

## Original Article

# ROR promotes the proliferation and migration of esophageal cancer through regulating miR-145/LMNB2 signal axis

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**Abstract:** Objective: LMNB2 is a protein that belongs to the RAB family. It is correlated with the tumorigenesis and development of several human cancers. The effect of LMNB2 on esophageal cancer (EC) has not yet been reported. The previous study showed that lncRNA ROR could promote the proliferation of EC. The current study aimed at exploring the correlation between ROR with LMNB2 and the role of ROR and LMNB2 in proliferation and migration of EC. Methods: This study performed dual luciferase reporter assay to evaluate the binding between miR-145 and ROR as well as miR-145 and LMNB2. Gene expression in EC tissues and cells were detected using quantitative real-time PCR (qRT-PCR) assay. The effect of ROR or miR-145 on LMNB2 expression was detected using western blot (WB) assay. Cells proliferation was detected by CCK8 and clone formation assay. Transwell and wound healing assay were carried out to determine the cells migration. Mouse xenograft assay was performed to detect the effect of LMNB2 on tumor growth in vivo. Results: This study demonstrated that miR-145 directly targets ROR and LMNB. ROR and LMNB2 were up-regulated and miR-145 was down-regulated in EC tissues and cells. The proliferation and migration of EC cells were promoted by overexpression of ROR or LMNB2. MiR-145 was capable of reversing the effect of ROR. The results also determined that down-regulation of LMNB2 had inhibitory effects and up-regulation of LMNB2 had catalytic effects on tumor growth in vivo. Conclusion: LMNB2 which is regulated by ROR and miR-145 was highly expressed in EC and promoted the proliferation and migration of EC in vitro and in vivo. The study suggests that ROR and LMNB2 could be potentially the therapeutic targets of EC.

**Keywords:** ROR, esophageal cancer, miR, proliferation, migration

## Introduction

Esophageal cancer (EC) is one of the most common lethal cancers with probably 509,000 deaths cases each year [1]. Globally, the mortality of EC is ranked at fifth place out of all the malignant tumors [2]. In spite of the advance in developing effective medical and surgical treatments, the 5-year survival rate of EC is low according to the global surveillance of trends in cancer survival 2000-2014 [3, 4]. There is limited understanding of the underlying mechanism of the initiation or progression of esophageal cancer is still limited making it difficult to develop effective diagnosis and therapy methods for EC.

Long non-coding RNAs (lncRNAs) are a novel group of ncRNAs with the length of more than

200 nucleotides [5]. They have vital roles in various pathological process such as autophagy, necrosis, and apoptosis [6, 7]. lncRNAs have been confirmed to play vital roles in cancer progression, metastasis, prognosis and drug resistance in EC [8].

lncRNA regulator of reprogramming (lncRNA ROR) which was firstly identified as a modulator for reprogramming of human induced pluripotent stem cells [9]. It located at chromosome 18q21.31 which consisted of 4 exons and has been confirmed to have strong effect on self-renewal and differentiation of human embryonic stem cells [10]. Numerous studies have demonstrated the crucial role of ROR in tumorigenesis and EMT in many malignancies including breast, liver, pancreatic, and colon cancers

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[11-14]. As in EC, ROR was also found to be up-regulated in esophageal squamous cell carcinoma [15]. Moreover, it was reported that Lnc-ROR promotes esophageal squamous cell carcinoma progression through the derepression of SOX9 [16]. However, further understanding of the function and underlying mechanism of ROR in EC requires more exploration. It is well known that lncRNAs may function as a competitive endogenous RNA (ceRNA) which is known as “microRNA (miRNA) sponge”, to block the function of corresponding miRNA [17].

MiR-145 is widely studied in various cancer including EC which was reported to be down regulated in all grades of EC samples [18] and inhibit the proliferation, migration, invasion and EMT through targeting and reduce the expression level of its target gene such as Sp1, CTGF, c-MYC [19-21]. ROR/miR-145 is a classic interaction relationship occurs in human cancers. ROR/miR-145 promotes invasion and metastasis in triple-negative breast cancer via targeting MUC1 [22]. It also promotes radioresistance in hepatocellular carcinoma cells regulating RAD18 expression [23]. In addition, recently, ROR/miR-145 was further indicated to prevent cardiac ischemia injury [24]. These findings indicated the critical effect of ROR/miR-145 signal axis.

In our study, we proved that ROR promotes the proliferation and migration of EC cells, moreover, miR-145 targets ROR directly in EC. Moreover, we predicted and verified a novel target gene of miR-145 that is LMNB2. Function and rescue study demonstrated the oncogenetic effect of LMNB2 which can be regulated by ROR and miR-145. In summary, our study extended the understanding of the function and mechanism involved in ROR/miR-145 and LMNB2 in EC which may serve as a promising therapeutic target of EC.

### Materials and methods

#### *Clinical tissues collection*

We collected 25 paired EC and adjacent normal tissues from patients at Zhongda Hospital, Southeast University. These patients were diagnosed with EC and did not receive radiotherapy or chemotherapy prior to surgery. The tissues were stored in liquid nitrogen and transferred to laboratory. The informed consent was ob-

tained from all patients with EC and the study was approved by the Ethics Committee of the Southeast University.

#### *Cell culture*

The human normal esophageal epithelial cell line HET-1A and EC cell lines (EC9706, EC109, KYSE70, KYSE450 and EC1) were purchased from American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI-1640 (GIBCO, NY, USA) and supplemented with 10% fetal bovine serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 mg/ml). All cells were maintained in an environment of 37°C and 5% CO<sub>2</sub>.

#### *Quantitative reverse transcription PCR (qRT-PCR)*

For mRNA qRT-PCR analysis, total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) and was reverse transcribed using a PrimeScript RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used to perform quantitative RT-PCR in a 20 ul reaction and the reaction was proceeded in a real-time PCR detection system (Applied Biosystems, USA). The qRT-PCR assay of miRNA was performed using the All-in-One miRNA qRT-PCR detection kit (Genecopeia, MD, USA). The qRT-PCR primer sequences were as follows: miR-145-forward: 5' TGCGGTCAGTGCCTACAGAAC 3'; miR-145-forward: 5' CCAGTGCAGGGTCCGAGGT 3'; ROR-forward: 5' CTTTCAGCACTCTGGACGGACTTG 3'; ROR-reverse: 5' GATCTATGGCATGGCAGGGTAG 3'; LMNB2-forward: 5' TGCACGGGCACAAAGACTTCCAC 3'; LMNB2-reverse: 5' CCCAGCACCTCAAATCGTTCCAT 3'; U6-forward: 5' GTCAGCGCGTGCTCGCTTCG 3'; U6-reverse: 5' GTGCAGGGTCCGAGGT 3'; β-actin-forward: 5' GGGAAATCGTGCCTGACATTAAG 3'; β-actin-reverse: 5' TGTGTTGGCGTACAGGTCTTTG 3'.

