Tumor cell-derived exosomal lncRNA LOC441178 inhibits the tumorigenesis of esophageal carcinoma through suppressing macrophage M2 polarization

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ABSTRACT

Esophageal carcinoma (EC) is a highly malignant type of tumor. In a previous study, the authors found that long non-coding RNA (lncRNA) LOC441178 inhibited the tumorigenesis of EC. Moreover, exosomes derived from tumor cells containing lncRNAs were found to play a key role in the tumor environment; however, whether exosomes can affect the tumor microenvironment by carrying LOC441178 remains unclear. Thus, the present study aimed to clarify this. In order to assess the effects of exosomal LOC441178 in EC, cell invasion and migration were examined using the Transwell assay. Exosomes were identified using transmission electron microscopy, Western blot analysis and nanoparticle tracking analysis. Furthermore, macrophage surface makers (CD206 and CD86) were analyzed using flow cytometry. Moreover, a subcutaneous xenograft mouse model was constructed to assess the role of TE-9 cells-derived exosomal LOC441178 in EC. The results revealed that LOC441178 overexpression notably suppressed the metastasis of EC cells. In addition, exosomes were successfully isolated from EC cells, and LOC441178 level was upregulated in exosomes derived from LOC441178-overexpressed EC cells. Exosomal LOC441178 also suppressed macrophage M2 polarization, and the polarized macrophages decreased EC cell invasion. Exosomes containing LOC441178 notably inhibited the growth of EC in mice. On the whole, the present study demonstrated that the delivery of LOC441178 by EC cell-secreted exosomes inhibited the tumorigenesis of EC by suppressing the polarization of M2 macrophages. These findings may provide a new theoretical basis for discovering new strategies against EC.

Key words: EC; exosome; LOC441178; EMT; STAT6.

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Introduction

Esophageal carcinoma (EC) is one of the most common malignant tumors, which includes adenocarcinoma and squamous cell carcinoma.[1] It has been reported that the incidence of EC is extremely high in China.[2] At present, radiation, surgery and chemotherapy are the main treatment strategies for EC, while the outcomes remain poor.[3] Therefore, it is essential to explore new effective methods for the treatment of EC.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNA transcripts.[4] lncRNAs are crucial modulators that participate in the progression of EC.[5,6] For instance, the overexpression of lncRNA SNHG7 has been shown to facilitate the metastasis of EC cells by regulating miR-625.[7]. Liang *et al.*[8] indicated that LINC00239 was able to enhance the c-Myc level in EC by interacting with c-Myc promoter-binding protein-1. Moreover, a previous study by the authors indicated that LOC441178 overexpression inhibits the tumorigenesis of EC,[9] which suggests that LOC441178 may function as a key mediator in EC.

Exosomes (70 to 120 nm in diameter) are microvesicles which are secreted from multi-vesicular bodies.[10] It has been reported that exosomes participate in the communication of cells by transferring proteins.[11,12] In addition, various exosomal lncRNAs can modulate tumor progression. For example, exosomal lncRNA UCA1 can modulate stem cell differentiation by miR-122-5p.[13] In addition, another study demonstrated that exosomal lncRNA AGAP2-AS1 enhanced radiotherapy immunity in non-small cell lung cancer by regulating the miR-296/NOTCH2 axis.[14] It has been revealed that the cross-talk between stroma cells and tumor cells plays an essential role in mediating cancer development.[15,16] In the tumor microenvironment, macrophages communicate with cancer cells to modulate the tumorigenesis of cancers.[17,18] Additionally, macrophages that exert oncogenic effects are regarded as tumor-associated macrophages, and they exhibit the M2 phenotype.[19] Furthermore, exosomal lncRNAs have been reported to regulate macrophage M2 polarization.[15,16] Nevertheless, whether exosomal LOC441178 derived from EC cells can mediate macrophage M2 polarization in EC remains largely unknown. The present study aimed to detect the function of exosomal LOC441178 in EC. In addition, the present study sought to explore the association between exosomal LOC441178 and macrophage M2 polarization in EC. It is hoped that the findings might shed a new light for clinicians to find new strategies against EC.

Materials and Methods

Cells and cell culture

EC cells (Eca-109 and TE-9) and THP-1 cells were purchased from ATCC. The cells were cultured in DMEM (Invitrogen, Thermo Fisher Scientific, Inc., Shanghai, China), supplemented with 10% FBS, 1% streptomycin and penicillin (Invitrogen) in 5% CO_2 at 37˚C.

Reagents

IL-4 (cat.no HY-P78549) and L-13 (cat.no HY-P70460) were purchased from MCE (Shanghai, China).

Cell transfection

The EC cells were transfected with pcDNA3.1 (GenePharma, Inc., Shanghai, China) or pcDNA3.1-LOC441178 for 48 h using Lipofectamine 2000 (Invitrogen). Following transfection, the cells were used in the subsequent analyses.

