonucleotide sequences were as follows:

miR-145 mimics: 5′-GUCCAGUUUUCCCAGGAAU CCCU-3′ and 5′-GGAUUCCUGGGAAAACUGGACUU -3′; miR-145 inhibitor: 5′-AGGGAUUCCUGGGAAAAC UGGAC-3′; negative control: 5′-UUCUCCGAACGUGU CACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAAT T-3′.

Wound Healing Assay
The cell migration ability was determined using the wound healing assay. Cells were plated in 6-well plates at 3×10^5 cells/well, and then transfected with miR-145 mimics, inhibitor, or control. Yellow pipette tip was used to create a wound in the cell monolayer. After washing with phosphate-buffered saline, the wound area was photographed under an inverted light microscope (Olympus IX51, Olympus, Center Valley, PA, USA) at 0 h and 48 h. The ratio of residual wound area to initial wound area was calculated and the wound area was quantified by Image-Pro Plus v. 6.0 (Media Cybernetics, Bethesda, MD, USA).

Transwell Invasion Assay
Cell invasion was examined in 24-well Transwell chambers (8 μm; Corning, Inc.). First, we used Matrigel (10 μg/ mL; BD Biosciences, San Jose, CA, USA) to cover the reverse side of the upper chambers and the surface was coated with 70 μL Matrigel (1 mg/mL). CRC cells (5×10^4) in 200 μl FBS-free medium were seeded in the upper chamber and 700 μL complete medium (with 10% FBS) was added to the bottom well. After 24-h incubation, cells that had invaded to the lower surfaces were gently cleaned and fixed with methanol for 10 min and stained with 0.1% crystal violet for 10 min. Five random fields were observed from the membrane, and the number of migrated cells was counted under an inverted phase contrast microscope (Olympus; ×40 magnification) and photographed.

Western Blotting

The cell proteins were extracted using cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA), after which the protein concentration was determined by a bicinchoninic acid protein assay kit (BCA, Sigma-Aldrich; Merck KGaA). Protein samples (40 μg/lane) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with Tris-buffered saline and 0.1% Tween 20 (TBS-T) containing 5% bovine serum albumin, then incubated with primary antibody against E-cadherin, vimentin, and TWIST1 (diluted 1:1000 in TBS-T; Abcam, Cambridge, MA, USA). The membranes were incubated at 4°C overnight. After washing with TBS-T three times, the membranes were incubated with the appropriate horseradish peroxidase–labeled secondary antibody (diluted 1:2000 in TBS-T; Abcam) at 37°C for 2 h. GAPDH was used as the internal control. The proteins were examined using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA) and the band density was scanned using Image Lab 5.0 (Bio-Rad, Hercules, CA, USA).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated using the TRIzol one-step method (Invitrogen). RNA (1 μg) was used, and complementary DNA (cDNA) was obtained by reverse transcription with a PrimeScript RT reagent kit according to the manufacturer's instructions (Takara Biotechnology, Dalian, China). qPCR was performed on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq II kits (Takara Biotechnology) according to the manufacturer's instructions. The U6 RNA level was used as the internal control for miRNAs. Relative expression was calculated using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.

Analysis of Lung Metastasis in Nude Mice

pCDH-CMV-luc-P2A-copGFP-T2A-Puro (Hangzhou Shiyu Biotechnology, Hangzhou, China) virus was transfected into a 6-well plate of LOVO cells. Stable strains were screened using 10 μg/mL puromycin, and observed under fluorescence microscopy. The cells were expanded to sufficient amounts, and then transfected with LV-miR-NC, LV-mimics-145, or LV-inhibitor-145. Female BALB/c nu/nu mice (5–6 weeks old) were randomly divided into four groups (n = 8 per group): normal saline, NC (negative control), miR-145, and miR-145 sponge. For lung metastasis analysis, 2×10^6 cells

per mouse were injected into the tail vein of each nude mouse. The mice were weighed every other day, and changes in body weight were observed. After 4 weeks, the mice were injected intraperitoneally with D-luciferin. Then, they were anesthetized by isoflurane and photographed in an IVIS imaging system. The lungs were removed and fixed in 10% formalin. Subsequently, consecutive tissue sections were made from each lung block, and stained with hematoxylin–eosin (H&E). The experimental procedures and use of laboratory animals were approved by the Medical Ethics Committee of Tongde Hospital of Zhejiang Province, Hangzhou, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications, No. 8023, revised 1978).