#### *Cell transfection*

The pcDNA3.1-ROR and pcDNA3.1-LMNB2 vector were established by General Biosystems (Anhui, China). MiR-145 mimics, miR-145 inhibitor, si-LMNB2 and the corresponding negative control were also purchased by General Biosystems. The shRNA of LMNB2 was designed and synthesized by Sigma (St. Louis, MO, USA).

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Transfections were carried out using lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's instructions.

### *Cell viability assay*

Following a transfection of 24 h, EC9706 and EC1 cells were seeded in 96 well plates (1,000 cells/well). The cell viability assay was conducted by CCK8 according to the manufacturer's protocol. The original medium was removed, and it was replaced with culture medium containing 10% CCK8. The replacing was performed at intervals of 12, 24, 48 and 72 hours after cells were seeded. After 4 h of incubation, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad, CA, USA). Each test was performed in triplicate.

### *Colony formation assay*

500 cells of transfected EC9706 and EC1 were seeded in 12-well plates. Following 14 days of incubation, the cells were fixed with 4% paraformaldehyde for 15 minutes and then stained with 0.1% crystal violet for 30 min. The clones were photographed with a microscope. The number of visible clones indicated the ability of cell clone formation. The assay was conducted three times independently.

### *Transwell assay*

$1 \times 10^5$  cells were seeded onto the upper chamber of each 24-well plate (Corning, NY, USA) with serum-free medium. The pore size of upper chamber was 8.0  $\mu\text{m}$ . The lower chamber was filled with 600  $\mu\text{l}$  of medium with 10% FBS. After the cells were incubated for 24 hours, the cells attached to the reverse phase of the membrane were fixed with 4% paraformaldehyde for 15 min and the cells on the upper chamber were removed using cotton swabs. Then the cells located on the lower surface were stained with 0.1% crystal violet for 5 min. Cells were photographed at least five fields using a light microscope (Leica, Germany).

### *Wound healing assay*

The transfected cells were seeded in 24 well plates ( $1 \times 10^5$  cells/well). The seeded cells were cultured overnight and a scratch was introduced using a pipette tip. The cells were then washed three times with PBS. At 0 h and

48 h, the images were captured under a light microscope. The pictures were obtained at least five fields to count average distance of migration.

### *Dual-luciferase reporter assay*

Bioinformatics analysis tool targetscan7.6 was used to predict the binding sites between miR-145 and LMNB2 or miR-145 and ROR. The predicted binding sequence or mutant binding sites of ROR and LMNB2 were synthesized by General Biosystems and cloned into pmirGLO vector (Promega, Madison, USA). Wt or mutant luciferase reporter plasmid was co-transfected with miR-145 mimic or control in EC9706 and EC1 cells using Lipofectamine 2000 (Thermo Fisher Scientific, mutant binding sites) according to the manufacturer's protocols. The lysed cells were exploited for luciferase activities analysis using a Dual-Luciferase Assay Kit (GeneCopoeia, MD, USA) after 48 h.

### *RNA pull-down assay*

EC9706 and EC1 cells were transfected with biotinylated biotin-miR-145 or biotin-nc. We collected the cells after 48 h. The cells were lysed by specific lysis buffer for 10 min. M-280 streptavidin beads (Sigma, CA, USA) pre-coated with BSA and yeast tRNA were used to incubated with the lysate at 4°C for 4 h. Then the beads were washed with precooled lysis buffer for three times, washed with low salt buffer for three times and washed with high salt buffer for two times. Finally, We performed qRT-PCR to detect the enrichment of LMNB2.

### *Western blot*

The cells were collected and lysed in ice-cold RIPA lysis buffer (Thermo Fisher Scientific). The protein was quantified with the BCA kit (TAKARA, Dalian, China). A total of 40  $\mu\text{g}$  protein was separated by 10% (SDS)-polyacrylamide gel for electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membrane. The  $1 \times$  Tris-buffered saline with Tween (TBST) containing five percent nonfat milk was used to block the membranes for 1 h at room temperature. The membranes were then incubated with specific primary antibodies against LMNB2 (Abcam, 1:1000) and GAPDH (Abcam, 1:5000) at a temperature of 4°C for incubation stored in a refrigerator overnight. Subsequently, The 2

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h of incubation was conducted at room temperature with the corresponding secondary antibodies (Abcam, 1:10000). The bands were visualized with an ECL kit (Pierce, USA) in a darkroom and analyze the gray scale of the strip by lab V 2.01 image analysis software (Total Lab, England).

### *Immunofluorescence*

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 15 min. Next, the cells were incubated with 5% normal goat serum that matched the species used to generate the secondary antibody, followed by incubation with  $\alpha$ -SMA primary antibody overnight at 4°C. Then, cells were then incubated in fluorochrome-conjugated secondary antibodies for 2 h at room temperature. Subsequently, DAPI was used to counterstain the cell nuclei. Images were captured with a Carl Zeiss Axioskop microscope (Carl Zeiss AG; magnification,  $\times$  400) Three fields of view were analyzed per sample.

### *Nude mice xenograft assay*

A total of 20 male BALB/c nude mice (4-6 weeks old, 18-22 g) were purchased by Vital River Laboratory Animal Technology Company (Beijing, China) and randomly divided into four groups (pcDNA3.1-ROR, pcDNA3.1 vector, sh-ROR and sh-nc, N = 5/group). The mice were housed in specific pathogen-free environment with a temperature of 25°C and 60% relative humidity.  $5 \times 10^6$  transfected EC9706 cells were suspended in 100  $\mu$ l PBS and inoculated subcutaneously into the dorsal flanks of nude mice. Tumor growth was monitored every two days after the tumor appears and the volume was calculated by the following equation: Tumor volume =  $1/2 \times (\text{width})^2 \times \text{length}$ . The mice were killed and the tumors were weighed after 23 d of cell inoculation.

