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Inhibition effect of exosomes-mediated Let-7a on the development and metastasis of triple negative breast cancer by down-regulating the expression of c-Myc

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Abstract. – OBJECTIVE: Triple negative breast cancer is typically characterized by high malignancy, easy recurrence and metastasis, and poor prognosis. However, effective treatment for triple negative breast cancer has not yet been identified. Our research explores the underlying mechanisms of exosomes in transporting Let-7a and regulating c-Myc gene and their roles in the development of triple negative breast cancer, and to provide new ideas for targeted therapy of triple negative breast cancer.

PATIENTS AND METHODS: Based on previous studies that focused on the roles of c-Myc and Let-7a in the development of triple negative breast cancer, triple negative breast cancer cell lines have been constructed by c-Myc knockout and overexpression of Let-7a, respectively, to evaluate the effects of c-Myc, and Let-7a, as well as exosomes transmitting Let-7a on the development of triple negative breast cancer.

RESULTS: Let-7a, which is mediated by exosomes, inhibited proliferation, migration, and invasion of MDA-MB-231 cells by binding on the 3'UTR region of the c-Myc gene and silencing of the c-Myc expression.

CONCLUSIONS: Our study revealed the role of c-Myc, Let-7a, and exosomes in the development of triple negative breast cancer, which lay the theoretical foundation for further usage of exosomes to construct tumor killing vectors and for exploring specific targets for triple negative breast cancer.

Key Words:
Triple negative breast cancer, c-Myc, Let-7a, Exosomes.

Introduction

Breast cancer is the most commonly found malignant tumor in women. Among all types of breast cancer, triple negative breast cancer (TNBC) accounts for 15%-20% of the total incidence[1,2]. Most patients are young women whose tumors have a high histological grade (grade III or higher)[3]. In contrast with other types of breast cancer, TNBC is the most aggressive type[4], and more prone to local recurrence and distant metastasis. Recurrence and metastasis usually occur 1-3 years after surgery, and most patients die in the first 5 years after treatment. Thus, the death rate of TNBC is significantly higher when compared to that of others breast cancer subtypes[5]. Currently, no effective drug is available to treat triple negative breast cancer. Therefore, studies on targeted therapy for triple negative breast cancer has become the hotspot and challenge in the cancer field.

In previous studies, it has been reported that the protein phosphorylation level and mRNA expression level of the phosphatidylinositol 3-kinase (PI3K) molecule in the P13K/protein kinase B (PKB or AKT)/mammalian target of rapamycin (mTOR) signaling pathway and its downstream molecules, including mTOR, the eukaryotic initiation factor 4E binding protein 1 (4EBP1), and selective inhibitor of p70 ribosomal S6 kinase 1 (S6K1) in breast cancer tissues are higher compared to adjacent tissues[6], thereby suggesting that activation of the PI3K/AKT/mTOR signaling pathway may be closely related to the occurrence and development of breast cancer. Studies on single use or combination use of LY294002 and RAD001 as specific inhibitors of PI3K and mTOR *in vitro* indicated that they inhibited the growth of MCF-7 and MDA-MB-231 cell lines in *in vitro* studies[7]. In addition, targeted inhibi-

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tion of the P13K/AKT/mTOR signaling pathway significantly inhibited the proliferation of human breast cancer cells, blocked the cell cycle, and induced apoptosis. Therefore, it is suggested that anti-tumor effects would be greatly improved if combined inhibition of multiple targets in the P13K/AKT/mTOR pathway was performed, especially for triple negative breast cancer cell lines.