Statistical Analysis

All experimental data are reported as the mean ± standard deviation (n = 3); the data were analyzed using Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistical differences between two groups were detected using the Student's t-test; comparisons among multiple groups were performed using one-way analysis of variance, followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-145 Regulated CRC Cell Migration and Invasion

To explore whether miR-145 affects cell migration and invasion in CRC, we transfected the cells with miR-145 mimics or inhibitor, and then examined them using Transwell invasion and wound healing assays. miR-145 overexpression inhibited CRC cell migration ability, whereas miR-145 inhibitor enhanced it (Figure 1A and B). The Transwell invasion assay indicated that compared with the negative control, few cells crossed the membrane after miR-145 mimics transfection, but more cells crossed the membrane following miR-145 inhibitor transfection. qRT-PCR determined the interference efficiency of miR-145 following transfected with miR-145 mimic or inhibitor (Figure 1C). The results confirm that miR-145 can regulate CRC cell migration and invasive ability.

TWIST1 Was A Direct Target Gene of miR-145

We hypothesized that miR-145 regulates TWIST1. To verify this, we used TargetScan (www.targetscan.org) to predict
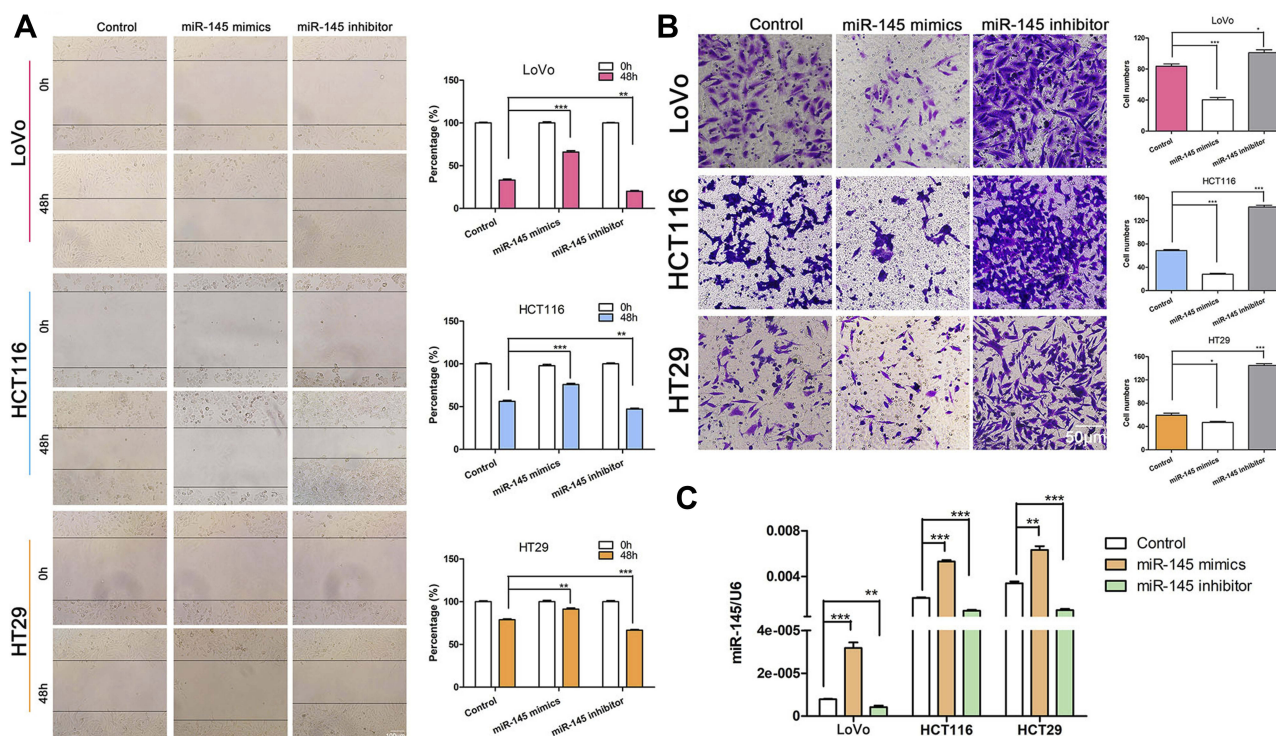
Figure 1 miR-145 regulated CRC cell invasion and migration. (**A**) Wound healing assay of CRC cell migration capability following transfection with miR-145 mimics or inhibitor compared with negative control (Control). **$P < 0.01$, ***$P < 0.001$. (**B**) Transwell invasion assay determination of the number of CRC cells that crossed the Matrigel layer after transfection with miR-145 mimics, inhibitor, or negative control (Control). *$P < 0.05$, ***$P < 0.001$. (**C**) qRT-PCR detection of miR-145 levels in CRC cells. **$P < 0.01$, ***$P < 0.001$.