### *Statistical analysis*

All data are presented as the means  $\pm$  standard deviation (SD). The date statistical significance analyses were performed using Prism GraphPad 5.0 and SPSS 20.0 software. Data were analysed using one-way ANOVA and Student's t-test. Linear correlation analyses were performed to determine correlations between PVT1, miR-148 and RAB34 expression levels.  $P < 0.05$  was considered to be statistically significant.

## Results

### *The expression of ROR is up-regulated in EC and associated with poor prognosis*

Previously, it was revealed that ROR was up-regulated in EC. In order to verify this result and lay the foundation for the subsequent experiments, 25 paired EC tissues and matched non-tumorous tissues were collected for evaluating the expression of ROR by qRT-PCR. The results revealed that ROR was up-regulated in EC tissues (**Figure 1A**). Compared with the NHBE group, the qRT-PCR assay in cells indicated that the expression level of ROR in EC cell lines including EC109, EC1, EC9706, KYSE70 and KYSE450 were all up-regulated in different degrees. ROR has the highest expression in EC-9706 (**Figure 1B**). Moreover, we found that ROR was up-regulated in EC tissues compared with normal tissues via analyzing the profiles of EC patients from the STATBASE dataset (**Figure 1C**).

### *Cell proliferation and migration were promoted by over-expression of ROR in EC cells*

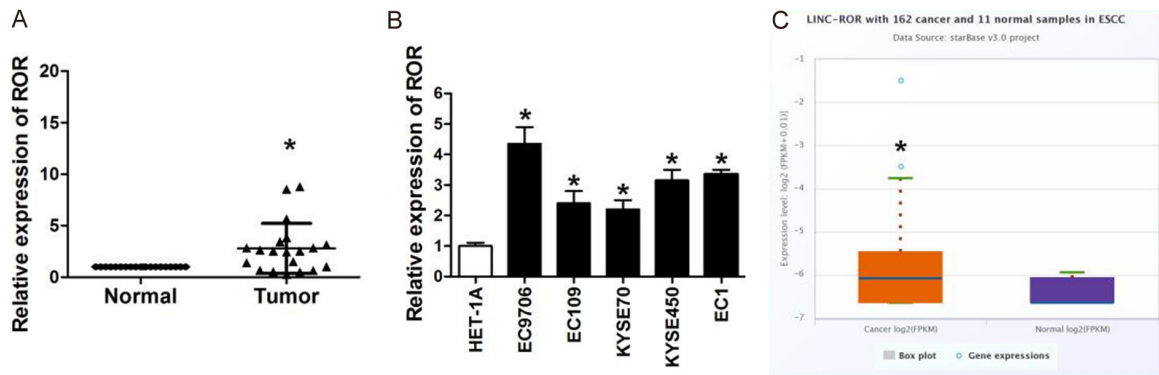
EC9706 and EC1 cell lines were chosen to investigate the effect of ROR on EC cells proliferation and migration. The over-expression vector pcDNA3.1-ROR and the corresponding negative control (pcDNA3.1) were transfected into EC9706 and EC1 cells. The effect of transfection was verified by qRT-PCR (**Figure 2A**). The effect of ROR on cell proliferation was demonstrated by CCK8 assay. The results revealed that over-expression of ROR promoted the cells viability in EC9706 and EC1 (**Figure 2B**). Moreover, the transwell assay and wound healing assay revealed that the migration of EC9706 and EC1 cells was accelerated by transfecting pcDNA3.1-ROR (**Figure 2C, 2D**). Overall, the results indicated that ROR promoted EC cells proliferation and migration.

### *MiR-145 is a direct target of ROR and is down-regulated in EC*

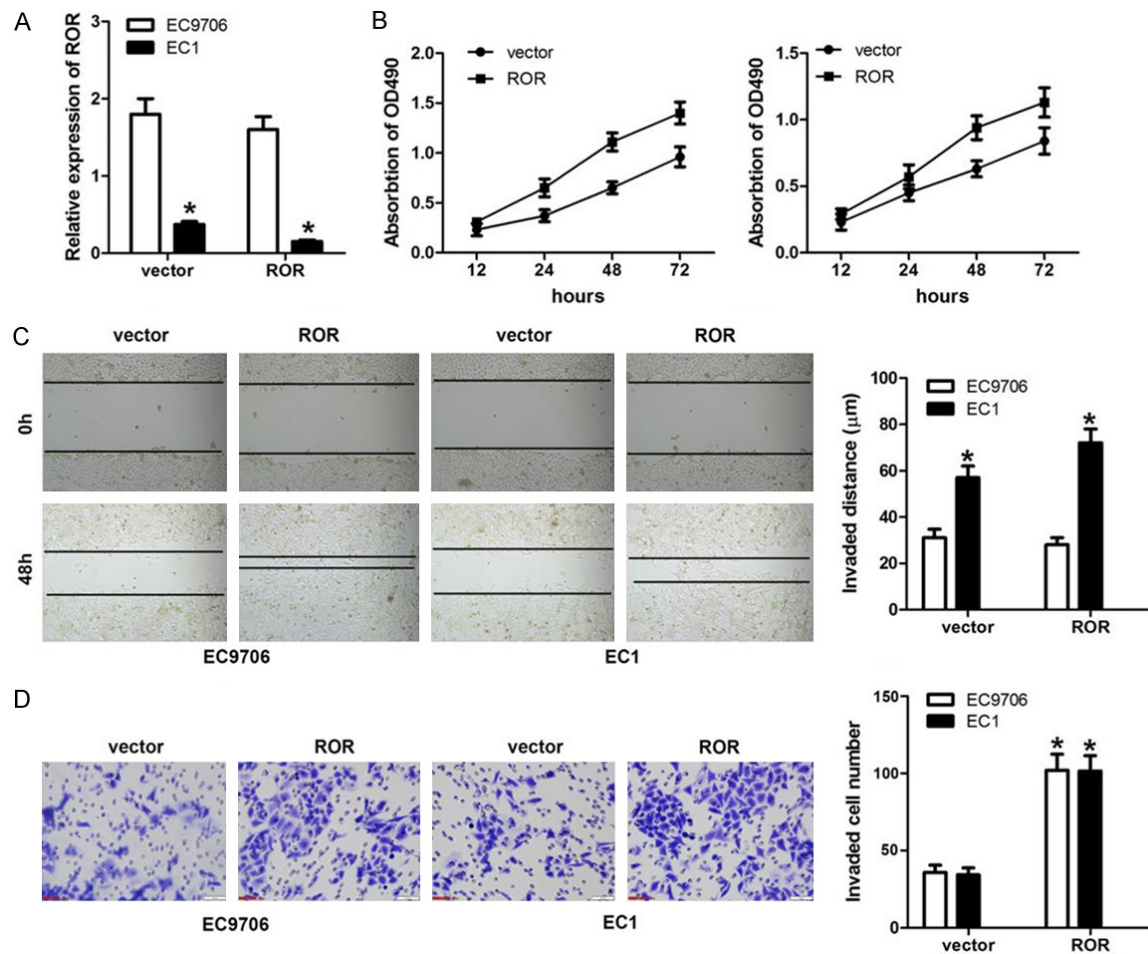
To explicate the underlying mechanisms of ROR effects in EC, we conducted bioinformatics analysis to screen of the targets of ROR. The screening was based on the database of StarBase and miR-145 was selected as the potential one. We designed the wild and mutant luciferase reporter of ROR according to the binding site predicted (**Figure 3A**). The luciferase