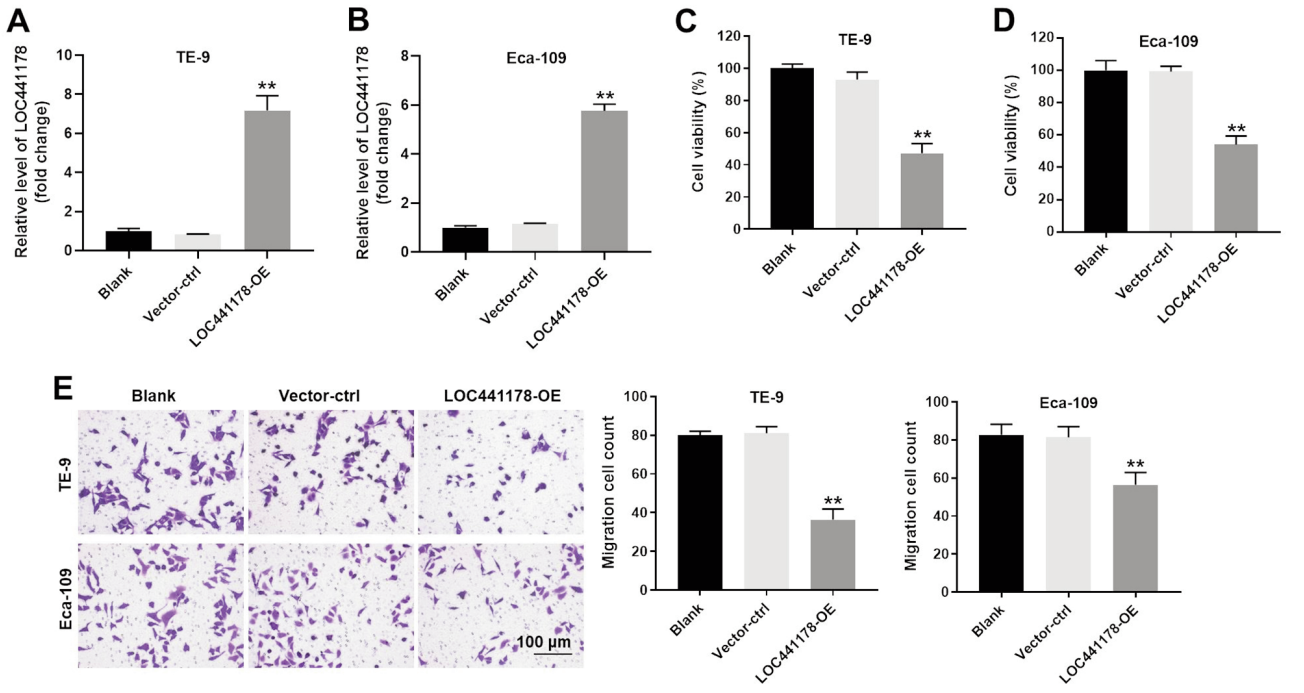

Figure 1. Overexpression of LOC441178 significantly inhibited the viability and migration of EC cells. EC cells were transfected with vector-control and pcDNA3.1-LOC441178. A,B) The level of LOC441178 in TE-9 or Eca-109 cells was investigated by RT-qPCR. C,D) The viability of TE-9 or Eca-109 cells was tested by CCK-8 assay.(E) The migration and invasion of TE-9 or Eca-109 cells were detected by Transwell assay. **p<0.01 compared to control.

Exosome extraction and identification

When the cell confluency reached 80%, the complementary medium was replaced with the defined medium (without FBS). The supernatants were centrifuged (300 x g for 15 min, 12,000 x g for 15 min and 10,000 x g for 30 min) following 48 h of culture. The supernatants were collected for the isolation of exosomes by ultracentrifugation (120,000 x g, 70 min). Transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and Western blot analyses were applied for identifying the exosomes. The procedure for TEM and NTA detection was as previously described.[15]

TEM

The exosome pellet was incubated for 5 min and subsequently immersed in 2% phosphotungstic acid solution for 1 min. In addition, pellet was fixed using 2.5% of glutaraldehyde (pH 7.2) at 4°C overnight. A 100 µl drop of the suspension was placed on a parafilm sheet, and a copper grid coated by carbon was placed onto the drop for 10 sec and then removed. Uranyl acetate and phosphotungstic acid (0.5 µg, 2%) were added onto the grid at room temperature for 5 s. Following the removal of excess liquid using filter paper, the grid was dried for 10 min at room temperature and the observed using a transmission electron microscope.

Nanosight tracking analysis

The hydrodynamic radius and concentration of exosomes were detected *via* NTA. In brief, a total of ~0.3 mL supernatant was loaded into the sample chamber of an LM10 Nanosight unit (Nanosight, Ltd., Salisbury, UK) and three videos of either 30 or 60 sec were recorded of each sample. Data analysis was performed using NTA 2.1 software (Nanosight, Ltd., Shanghai, China). In NTA, the paths of unlabeled particles acting as point scatterers, undergoing Brownian motion in a 0.25 mL chamber through which a 635-nm laser beam is passed, is determined from a video recording, with the mean squared displacement determined for each possible particle. The diffusion coefficient and sphere-equivalent hydrodynamic radius are subsequently determined using the Stokes-Einstein equation.

Macrophage M2 polarization

The THP-1 cells were exposed to 100 ng/mL PMA for 2 h. The cells were exposed to TE-9-Exo-NC, TE-9-Exo-LOC441178 OE or IL4/IL-13 for 24 h. Subsequently, CD206 (eBioscience, Thermo Fisher Scientific, Inc.) and CD86 (eBioscience; Thermo Fisher Scientific, Inc.) were assessed using flow cytometry (BD Biosciences, Franklin Lake, NJ, USA).