whether *TWIST1* is a target of miR-145 (Figure 2A), and the results were as we had expected. Next, we examined TWIST1 protein and miR-145 levels using Western blotting and qRT-PCR, respectively, and found that TWIST1 expression correlated negatively with miR-145 expression (Figure 2B and C). Then we transfected CRC cells with miR-145 mimics, inhibitor, or negative control and detected TWIST1 protein expression. miR-145 significantly down-regulated TWIST1 levels, but the miR-145 inhibitor had the opposite effect (Figure 2D). These findings suggest that *TWIST1* is a target gene of miR-145 in CRC cells.

TWIST1 siRNA Reduced CRC Cell Migration and Invasive Capability

Increasing evidence suggests that TWIST1 is related with cell invasion and metastasis in various tumors, such as pancreatic cancer, ovarian cancer, and non–small cell lung cancer (NSCLC).[21–23] To assess the role of TWIST1 in CRC cells, we transfected CRC cells with TWIST1 siRNA or negative siRNA, and determined the interference efficiency of TWIST1 siRNA using Western blotting (Figure 3C). The wound healing assay determined that, compared with negative

siRNA, TWIST1 knockdown increased cell motility significantly and weakened CRC cell migration ability significantly (Figure 3A); the Transwell assay demonstrated significantly fewer invaded cells among the cells transfected with TWIST1 siRNA as compared with cells transfected with negative siRNA (Figure 3B), indicating that inhibiting TWIST1 suppresses CRC cell migration and invasive ability significantly.