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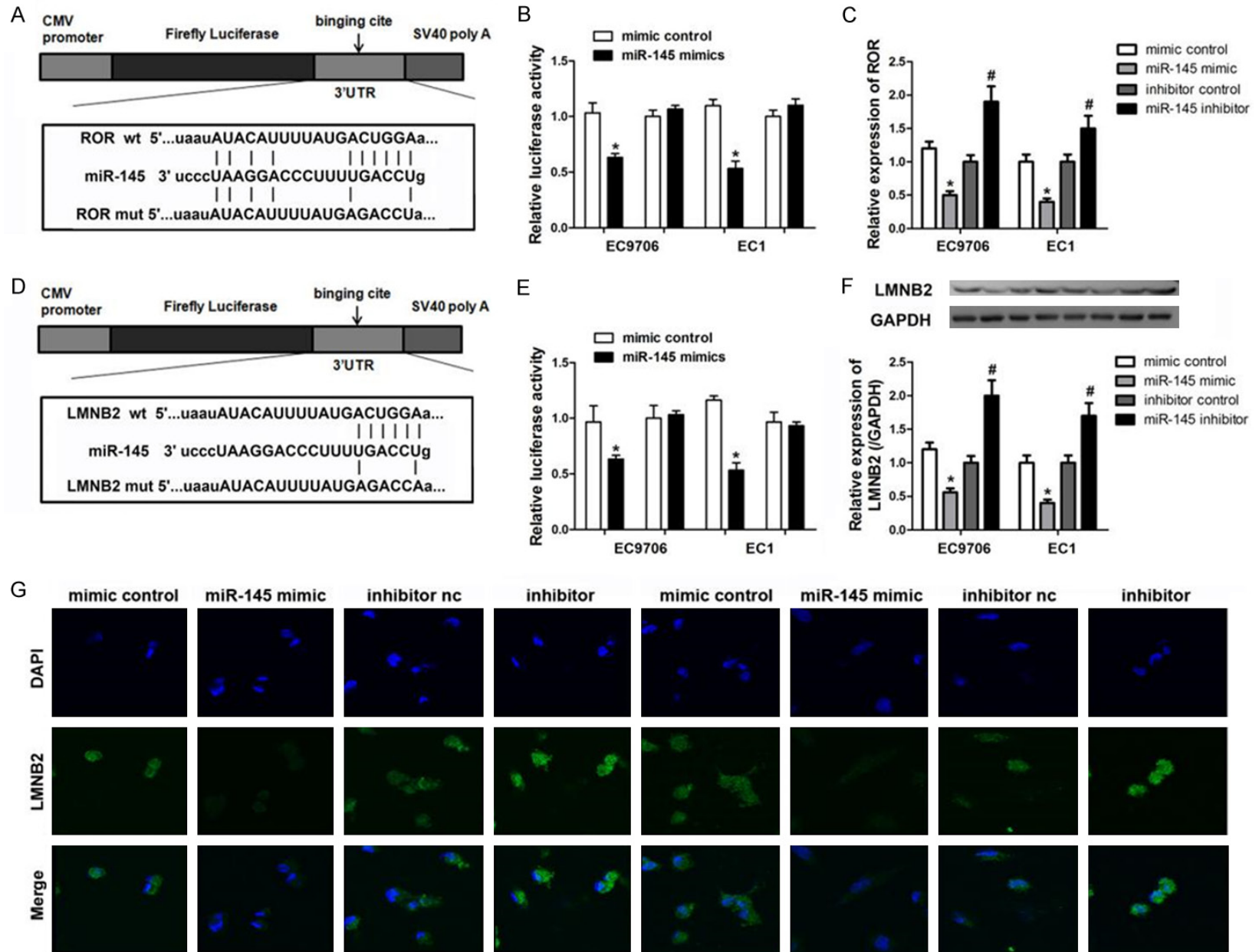


**Figure 1.** LncRNA ROR expression is up-regulated in EC tissue and cell lines. A. ROR expression in 25 paired of EC tissues and normal tissues was detected by qRT-PCR assay. B. ROR expression was examined by qRT-PCR in EC cell lines (EC9706, EC1, SPCA1, H2279, H1975, H1650 and HC82) and esophageal epithelial cell line HET-1A. C. Relative expression of ROR in the profiles of EC patient tissues from StarBase. \*P<0.05 vs normal and HET-1A groups respectively.