Phosphorylation of PI3K further activates downstream signaling molecules, such as AKT, that subsequently transmit cellular signals to the nucleus. The c-Myc gene plays a key role as a node of multiple signaling pathways, which mediate PI3K pathway to transmit cellular signals to the nucleus. In previous studies, it has been shown that the transcription factor c-Myc plays an important role in the development of many malignant tumors[8]. c-Myc is involved in the regulation of cell proliferation, differentiation, growth, apoptosis, cell cycle regulation, and cellular malignant transformation. Moreover, c-Myc is the endpoint of many cellular signaling pathways and it is involved in the transduction of multiple pathways. Therefore, c-Myc is crucial in tumor proliferation and differentiation. According to several studies, increased c-Myc expression suggested poor prognosis and consequently tumors were more prone to recurrence and metastasis. Some studies revealed that amplification of the c-Myc gene often occurred in ER/PR-negative breast cancer. In particular, abnormally high expression of the c-Myc gene in triple negative breast cancer was closely related to its recurrence and metastasis[9]. However, how c-Myc regulates the recurrence and metastasis of triple negative breast cancer is unknown.

c-Myc is an important tumor-associated gene that is regulated by post-transcriptional regulation of miRNAs with tumor suppression. However, due to the wide distribution of c-Myc binding sites in gene promoter regions, transcriptional regulation of c-Myc genes by miRNAs is more important in malignant tumor development[10]. By regulating the expression of targeted genes, miRNA plays a significant role in the invasion and metastasis of breast cancer. Therefore, identifying the regulatory mechanisms of miRNA on their target genes has great clinical value for finding targets for early diagnosis, prognosis, and treatment of breast cancer. Let-7a is a well-studied miRNA in breast cancer. Thakur et al[11] found that the expression of Let-7a was significantly decreased in triple negative breast cancer. Let-7a regulated the cell cycle and cellular differentiation by regulating the expression of proto-oncogenes (such as KRAS, c-Myc), and cell cycle-associated genes (cyclinD1/2), which are important in tumor growth[12].

In recent studies, it was suggested that exosome transmitting miRNAs may be critical in the regulation of gene expression by miRNAs[13]. Exosomes release their contents to recipient cells by cell fusion or endocytosis. Then, the transferred nucleic acids will be transcribed and translated in recipient cells, thereby regulating the expression of target genes in recipient cells[14,15]. However, the regulation mechanism of exosomes, such as Let-7a and c-Myc in the development of triple negative breast cancer is still unclear. Therefore, we analyzed gene expression levels in clinical samples of triple negative breast cancer and constructed gene knockout and over-expressing breast cancer cell lines. This to demonstrate the underlying mechanisms of exosomes in transporting Let-7a and regulating c-Myc gene and their roles in the development of triple negative breast cancer, and to provide new ideas for targeted therapy of triple negative breast cancer.

Patients and Methods

Human Tissue Sampling

The current study was approved by the Human Ethics Committee of the 3rd Affiliated Teaching Hospital of Xinjiang Medical University (Affiliated Cancer Hospital). Tissue samples were obtained from biopsies of 14 breast cancer patients and were confirmed by Pathology and Immunohistochemistry at the Affiliated Tumor Hospital of Xinjiang Medical University (Xinjiang, China). All participants provided written informed consent and informed consent for the use of tissue to conduct comprehensive breast cancer experiments. After tissue biopsy, samples were immediately frozen in liquid nitrogen, and stored at -70°C until further use.

Cell Culture

Human breast cancer cell lines MCF-7, T47D, sk-br-3, and MDA-MB-231 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium (HyClone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37°C under 5% CO_2.

Real-Time PCR

Total RNA was extracted from human tumor and normal tissues, or cultured cells using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and 1 μg of RNA was reverse-transcribed into cDNA using reverse transcriptase (Vazyme, Nanjing, China). Real-time PCR was performed on a Bio-Rad CFX96 detection system using the SYBR Green I Real-Time Detection Kit (CW-BIO, Beijing, China). As a quantification reference, β-actin was used. RT-PCR primer sequence and 3'UTR fragment primer sequence: β-actin: F: 5›-CACGAAACTACCTTCAACTCC-3›, R: 5'-CATACTCCTGCTTGCTGATC-3'. c-Myc: F: 5'-TACGAATCTCCGACCACCA-3', R: 5'-GGACCAGACATCACCAAGC-3'. Let-7a: F: 5'-CCGAGTGACAAGCCTGTAGC-3', R: 5'-AGGAGGTTGACCTTGGTCTG-3'.