miR-145 Suppressed CRC Cell Migration and Invasion by Targeting TWIST

We demonstrated that miR-145 and TWIST1 can regulate cell invasion and migration and that miR-145 can regulate TWIST1 levels. We hypothesized that miR-145 regulates CRC cell invasion and migration by regulating TWIST1. To prove this, we silenced TWIST1 and then transfected CRC cells with miR-145 mimics or inhibitor. The results showed that there was no significant difference among the three groups, i.e., TWIST1 siRNA only, TWIST1 siRNA+miR-145 mimics, and TWIST1 siRNA+miR-145 inhibitor (Figure 4A and B). Western blotting confirmed TWIST1 expression following treatment with TWIST1 siRNA or negative control siRNA (Figure 4C).
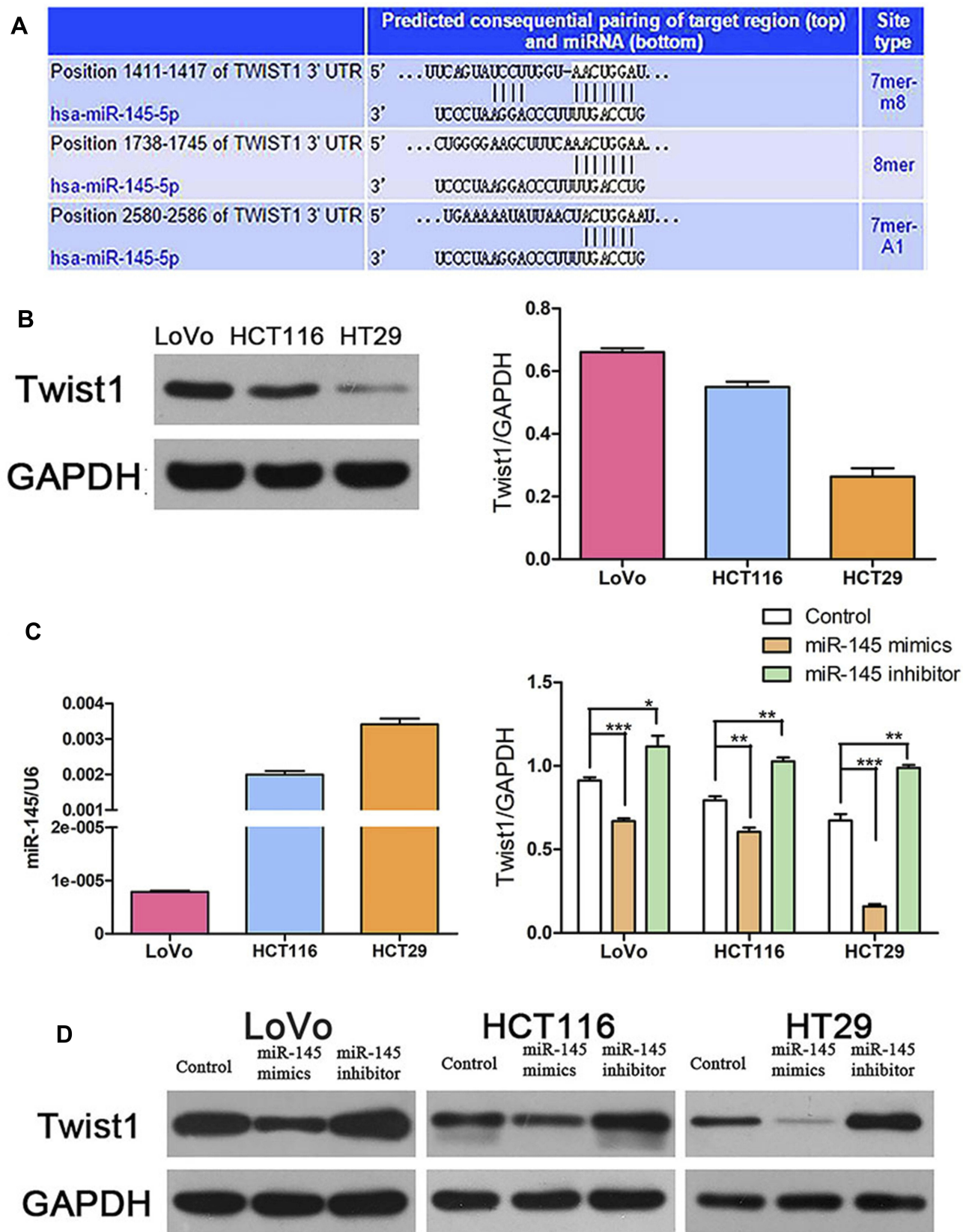