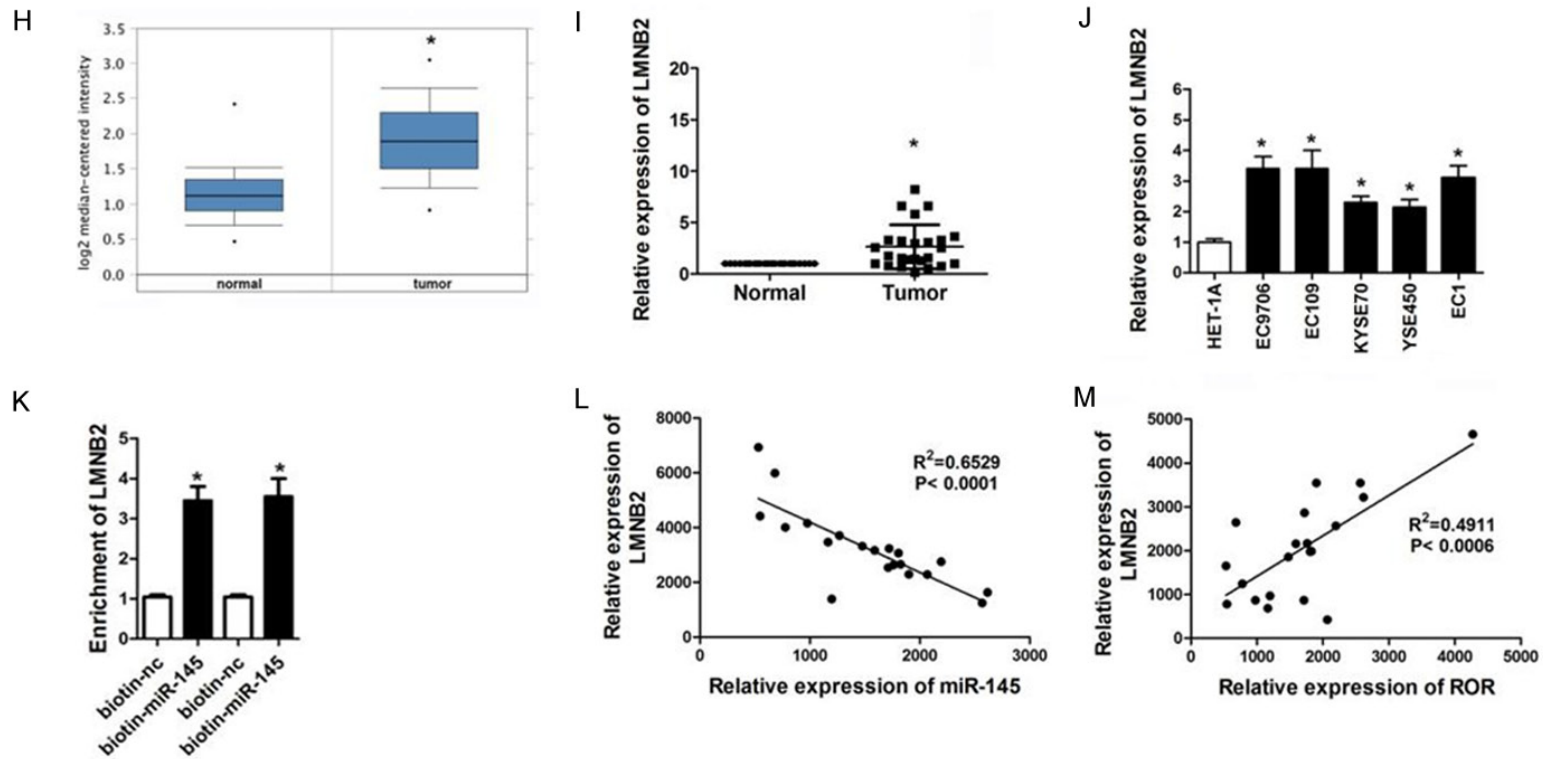


**Figure 2.** ROR promotes the proliferation and migration of EC cell lines in vitro. EC9706 and EC1 cells were transfected with the overexpressing ROR vector or the vector control. (A) Transfection efficiency of ROR overexpressing vector (pcDNA3.1/ROR) was detected by qRT-PCR assay. (B) Proliferation ability of each group was measured by CCK-8 assay. Migration ability of each group was detected by (C) transwell assay and (D) wound healing assay. \*P<0.05 vs vector group.

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**Figure 3.** MiR-145 targets ROR along with LMNB2 in EC9706 and EC1 cells. A. Schematics of the predicted binding sequences of miR-145 in the wild-type and mutant (in red) targeting region of ROR. B. The double luciferase assay was performed to prove the relationship between miR-145 and ROR. C. After transfection of miR-145 mimics or inhibitor, the expression of ROR in EC9706 and EC1 cells was tested by qRT-PCR after the transfection of miR-145-mimics or miR-145-inhibitor. D. Schematics of the predicted binding sequences of miR-145 in the wild-type and mutant (in red) 3'UTR of LMNB2. E. Double luciferase assay was performed to prove the relationship between miR-145 and LMNB2. F. The protein expression of LMNB2 in EC9706 and EC1 cells was tested by WB after the transfection of miR-145 mimics or miR-145 inhibitor. G. The protein expression of LMNB2 was tested by immunofluorescence. H. Relative expression of LMNB2 in the profiles of EC patient tissues from Oncomine database. I, J. LMNB2 expression was examined by qRT-PCR in EC tissues and cell lines along with control. K. The enrichment of lncRNA ROR was detected by RNA pull-down. L, M. Pearson analysis of EC tissues was performed to detect the correlation between miR-145 and ROR and between miR-145 and LMNB2. \* $P<0.05$  vs mimic control, normal, HET-1A and biotin-nc group. # $P<0.05$  vs inhibitor control group.

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ase reporter assay showed that co-transfection of miR-145 mimics largely decreased the activity of the reporter plasmid with ROR-wt. However, ROR-mut did not have a similar effect (**Figure 3B**). The effect of miR-145 on the expression of ROR was performed by qRT-PCR. The results suggested that over-expression of miR-145 inhibited the expression of ROR, inversely, inhibition of miR-145 promoted the expression of ROR (**Figure 3C**).