Western Blot Analysis

Cells or tissues were lysed in Radio Immunoprecipitation Assay (RIPA) buffer containing protease inhibitors. Quantitative analysis of the proteins was carried out by Bicinchoninic Acid (BCA) protein quantitative method. Equal amounts of protein were separated by 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to PVDF membranes, which were blocked with 5% skim milk in room temperature for 1 h and then incubated with the primary antibodies below overnight at 4°C. Antibodies directed against c-Myc, CD9, and CD81 were purchased from Cell Signaling Technology (1:1000, Louis Park, MN, USA). After washing three times with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibody (1:5000, Bioworld Technology Inc., St. Louis Park, MN, USA) for 2 h at room temperature. GAPDH was employed as a loading control at 36 KDa.

Lentivirus Transfection Experiment

MDA-MB-231 tumor cells were cultured to the logarithmic growth phase. Then, 1×10^5 cells were harvested and plated in a six-well plate, then fresh complete medium containing 6 μg/mL polybrene was added to each well, together with 50 μL virus. After incubation for 24 h, culture medium was replaced with fresh complete medium. At 5 days after transduction, stable cells were selected by maintaining the cells in culture medium containing 2 ug/mL puromycin.

Cell Proliferation Assay

MDA-MB-231 cells were seeded at a density of 4×10^3 cells/well in 96 well plate. Briefly, 20 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added to each well and incubated for 4 h. Then, the media was carefully removed followed by adding 150 μL of dimethyl sulfoxide. The plate was placed on a low-speed shaker to completely dissolve the crystals. The OD490 nm value was measured using a microplate reader (SpectraMax i3x system, MD, USA) and experiments were performed in triplicate.

Cell Invasion Assay

Cell invasion assay was performed in a 24-well Boyden chamber with an 8 μm pore sized polycarbonate membrane (Corning, CA, USA). Matrigel was thawed overnight at 2°C-8°C. Subsequently, 25 μL of diluted Matrigel was added to the upper transwell chamber to cover the entire polycarbonate membrane and incubated at 37°C for 30 min to allow the Matrigel to polymerize into gel. Next, MDA-MB-231 cells (2×10^4 in 100 μL of serum-free medium) were seeded into the upper chamber, and 500 μl of chemokine was added to the lower chamber. After incubation for 12 h, the cancer cells that remained on the upper surface of the membrane were removed. The migrated cells on the lower surface of the membrane were fixed by paraformaldehyde, stained with crystal violet, and counted using a microscope.

Cell Scratch Assay

MDA-MB-231 cells (1×10^5) were seeded into 24-well plates and incubated overnight. Cells were washed the next day and starved for 16-18 h in medium containing 1% FBS. When the cells reached confluence, the monolayer was scratched with a 1 ml pipet tip at the center of each well. After washing 3 times with PBS, complete medium was added to each well. Images were taken at 24/48 h using a Nikon TMS inverted microscope (10× magnification, Tokyo, Japan) and a Canon EOS camera (Tokyo, Japan). Cell-free areas were determined by ImageJ software.

Luciferase Reporter Assay

For luciferase reporter assays, MDA-MB-231 cells were seeded at a density of 5×10^4 cells/well in 24 well plate and co-transfected with 50 nmol/L Con mimics or Let-7a mimics. Thereinto, 500 ng of reporter molecules contain 3'UTR-WT or 3'UTR-MUT. Two days after transfection,

cells were lysed in Passive Lysis Buffer (Promega, Madison, WI, USA). Then, Dual-Luciferase® was added to the cell lysate to measure the luciferase activity using GloMax® 20/20 luminometer (Promega) according to the manufacturer's guidelines. The 3'UTR sequences were amplified from human cDNA by PCR using the following primers: forward, 5'-GCGCGAATTCACACGATGCGTATTTTAGTT-3'; reverse, 5'-GCGCAAGCTTAAGAATCACAGTTATGCCAA-3'. Relative luciferase activity = firefly luciferase / Renilla luciferase[14].