Reverse transcription-quantitative PCR (RT-qPCR)

TRIzol® reagent was applied for isolating total RNA. PrimeScript RT (ELK bioscience, Wuhan, China) was applied to reverse transcribe the total RNA into cDNA. qPCR analysis was then performed using SYBR-Green. All qPCR reactions were repeated three times in line with the following protocol: 94°C for 2 min, followed by 35 cycles (94°C for 30 s and 55°C for 45 s). The primer sequences used were as follows: LOC441178 forward, 5'-TGGGCAATAGGCGATACGAT-3' and reverse, 5'-GATAAAGGGAAAGCTCTGTGCC-3'; β-actin forward, 5'-GTCCACCGCAAATGCTTCTA-3' and reverse, 5'-TGCTGTCACCTTCACCGTTC-3'. The $2^{-\Delta\Delta cq}$ method was applied for data quantification.

CCK-8 assay

The EC cells ($5x10^3$ cells per well) were seeded overnight. They

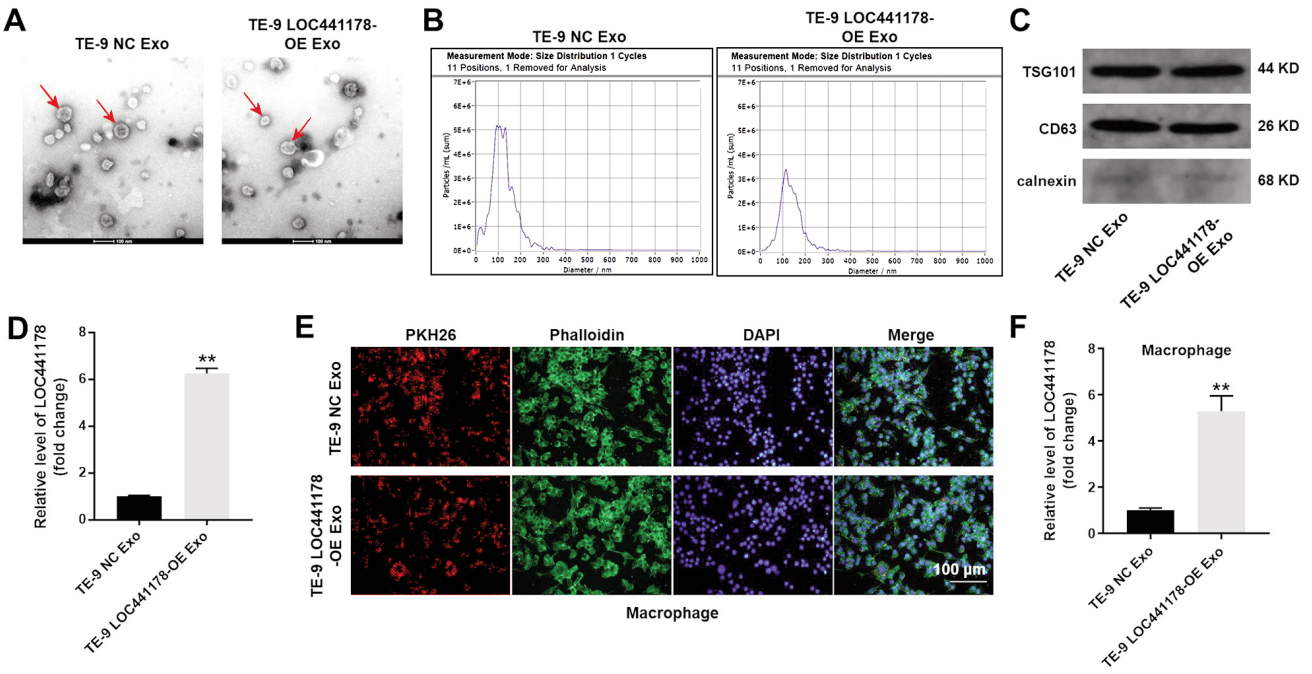

Figure 2. LOC441178 can be transferred from TE-9 cells to macrophages cells *via* exosomes. A) The isolated extracellular vesicle were observed by TEM; red arrows indicate the exosomes. B) The particle sizes of exosomes derived from TE-9 (TE-9 NC-exo) or exosomes derived from LOC441178-overexpressed TE-9 (TE-9 LOC441178-OE-exo) were measured by NTA. C) The expressions of TSG101, calnexin, and CD63 in TE-9 NC-exo or TE-9 LOC441178-OE-exo were detected by Western blot. D) The expression of LOC441178 in TE-9 NC-exo or TE-9 LOC441178-OE-exo was investigated by RT-qPCR. E) THP-1 cells were treated with 100 ng/mL PMA and then incubated with TE-9 cell-derived exosomes; then, the location of exosomes was observed by immunofluorescence staining. F) Macrophages were treated with TE-9 NC-exo or TE-9 LOC441178-OE-exo; the level of LOC441178 in macrophages was investigated by RT-qPCR. **p<0.01 compared to TE-9 NC-exo.

were then transfected with NC or LOC441178 OE. Subsequently, the EC cells were exposed to 10 μL CCK-8 for a further 2 h. A microplate reader was applied to measure the absorbance (450 nm).

Exosome labeling and uptake

Macrophages were cultured in a 4-well chamber for 24 h, then the PKH26 (Sigma-Aldrich)-labeled TE-9-derived exosomes were added into the wells and incubated with the cells for 12 h at 37°C. The cells were then washed three times with cold PBS following incubation, fixed in 2% paraformaldehyde for 10 min at room temperature. Next, cells were stained with FITC-labelled phalloidin (20 μg/mL) at room temperature for 10 min. Then, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Luois, MO, USA) for 2 min at room temperature, washed three times with PBS and stained with DAPI (10 μg/mL) at room temperature for 5 min. Images were then captured using a fluorescence microscope (200x, Olympus, Tokyo, Japan). The filter sets were as follows: for DAPI, exc. 420 nm, em. 485 nm; for FITC, exc. 460 nm, em. 550 nm; for PKH26, exc. 515 nm, em. 550 nm.