Figure 2 *TWIST1* was a direct target gene of miR-145 in CRC cells. (**A**) TargetScan prediction matching miR-145 to the *TWIST1* 3′UTR. (**B**) Western blot detection of TWIST1 expression. (**C**) qRT-PCR detection of miR-145 and *TWIST1* expression. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (**D**) Western blot detection of TWIST1 expression following transfection with miR-145 mimics or inhibitor.
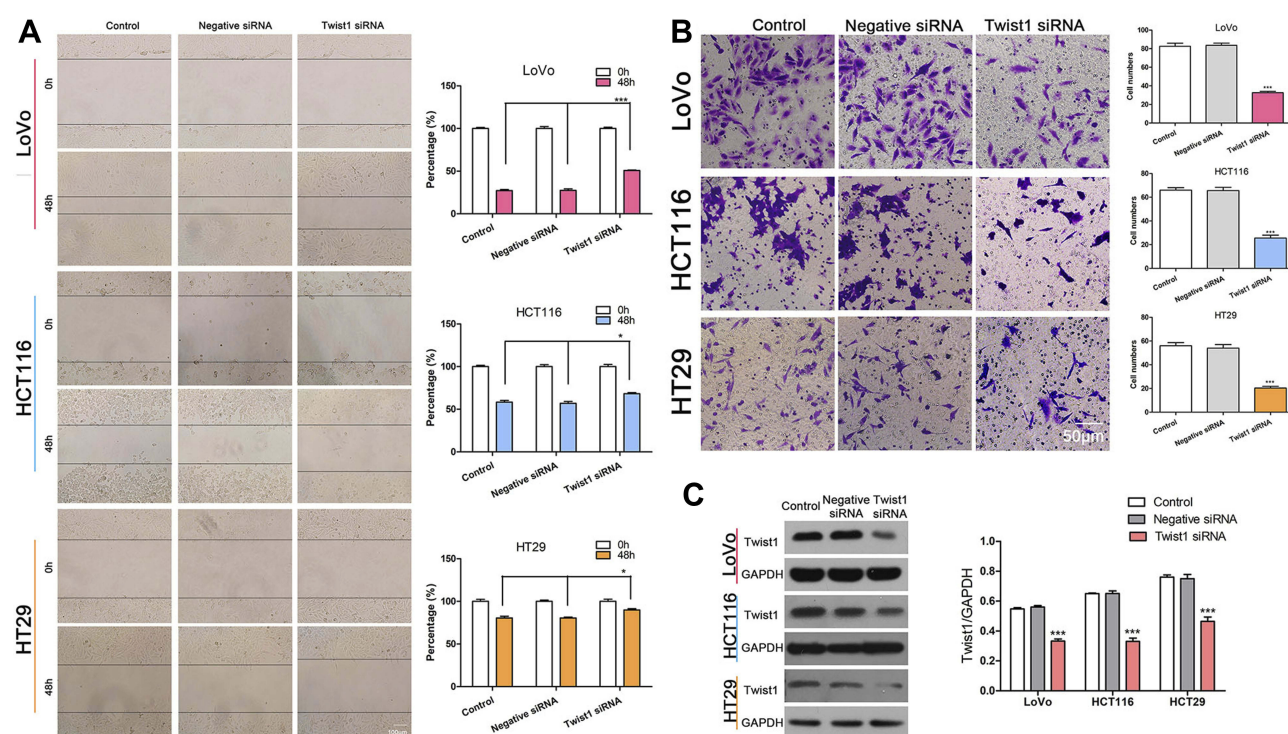
Figure 3 *TWIST1* knockdown reduced CRC cell invasive and migration capability. (**A**) Wound healing assay determining the cell migration ability following transfection with TWIST1 siRNA or negative siRNA. *$P < 0.05$, ***$P < 0.001$. (**B**) The Transwell assay showed that the number of invaded cells following transfection with TWIST1 siRNA or negative siRNA. ***$P < 0.001$. (**C**) TWIST1 expression following transfection with TWIST1 siRNA or negative siRNA. ***$P < 0.001$.
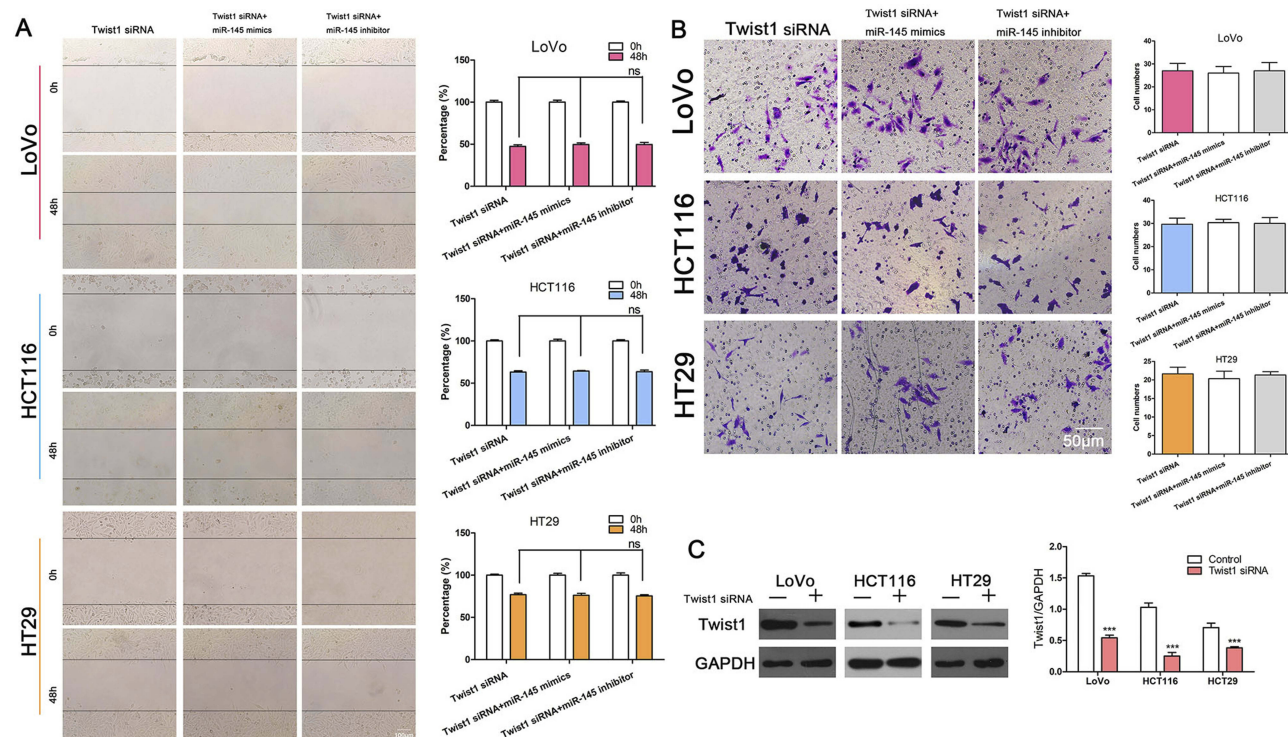