### *MiR-145 directly target LMNB2 which is up-regulated in EC*

MiRs target and suppress the expression level of their target genes to participate in the regulation of cell process. To clearly explicate the underlying mechanisms of ROR effects in EC, we predicted target genes of miR-145 based on the database of targets can7.6 and LMNB2 was selected as the potential target. We designed the wild and mutant region from the 3'UTR of LMNB2 according to the binding site predicted above (**Figure 3D**). The luciferase reporter assay indicated that the activity of the reporter plasmid carrying LMNB2-wt was largely decreased by co-transfection of miR-145 mimics. However, but not LMNB2-mut (**Figure 3E**). The effect of miR-145 on expression of ROR was investigated by western blot and immunofluorescence assay. The results suggested that miR-145 inhibited the expression of LMNB2, inversely, inhibition of miR-145 promoted the expression of LMNB2 (**Figure 3F, 3G**). Furthermore, analysis of the data using the Oncomine database indicate an up-regulated level of LMNB2 in EC tissues (**Figure 3H**). Then, we detected the LMNB2 expression of EC tissue and cell lines using qPCR. Accordingly, the results revealed that LMNB2 level was elevated in EC tissue and cell lines (**Figure 3I, 3J**). Furthermore, RNA pull-down assay demonstrated that LMNB2 was co-precipitated with miR-145. This indicates that miR-145 can bind with LMNB2 (**Figure 3K**). Finally, we analyzed the correlation between ROR and miR-145 as well as miR-145 and LMNB2 expression in EC tissues. The results suggested a significantly negative correlation between miR-145 and LMNB2 and a positive correlation between ROR and miR-145 (**Figure 3L, 3M**).

### *MiR-145 reversed the effect of ROR in EC*

In order to verify the relationship between miR-145 and ROR. A rescue experiment was carried

out. Overexpressing vector of ROR and miR-145 mimic were co-transfected into EC9706 and EC1 cells. CCK8 assay revealed that over-expression of miR-145 by transfection of miR-145 mimic inhibited the viability of EC9706 and EC1 cells compared to the ROR group (**Figure 4A, 4B**). Similarly, transwell assay and wound healing assay both revealed that miR-145 reversed the function of ROR on the migration of EC9706 and EC1 cells (**Figure 4C, 4D**).

### *LMNB2 promotes the proliferation and migration of EC cells*

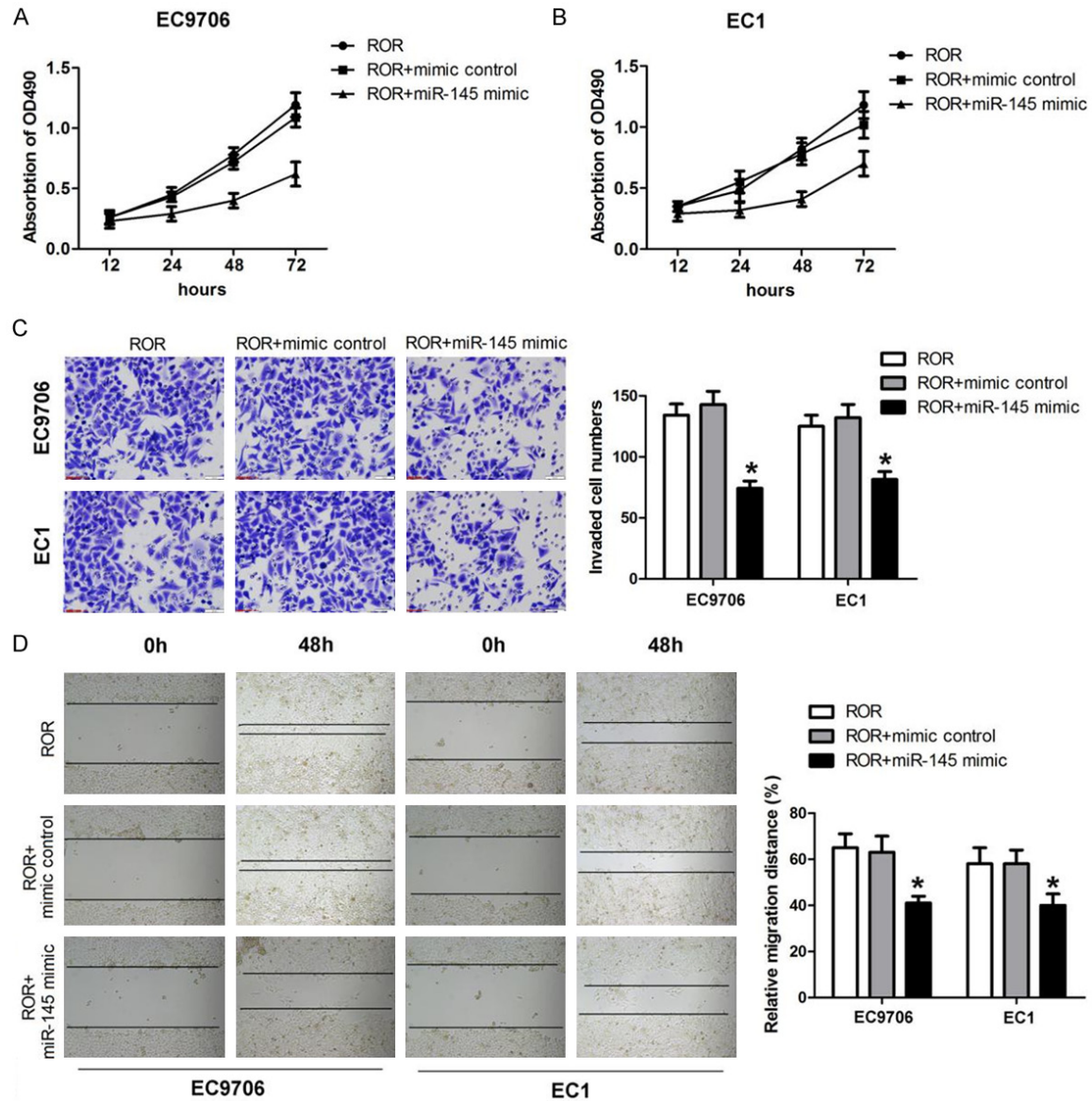
The results of **Figure 2** demonstrated that over-expression of ROR promoted the proliferation and migration of EC9706 and EC1 cells and the results of **Figure 4** revealed that the expression levels of ROR and LMNB2 are positively correlated. Therefore, we suspected that the expression of LMNB2 could also promote the proliferation and migration of EC9706 and EC1 cells. On this basis, pcDNA3.1-LMNB2 or si-LMNB2 was transfected into EC9706 and EC1 cells, and the efficiency of the over-expression and interference vector were detected by qRT-PCR (**Figure 5A**). As shown by the result of CCK8 assay, over-expression of LMNB2 increased cell viability, while knockdown of LMNB2 reduced cell viability (**Figure 5B**). The effects of LMNB2 on migration capacity of EC9706 and EC1 cells were detected by transwell and wound healing assay. The results showed that up-regulation of LMNB2 promoted the migration of EC9706 and EC1 cells while the down-regulation of LMNB2 inhibited the cell migration (**Figure 5C, 5D**).