Western blot analysis

RIPA (Genepharma) was used for extracting total protein. BCA was used for total protein quantification. SDS-PAGE (10%) was used to separate the proteins. The proteins were then transferred onto PVDF membranes (MilliporeSigma). Primary antibodies targeted against CD63 (cat. no. ab134045, 1:1,000; Abcam, Cambridge, MA, USA), TSG101 (cat. no. ab125011, 1:1,000; Abcam), p-STAT6 (cat. no. ab263947, 1:1,000; Abcam), STAT6 (cat. no. ab32108, 1:1,000; Abcam), N-cadherin (cat. no. ab76011, 1:1,000; Abcam), E-cadherin (cat. no. ab40772, 1:1,000; Abcam), calnexin (cat. no. ab133615, 1:1,000; Abcam), PTEN (cat. no. ab267787, 1:1,000; Abcam), Akt (cat. no. ab8805, 1:1,000; Abcam), p-Akt (cat. no. ab38449, 1:1,000; Abcam) and β-actin (cat. no. ab8226, 1:1,000; Abcam) were used to incubate the membranes at 4°C overnight after blocking for 1 h with skimmed milk (5%). HRP-conjugated secondary antibodies (cat. no. ab288151, 1:5,000; Abcam) were used to incubate the membranes. The protein bands were visualized using ECL (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). IPP 6.0 (Image-Pro Plus 6.0) was applied for densitometric analysis.

Transwell assay

Matrigel (100 μL) was applied to pre-treat the upper chamber (Matrigel was not included in the migration analysis). The EC cells ($1.0x10^6$) were cultured in the upper chamber (1% FBS). The lower chamber supplemented with RPMI-1640 medium containing 10% FBS. The chamber was incubated at 4°C. The cells were then stained with crystal violet (0.1%) for 20 min. The cells were observed under a light microscope (200x, Olympus, Japan).

Wound healing assay

The TE-9 cells were underlined perpendicular to the cell culture plate with a small pipette head after the cells reached 80% confluency. Serum-free medium was used for further culture after the cells were washed three times, and the scratch widths were calculated under a microscope at 0 and 48 h.

ELISA

The IL-10 (cat.no EK110) and TGF-β (cat.no EK981) levels in EC cell supernatants were investigated using an ELISA kit according to the protocol of manufacturer (Multisciences (Lianke) Biotech, Co. Ltd, Hangzhou, China).

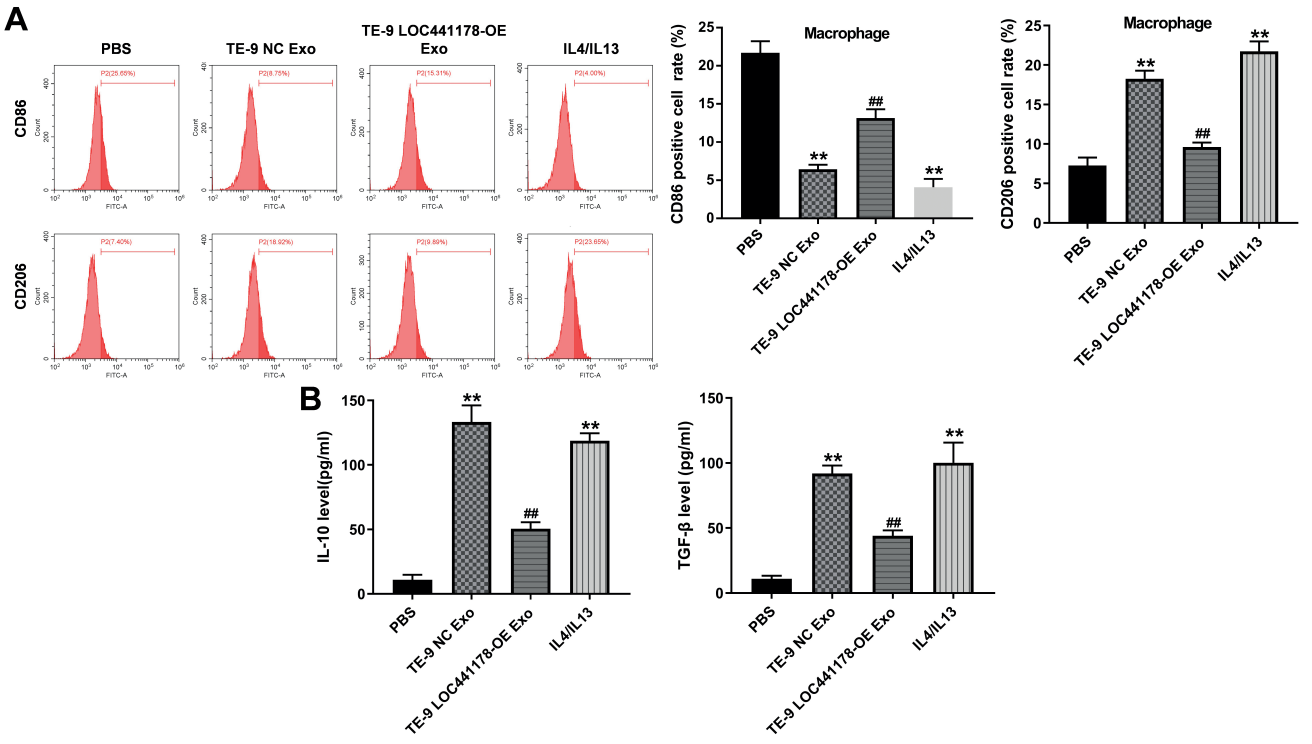

Figure 3. Exosomal LOC441178 inhibited M2 polarization in macrophages. A) Macrophages were treated with TE-9 NC-exo, TE-9 LOC441178-OE-exo or IL-4/IL-13 (20 ng/mL) for 24 h; then, the rate of CD86 or CD206 distribution in macrophages was detected by flow cytometry. B) The levels of TGF-β and IL-10 in supernatants of macrophages were detected by ELISA. $^{**}p<0.01$ compared to PBS; $^{\#\#}p<0.01$ compared to TE-9 NC-exo.