Figure 4 miR-145 suppressed CRC cell migration and invasion by targeting TWIST1. (**A**) Wound healing assay showing no significant difference among cells transfected with TWIST1 siRNA alone, or with TWIST1 siRNA and miR-145 mimics or miR-145 inhibitor. (**B**) Transwell assay after treatment with TWIST1 siRNA alone, or with TWIST1 siRNA and miR-145 mimics or miR-145 inhibitor. (**C**) Western blot showing the interference efficiency of TWIST1 siRNA. ***$P < 0.001$.

miR-145 Overexpression Inhibited EMT in CRC Cells

Much evidence has indicated that EMT is associated with cell invasive and migration capability in different cancers, including hepatocellular carcinoma, pancreatic cancer, NSCLC, gastric cancer, and colon cancer.[24–28] Here, we found that, compared with negative siRNA transfection, TWIST1 knockdown significantly upregulated E-cadherin expression and downregulated the level of vimentin in the CRC cells (Figure 5A). Furthermore, we found that miR-145 overexpression increased E-cadherin levels and decreased vimentin expression significantly, whereas miR-145 inhibition had the opposite effect (Figure 5B). Furthermore, we detected the expression of MMP-9 following transfection with miR-145 mimic or inhibitor in Figure S. These results indicate that miR-145 can regulate E-cadherin and vimentin levels in CRC cells.

miR-145 Regulated Metastasis in vivo

To further research the effect of miR-145 on CRC metastasis in vivo, we transfected LOVO cells with pCDH-CMV-LUC-P2A-copGFP-T2A-Puro and later transfected them with NC, miR-145 mimic, or miR-145 inhibitor to obverse lung metastasis in vivo. qRT-PCR determined the transfection efficiency of miR-145 treatment with miR-145 mimic or inhibitor (Figure 6A). Figure 6B shows the

changes in body weight. Luciferase signals from the lungs of the miR-145 inhibitor group indicated higher metastasis ability compared with that of control group at 4 weeks post-injection (Figure 6C and D). H&E staining also showed that the capability for lung metastasis was faster following transfection with miR-145 inhibitor (Figure 6E and F). These results were consistent with the in vitro results.

Discussion

miRNAs can regulate 30% of human genes post-transcriptionally, suggesting that they may play a key role in physiological and pathological processes, including even human carcinogenesis.[29] Recently, many reports have proven that miRNA expression is closely related to the occurrence, progression, metastasis, and prognosis of CRC.[30,31] Accordingly, the identification of CRC-related miRNAs will facilitate understanding of the etiology of CRC and elucidate their biological effects in the diagnosis and prognosis. miR-145 can inhibit the migration and invasive ability in some cancer cells, such as breast cancer cells, glioma stem cells, prostate cancer cells, and NSCLC cells.[8,32–34] In the present study, we prove that miR-145 overexpression inhibited CRC cell invasion and migration, while miR-145 inhibitor had the opposite effect. These findings indicate that miR-145 plays a vital role in regulating CRC cell invasion and migration.
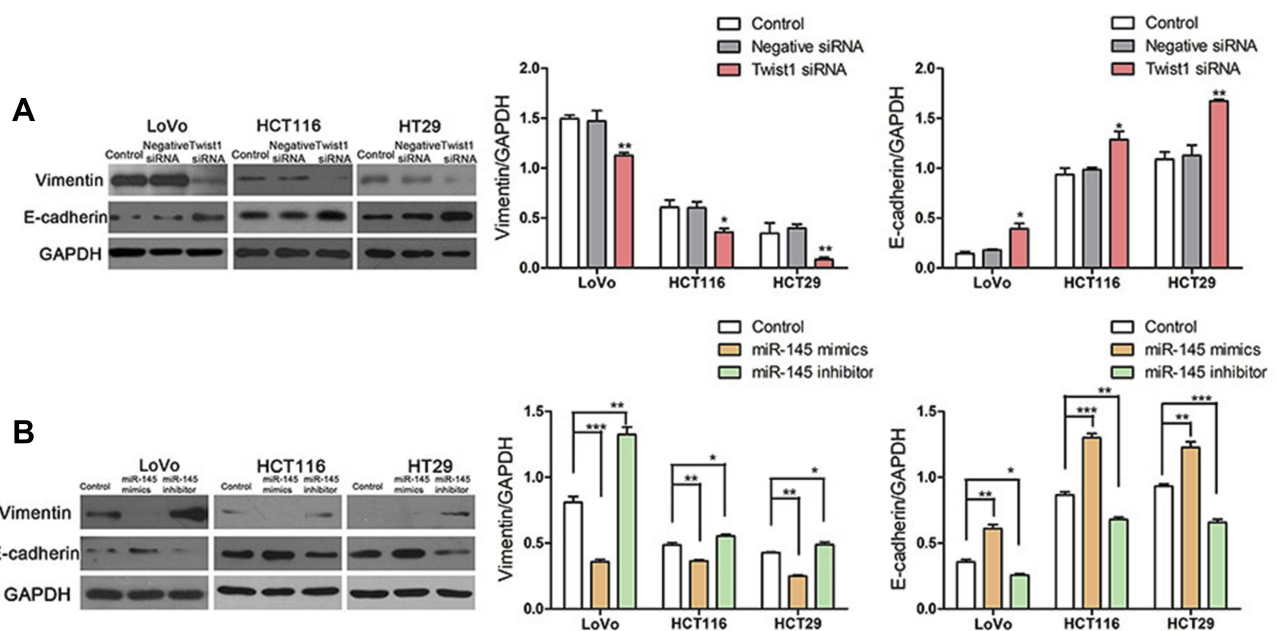