### *LMNB2 promotes tumorigenesis of EC cell in vivo*

To further investigate the effect of LMNB2 on the tumor growth in vivo, EC9706 cells that stably transfected with pcDNA3.1-LMNB2, sh-LMNB2 or their negative controls were injected into the flanks of nude mice subcutaneously, respectively. The results revealed that the tumor volume in the mice with LMNB2 over-expressing cells was increased compared with the control group, while the mice with LMNB2 inhibiting cells had smaller tumor volumes (**Figure 6A, 6B**). At last, the tumors were removed and weighed. The mean tumor weight of LMNB2 over-expressing group was significantly higher than that of the control group and LMNB2 down-regulating group was lower than



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**Figure 4.** MiR-145 reversed the effect of ROR in EC cells. EC9706 and EC1 cells were transfected with pcDNA3.1/ROR and mimic control or pcDNA3.1/ROR and miR-145 mimic. A, B. Proliferation ability of each group were measured by CCK-8. C, D. Migration ability of each group were detected by transwell assay and wound healing assay. \*P<0.05 vs ROR + mimic control group.

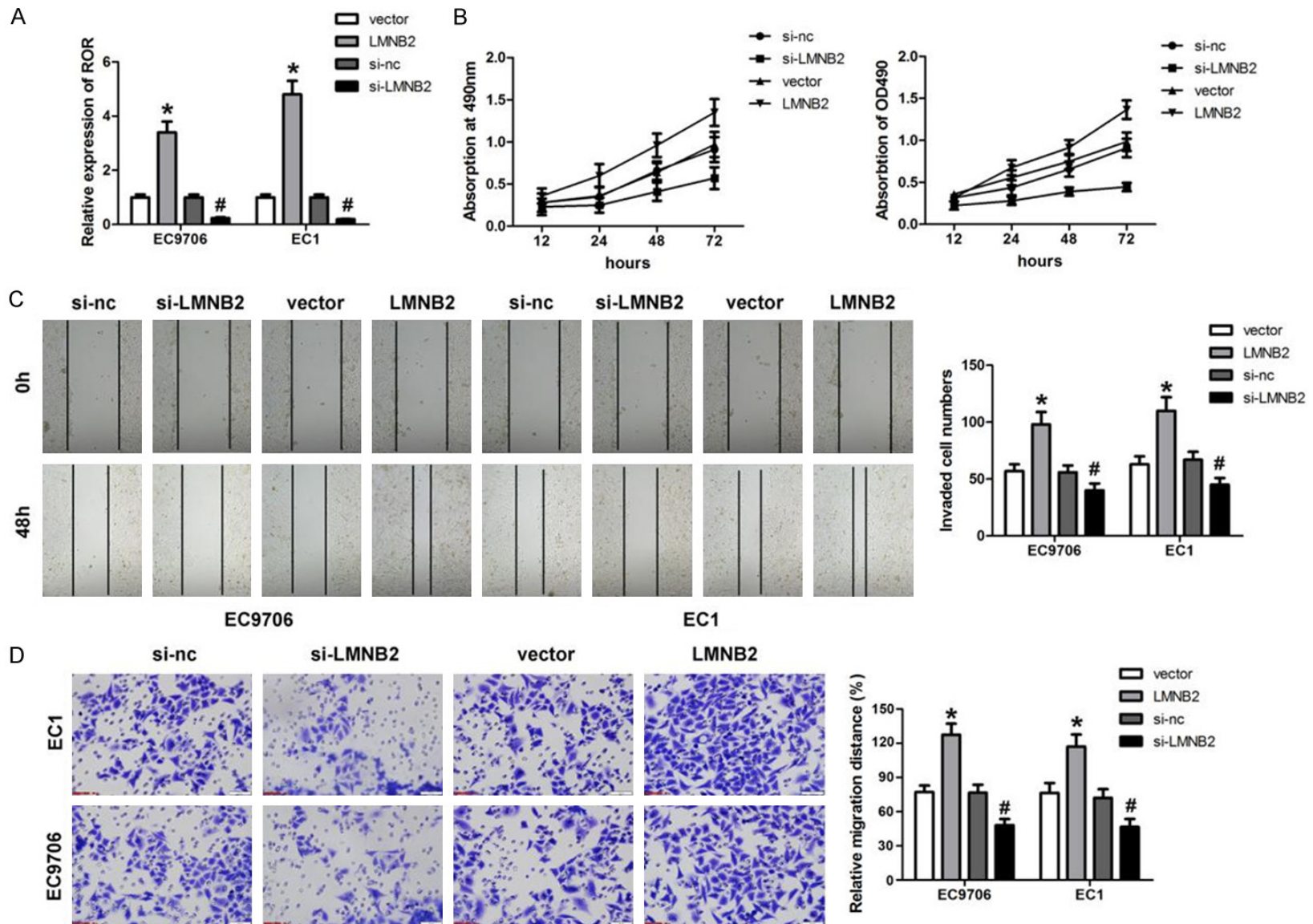
the tumors in control group (Figure 6C). IHC was performed to assess the expression of LMNB2 in the tumor tissues of each group. The results indicated that LMNB2 expression was notably u-regulated in the LMNB2 over-expressing group while down-regulated in the LMNB2 silencing group (Figure 6D). These results indicated the successful establishment of the stable cell line.

### Discussion

In this study, we aim to extend our knowledge about the function of ROR and miR-145 as well