In vivo experiment

BALB/c mice (n=16; Beijing Vital River Laboratory Animal Technology Co., Ltd., China) were placed according to the conditions of the SPF facility. The TE-9 cells co-cultured with macrophages, macrophages-exo, macrophages$^{exo\text{-}NC}$ or macrophages$^{exo\text{-}LOC441178\ OE}$ were transplanted into the mice subcutaneously. The tumor volume was detected once a week in line with the following equation: Length x width^2. Meanwhile, the tumor volume of living mice was measured using a Vernier caliper. Finally, the mice were sacrificed for tumor collection. The isolated tumors were then weighed. All the animal experiments were applied in line with the NIH guide. Moreover, the Ethics Committees of The Second Affiliated Hospital of Fujian Medical University approved the study.

Statistical analysis

In total, three independent experiments were applied in each group. Moreover, all data are expressed as the mean ± SD. GraphPad Prism 7 software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analyses. One-way ANOVA followed by Tukey's test (multiple groups) or the Student's *t*-test (only two groups) were used to compare the differences. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Overexpression of LOC441178 markedly suppresses the viability of EC cells

To assess the function of LOC441178 in EC, the TE-9 and Eca-109 cells were transfected with pcDNA3.1-LOC441178. As illustrated in Figure 1 A,B, the LOC441178 level in EC cells was significantly elevated following transfection with pcDNA3.1-LOC441178. In addition, LOC441178 upregulation significantly decreased the viability of EC cells (Figure 1 C,D). Consistently, the migration of EC cells was notably suppressed by pcDNA3.1-LOC441178 (Figure 1E). Taken together, LOC441178 upregulation attenuated EC cell viability. Since the TE-9 cells were more sensitive to pcDNA3.1-LOC441178 transfection compared with the Eca-109 cells, these cells were selected for further analysis.

LOC441178 can be transferred from TE-9 cells to macrophages cells *via* exosomes

Exosomes from tumors function as key modulators in tumor progression.[20,21] Thus, exosomes were extracted from TE-9 cells. As illustrated in Figure 2A, rounded particles (30-150 nm diameter) were observed, and NTA revealed the similar size distribution