Figure 5 Overexpression of miR-145 inhibited EMT in CRC cells. (**A**) Western blot was used to analyze E-cadherin and vimentin expression following transfection with TWIST1 siRNA, negative siRNA, or control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control. (**B**) Western blot determination of E-cadherin and vimentin levels. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
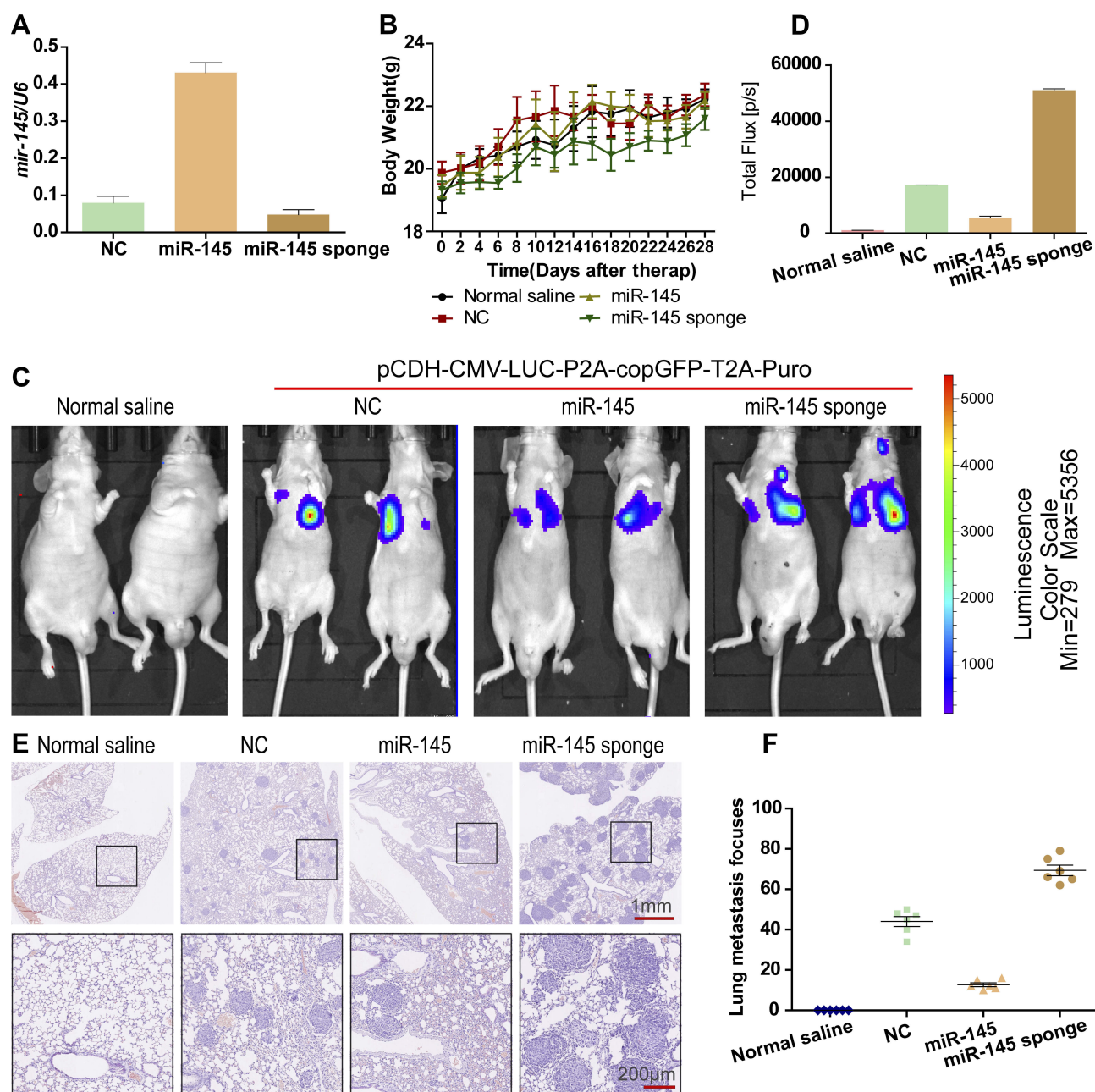
Figure 6 miR-145 regulated metastasis in vivo. (**A**) miR-145 levels were determined by qRT-PCR. (**B**) The body weight was measured every other day in the different treatment groups. (**C** and **D**) Luciferase expression from intrahepatic tumors of the different treatment groups (normal saline, NC, miR-145, miR-145 sponge). (**E** and **F**) Lung tissues were photographed, fixed, and stained with H&E.