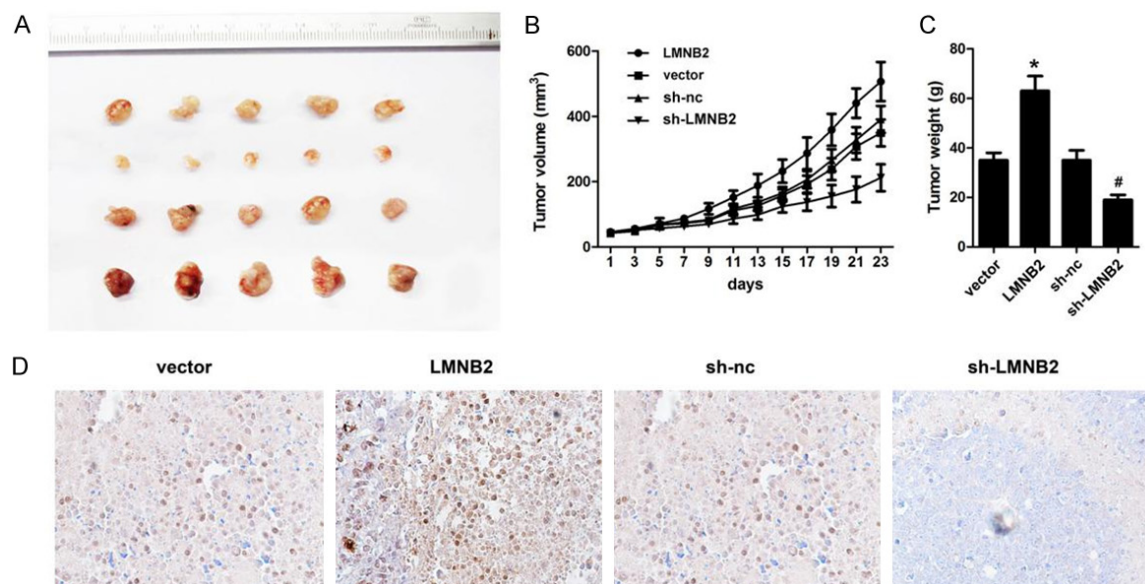
as the downstream target gene regulated by them. We found that the expression of lncRNA ROR was statistically higher in both EC tissues and cells and demonstrated that ROR promoted the proliferation and migration of EC cells which is consistent with the previous studies [25, 26]. Then we performed dual luciferase assay to reveal that miR-145 targeted the ROR and proved that miR-145 was down-regulated in EC tissues and cells. The down-regulation of miR-145 was related to poor prognosis of EC patients. Here, we found a question. Most studies revealed a anti-cancer role of miR-145 in

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**Figure 5.** LMNB2 promotes the proliferation and migration of EC cell lines in vitro. The EC9706 or EC1 cells were transfected with si-LMNB2 or LMNB2 overexpressing vectors. (A) The efficiency of over-expression and knockdown of LMNB2 was detected by qRT-PCR assay. (B) Proliferation ability of each group was measured by CCK-8. Migration ability of each group were detected by (C) wound healing assay and (D) transwell assay \* $P < 0.05$  vs vector group. # $P < 0.05$  vs si-nc group.

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**Figure 6.** LMNB2 promotes tumor growth of EC in vivo. A. The image of the tumors. EC9706 cell lines that stably expressed pcDNA3.1-LMNB2, sh-LMNB2 or corresponding control were injected into the flanks of nude mice subcutaneously. B. The mice were closely monitored for tumor growth and the tumor volume was measured at the indicated times (Tumor volume =  $1/2 \times (\text{width})^2 \times \text{length}$ ). C. The weight of tumor in each group. D. IHC was performed to evaluate the expression of LMNB2 in tumor tissues. \* $P < 0.05$  vs vector group. # $P < 0.05$  vs sh-nc group.

EC. For instance, microRNA-145 performs as a tumor suppressor in EC by targeting phospholipase C epsilon 1 [27]. miR-145-5p suppresses tumor cell migration, invasion and epithelial to mesenchymal transition by regulating the Sp1/NF- $\kappa$ B signaling pathway in esophageal squamous cell carcinoma [28]. Moreover, miR-145 regulates epithelial-mesenchymal transition (EMT) by targeting connective tissue growth factor (CTGF) in EC [29]. However, in a recent research work, the scientists found that miR-145 was up-regulated in EC and exert an oncogenic effect. Overexpression of miR-145 notably promoted migration and invasion in vitro. Mechanically, they identified SMAD5 as direct target of miR-145. In our study, we demonstrated that miR-145 was down-regulated in EC tissue and cell lines. It played an anti-cancer role which was consistent with most of the previous studies.

It is well known that ceRNA is the main mechanism involved in the function of lncRNAs. In this way, lncRNAs competes with the other genes to bind with miRs, this spared the negative regulation of miRs on their target genes. As ROR/miR-145 has been extensively investigated, we focused on the target gene of miR-145 which may exert as a ceRNA of ROR. Bioinformatics

prediction and luciferase activity assay verified that miR-145 directly targets LMNB2 in EC which has not been reported before. It was also determined that LMNB2 could reverse the function of miR-145 which confirmed their targeting relationship.

Lamins are intermediate filament proteins that form a scaffold known as the nuclear lamina [30]. They are involved in tissue homeostasis and it is well known that lamins are required for the maintenance of nuclear architecture and function [31, 32]. Thus, the lamins have multiple functions in the cell nucleus. They provide mechanical support for the nucleus and play a role in DNA replication and repair, transcription, chromatin organization and gene regulation [33]. There are two types of lamins in mammalian cells, namely, lamin A/C and lamin B, which are encoded by LMNA, LMNB1, and LMNB2 genes. Many recent studies have reported link between lamins and cancer. A-type lamins increase the invasiveness of colorectal cancer (CRC) by promoting a more stem cell-like phenotype, thereby decreasing survival times [34]. Low LMNA expression is associated with increased disease recurrence in stage II and III colon cancer patients.



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In spite of these findings, the function of LMNB2 in EC remains to be studied. In the present work, the expression of LMNB2 was verified to be up-regulated in EC tissues and cells. Meantime, it was positively regulated by ROR and negatively regulated by miR-145. To elucidate the effect of LMNB2 on EC, we over-expressed the LMNB2 by transient transfection of pcDNA3.1-LMNB2 and reduced the endogenous LMNB2 by transient transfection of siRNA-LMNB2 in EC cells. We firstly determined that LMNB2 could promote cell proliferation and migration in EC9706 and EC1 cells. The nude mice xenograft assay revealed that LMNB2 could promote tumor growth in vivo.

In conclusion, we demonstrated that ROR exert its oncogenic role by regulating the miR-145/LMNB2 signal axis. We firstly discovered the function of LMNB2 in EC. Our findings expand the understanding on the effect and mechanism of ROR, miR-145 and LMNB2. ROR may become a new biomarker and therapeutic target for EC. In addition, the ROR/miR-145/LMNB2 may become an important signal in EC. However, the rescue experiments of the LMNB2 and miR-145 as well as LMNB2 and ROR are required to further confirm the ceRNA mechanism between LMNB2 and ROR.

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### Disclosure of conflict of interest

None.

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