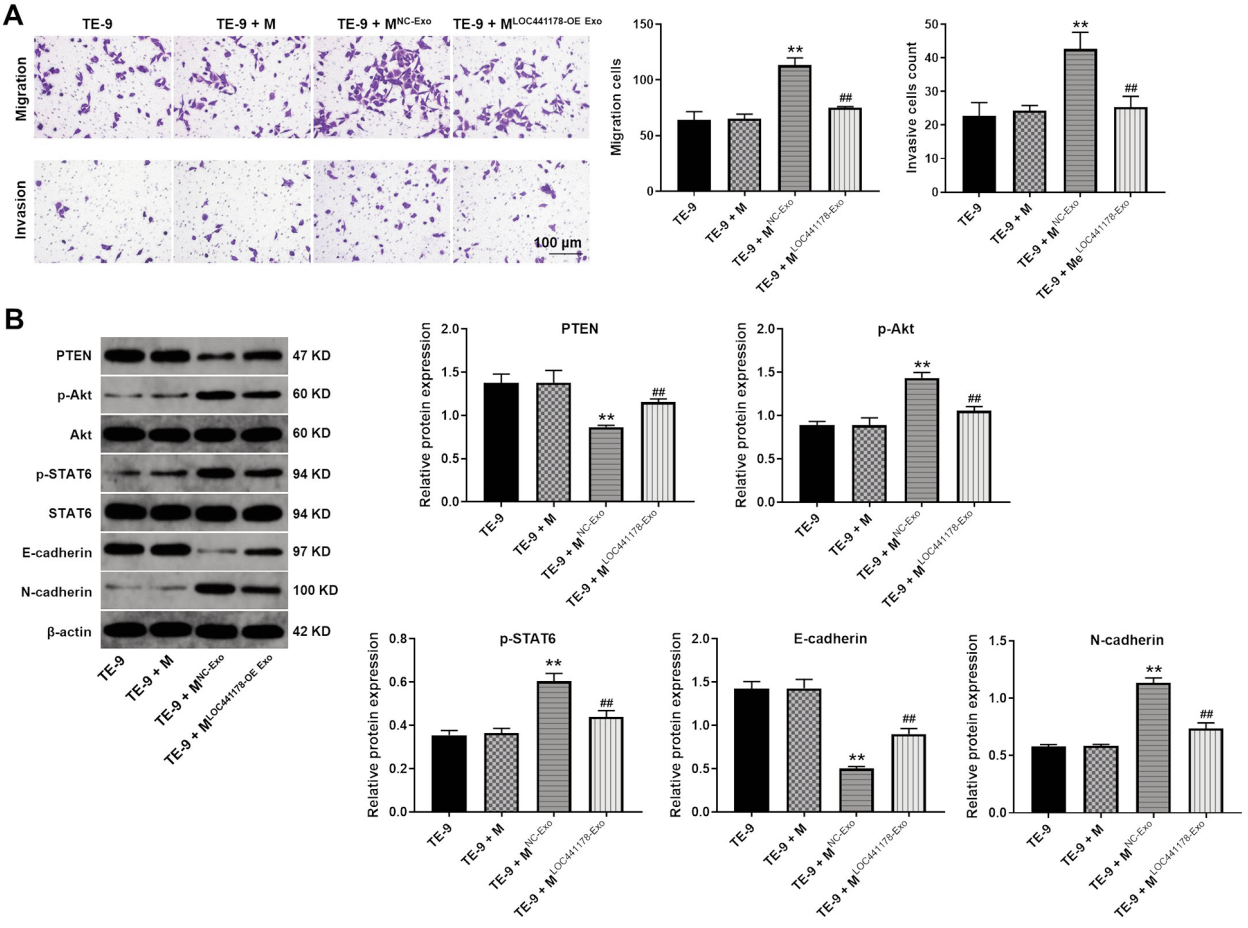

Figure 4. Exosomal LOC441178 notably suppressed the migration, invasion and EMT process of EC cells *via* inhibiting the macrophages M2 polarization. A) TE-9 cells were co-cultured with macrophages, $M^{exo\text{-}NC}$ or $M^{LOC441178\text{-}Exo}$; then, the migration and invasion of EC cells was investigated by Transwell assay. B) The protein levels of PTEN, Akt, p-Akt, E-cadherin, N-cadherin, p-STAT6 and STAT6 in TE-9 cells were tested by western blot; β-actin was used for normalization. $^{**}p<0.01$ compared to TE-9 cells; $^{\#\#}p<0.01$ compared to TE-9 + $M^{exo\text{-}NC}$.

(Figure 2B). Moreover, TSG101 and CD63 were highly expressed in exosomes containing pcDNA3.1-LOC441178 (TE-9$^{LOC441178\ OE}$-exo) or exosomes containing pcDNA3.1 (TE-9^{NC}-exo), while calnexin was rarely expressed (Figure 2C). The level of LOC441178 in TE-9$^{LOC441178\ OE}$-exo was markedly higher than that in TE-9^{NC}-exo (Figure 2D).

To investigate whether exosomes were able to mediate cell-to-cell crosstalk by transmitting LOC441178 among TE-9 cells and macrophages (PMA-differentiated human THP-1 monocytes), macrophages were exposed to TE-9 cell-derived exosomes. Following 48 h of incubation, PKH26 lipid dye was observed in macrophages, suggesting that TE-9^{NC}-exo or TE-9$^{LOC441178\ OE}$-exo was able to be delivered to macrophages (Figure 2E). Furthermore, the LOC441178 level in macrophages was evidently elevated by TE-9$^{LOC441178\ OE}$-exo (Figure 2F). On the whole, these results indicated that LOC441178 could be transmitted from tumor cells to macrophages.

Exosomal LOC441178 attenuates macrophage M2 polarization

To assess the effects of TE-9$^{LOC441178\ OE}$-exo on the M2 polarization of macrophages, macrophages were exposed to TE-9 cell-derived exosomes or IL-4/IL-13. The results demonstrated a $CD86_{low}/CD206_{high}$ phenotype in cells following exposure to TE-9^{NC}-exo, while the effects of exosomes were abolished by TE-9$^{LOC441178\ OE}$-exo (Figure 3A). Additionally, TE-9^{NC}-exo enhanced the levels of IL-10 and TGF-β in macrophages; however, the effects of TE-9^{NC}-exo were restored by TE-9$^{LOC441178\ OE}$-exo (Figure 3B). These results suggested that exosomal-LOC441178 was able to decrease macrophage M2 polarization.

Exosomal LOC441178 attenuates the invasion, and epithelial-mesenchymal transition (ETM) process of EC cells by suppressing macrophage M2 polarization

To verify whether exosomal LOC441178 can suppress the invasion and migration of EC cells by suppressing the M2 polarization, the Transwell assay was performed. It was found that the TE-9 cells co-cultured with M2 macrophages (TE-9 + M^{NC}-exo) exhibited a greater invasive and migratory ability, while the phenomena were alleviated by exosomes in which LOC441178 was overexpressed (LOC441178-modified exosomes) (Figure 4A). Since the EMT process is able to induce the migration and invasion of cancer cells,[22,23] the N-cadherin and E-cadherin levels were detected. The data revealed that macrophages incubated with TE-9^{NC}-exo (M^{NC}-exo) reduced the levels of PTEN, E-cadherin and upregulated the expression levels of p-Akt, N-cadherin and p-STAT6 in TE-9 cells, while these phenomena were partially restored by the LOC441178-modified exosomes (Figure 4B).