TWIST1 is a novel oncogene overexpressed in a variety of tumors.[35] Its activation inhibits apoptosis and promotes the induction of EMT and proliferation in human cancer cells, which contribute to metastasis.[36,37] Much evidence has indicated that some miRNAs, such as miR-186, miR-151-3p, miR-720, miR-548c, miR-33a, and miR-520d-5p, directly regulate TWIST1 in a variety of cancer cells.[38–43] miR-151-3p can repress invasion and metastasis in breast cancer by regulating *TWIST1* expression.[39] Here,

we explored the role of the molecular mechanism of miR-145. TargetScan identified TWIST1 as a direct functional downstream target of miR-145. Moreover, miR-145 expression was negatively associated with TWIST1 expression. TWIST1 knockdown inhibited CRC cell invasion and migration. To explore whether miR-145 actually suppressed CRC cell migration and invasion by targeting TWIST1, we silenced TWIST1, then transfected CRC cells with miR-145 mimics and inhibitor, found no significant difference in cell

migration and invasion compared with that of cells trans-fected with TWIST1 siRNA alone. These results indicate that the role of miR-145 in inhibiting CRC cell invasion and migration is associated with TWIST1 expression.

EMT is a complex transdifferentiation process that is accompanied by the loss of junctional adhesion and changes in mesenchymal phenotype and morphology, resulting in increased cell invasion and migration.[44] EMT activation causes tumor cell invasion and spread, so it is considered the first step of metastasis.[44] TWIST1 is a well-known transcription factor in EMT progression. Recently, it was reported that most miRNAs participated in tumor metastasis by targeting important EMT genes.[45] Liu et al and You et al have revealed that the overexpression of miR-132 or miR-204 suppresses lung cancer cell migration and invasion by targeting EMT.[27,46] miR-145 can also suppress breast cancer cell migration by targeting and inhibiting EMT.[8] Furthermore, others have reported that miRNAs can regulate cell invasion and migration by targeting TWIST1 and inhibiting EMT in cancer cells.[39,47] In the present study, we determined that miR-145 is involved in cell invasion and migration by regulating TWIST1 and EMT. Western blotting indicated that transfection with miR-145 mimics significantly upregulated the level of E-cadherin and downregulated vimentin expression, compared with the negative control, but the miR-145 inhibitor had the opposite effect. Moreover, the increased expression of matrix metalloproteinases (MMPs) is associated with increased potential for metastasis.[48] Therefore, we determined the expression of MMP-9 following transfection with miR-145 mimic or inhibitor, and show that miR-145 mimic downregulated MMP-9, while miR-145 inhibitor upregulated it (Figure S1).

In conclusion, we demonstrate that the overexpression of miR-145 can inhibit CRC cell invasion and migration by targeting TWIST1 and inhibiting EMT. These findings indicate that miR-145 plays an important role in regulating CRC cell invasion and migration.

Acknowledgements

Disclosure

The authors report no conflicts of interest in this work.

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