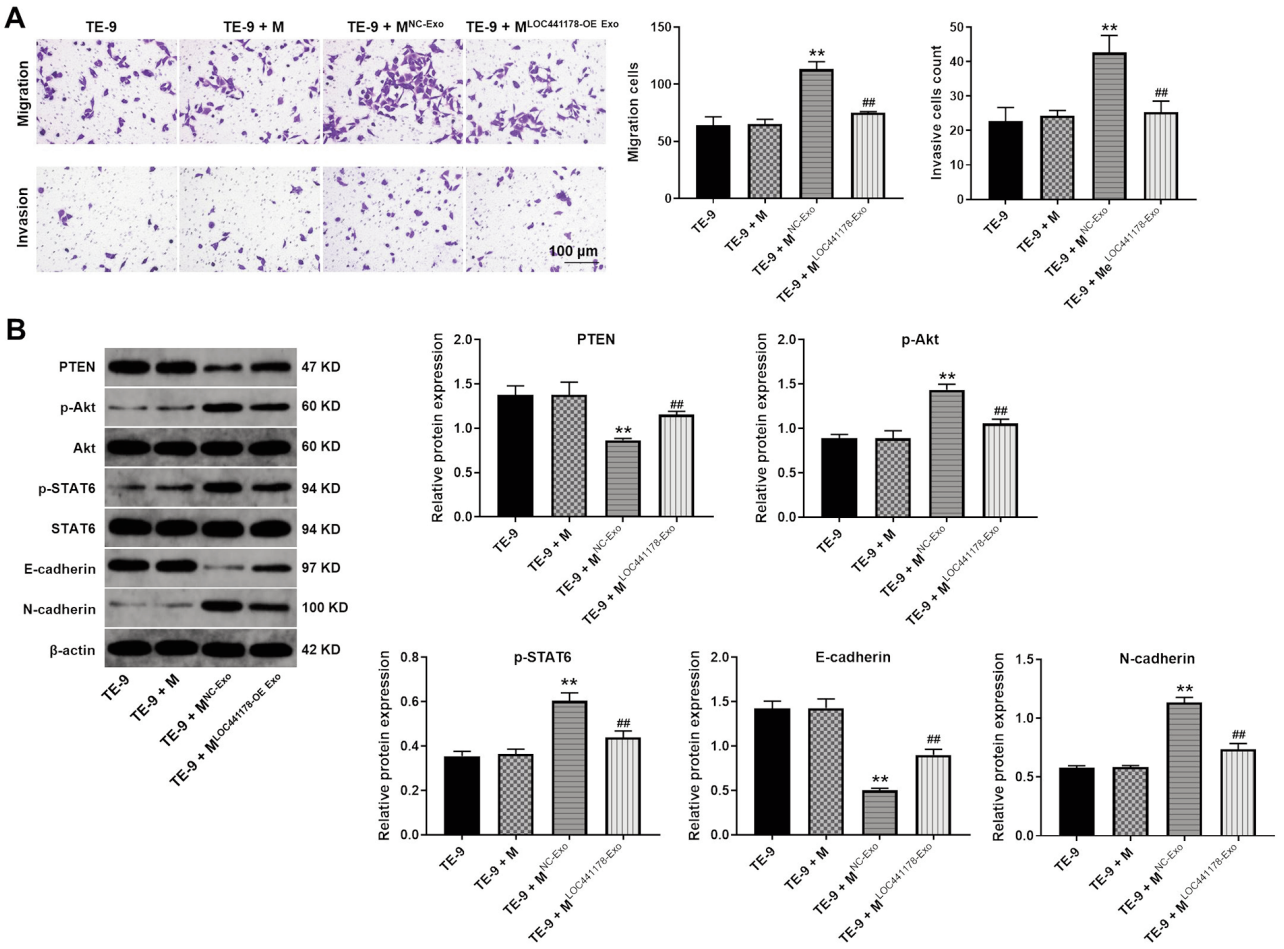

Figure 5. Exosomal LOC441178 significantly suppressed the wound healing rate of EC cells. The migration of EC cells was tested by wound healing assay. **p<0.01 compared to TE-9 cells; ##p<0.01 compared to TE-9 + M$^{exo\text{-}NC}$.

Furthermore, the M^{NC}-exo-induced increase in the wound healing rate in TE-9 cells was markedly attenuated by exosomes overexpressing LOC441178 (Figure 5 A,B). Taken together, the LOC441178-modified exosomes markedly suppressed the invasion and migration of EC cells by suppressing macrophage M2 polarization.

LOC441178-modified exosomes attenuate the growth of EC by suppressing macrophage M2 polarization

To further investigate the role of LOC441178-modified exosomes in EC *in vivo*, *in vivo* experiments were performed. As revealed in Figure 6 A-C, the TE-9^{NC}-exo-incubated macrophages enhanced the weight and volume of tumors in mice, while macrophages exposed to TE-$9^{LOC441178\ OE}$-exo significantly reversed these phenomena. The LOC441178 level in mice was notably increased by $M^{NC\text{-}exo}$, which was further increased in the presence of TE-$9^{LOC441178\ OE}$-exo (Figure 6D). Moreover, the TE-9^{NC}-exo-treated macrophages elevated the p-STAT6 and N-cadherin levels, and decreased the E-cadherin levels in tumor tissues of mice; these effects were significantly reversed by TE-$9^{LOC441178\ OE}$-exo (Figure 6E). On the whole, these results demonstrated that exosomal LOC441178 was able to attenuate EC growth *in vivo* by attenuating macrophage M2 polarization.

Discussion

It has been indicated that exosomes are associated with cancer progression.[24-26] In addition, it has been reported that exosomes can function as important mediators in the tumor microenvironment, and they can construct the association between tumor cells and stromal cells.[27,28] Moreover, M2 macrophages are known to be associated with the development of malignant tumors, as they can promote the metastasis of cancer.[29,30] In the present study, EC cell-derived exosomes were able to induce the M2 polarization of macrophages, and the polarized macrophages increased the metastasis of EC cells. Moreover, it was found that exosomes derived from EC cells functioned as crucial players in the tumor microenvironment, since they can play the role of a mediator among multiple cells. It has been reported that the dysregulation of lncRNAs can lead to the occurrence of multiple cancers.[31,32] It has also been revealed that LOC441178 has key biological functions during the tumorigenesis of cancer.[33] In addition, a previous study by the authors indicated that LOC441178 overexpression suppressed the migration and proliferation of EC cells.[9] In the present study, exosomal LOC441178 was able to reverse macrophage M2 polarization, which in turn inhibited the invasion, migration and EMT process of EC cells. Therefore, the present study firstly explored the function of exosomal LOC441178 in EC and the association between exosomal LOC441178 and M2 macrophages, suggesting that exosomal LOC441178 may play a key role in the tumor microenvironment.

The M2 polarization of macrophages has been confirmed to promote the progression of malignant tumors.[34,35] In addition, Akt and STAT6 have been reported to promote M2 polarization.[36,37] In the present study, exosomes overexpressing LOC441178 inactivated Akt/STAT6 signaling. Thus, it was suggested that exosomes overexpressing LOC441178 were able to inhibit macrophage M2 polarization *via* the inactivation of Akt/STAT6 signaling. Moreover, ROCK1 is known to be associated with the growth of cancer cells, which is positively regulated by PTEN.[38,39] Moreover, PTEN can negatively regulate Akt signaling.[40] A previous study indicated that LOC441178 overexpression inhibited the invasion and migration of squamous carcinoma cells, through indirectly tar-

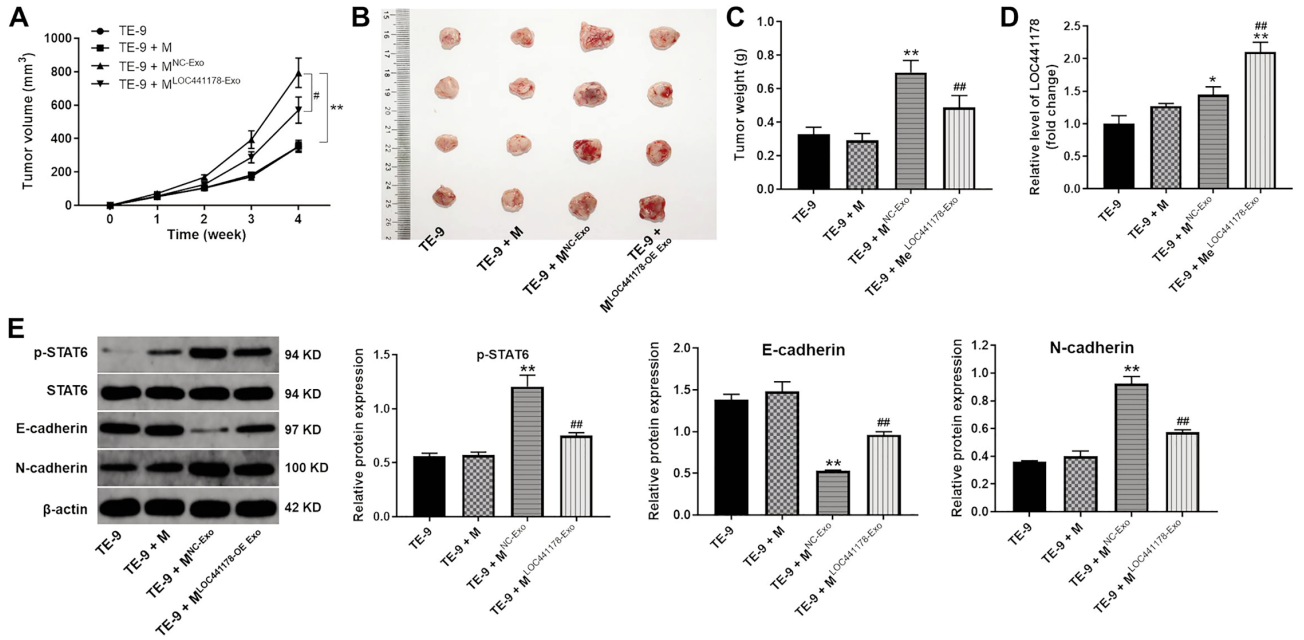

Figure 6. Exosomal LOC441178 significantly suppressed the tumor growth of EC *in vivo*. A) The tumor volume of mice was measured every week for four weeks. B) The tumor tissues of mice were pictured. C) Tumor weights of mice were measured. D) The expression of LOC441178 in tissues of mice was detected by RT-qPCR. E) The protein levels of E-cadherin, N-cadherin, p-STAT6 and STAT6 in tumor tissues were tested by Western blot; β-actin was used for normalization. *p<0.05; **p<0.01 compared to TE-9 cells; ##p<0.01 compared to TE-9 + $M^{exo\text{-}NC}$.

geting ROCK1.[33] Thus, exosomal LOC441178-mediated PTEN expression may occur due to the activation of ROCK1.

Notably, there are some limitations to the present study as follows: i) Other mechanisms through which exosomal LOC441178 regulates the tumorigenesis of esophageal carcinoma remain unexplored; ii) miRNAs sponged by LOC441178 need to be investigated; iii) the mechanism by which exosomal LOC441178 regulates STAT6 signaling remains unexplored. Thereby, further studies are required in the future.

In conclusion, tumor cell-derived exosomal lncRNA LOC441178 inhibits the tumorigenesis of esophageal carcinoma by suppressing macrophage M2 polarization. Thereby, the present study may provide a new theoretical basis for discovering new strategies against EC.

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