Separation of Exosomes

The cells were cultured in complete medium containing exosome-free FBS. When confluency reached 90%, cells were washed with PBS. Exosomes were isolated from the culture medium by differential centrifugation. Briefly, the medium was centrifuged at 300 g for 10 min at room temperature, then continually centrifuged at 2,000 g for 20 min at 4°C to remove cells. Then, cell debris was filtered through a 0.22 μm filter membrane (Millipore, Shanghai, China). The remaining medium was centrifuged at 100,000 g for 90 min by ultracentrifugation. The obtained exosomes were re-suspended in PBS and centrifuged again at 100,000 g for 90 min. The size and concentration of exosomes were determined by qNano (Izon Science, Christchurch, New Zealand).

Animal Experiment In Vivo

All animal experiments were approved by the Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University (Xinjiang, China). Luciferase-labeled MDA-MB-231 cells were injected subcutaneously into 6-week-old female NSG mice. Mice were divided into 3 groups, and PBS, Exo-WT, and Exo-Let-7a were administered through the tail vein at 2 μg each time, 2 times a week, and 6 times in total. When tumors became palpable, tumor sizes were assessed by caliper measurement using the formula ($width^2$ x length) / 2 (mm^3). At the same time, *in vivo* imaging was performed on each mouse on a weekly basis. At the end of the experiment, mouse tumors and peritumoral tissues were fixed in formaldehyde following the manufacturer's protocol (Beyotime, Shanghai, China), embedded in paraffin, and analyzed by Hematoxylin and eosin staining.

Statistical Analysis

The experimental data were analyzed by Student's *t*-test using SPSS 2.0 software (IBM Corp. IBM SPSS Statistics for Windows, Armonk, NY, USA), with $p<0.05$ as criteria for a statistically significant difference. Data are expressed as mean ± SD (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-tailed Student *t*-test).

Results

Let-7a and c-Myc Negatively Correlated with Breast Cancer

We first analyzed the GEO database (GSE27447), which included normal tissue and cancer tissues from 19 patients. Through bioinformatics analysis, we found that the expression of Let-7a was downregulated in tumor tissue when compared to normal tissue (Figure 1 A). Second, we analyzed the relationship between the expression of Let-7a and prognosis of triple negative breast cancer patients. As shown in Figure 1 B, the mean overall survival time of triple negative breast cancer patients with low Let-7a expression (median survival: 385 days) was significantly shorter when compared to patients with high Let-7a expression (median survival: 591 days; $p = 0.02$, log rank test). Subsequently, normal tissue and tumor tissue was collected from 14 breast cancer patients, respectively, and the expression of Let-7a and c-Myc in both types of tissue were analyzed by qPCR. The results showed that the c-Myc expression in tumor tissue was significantly higher when compared to those in normal tissue, while opposite results were observed for Let-7a expression, which was significantly lower in tumor tissue when compared to normal tissue (Figure 1 C, D). To validate the expression of Let-7a and c-Myc in triple negative breast cancer tissue, several triple negative breast cancer cell lines, including MCF-7, T47D, sk-br-3, and MDA-MB- 231 were selected to analyze the expression of the two genes in these breast cancer cell lines. Western blot analysis and qPCR results showed that the expression of Let-7a in breast cancer cells was low, while c-Myc was highly expressed (Figure. 1 E, F). In summary, we verified that the expression of Let-7a was low and that of c-Myc was high in both breast cancer cells and tissue, indicating that it is necessary to further explore the roles of Let-7a and c-Myc in the development of breast cancer.

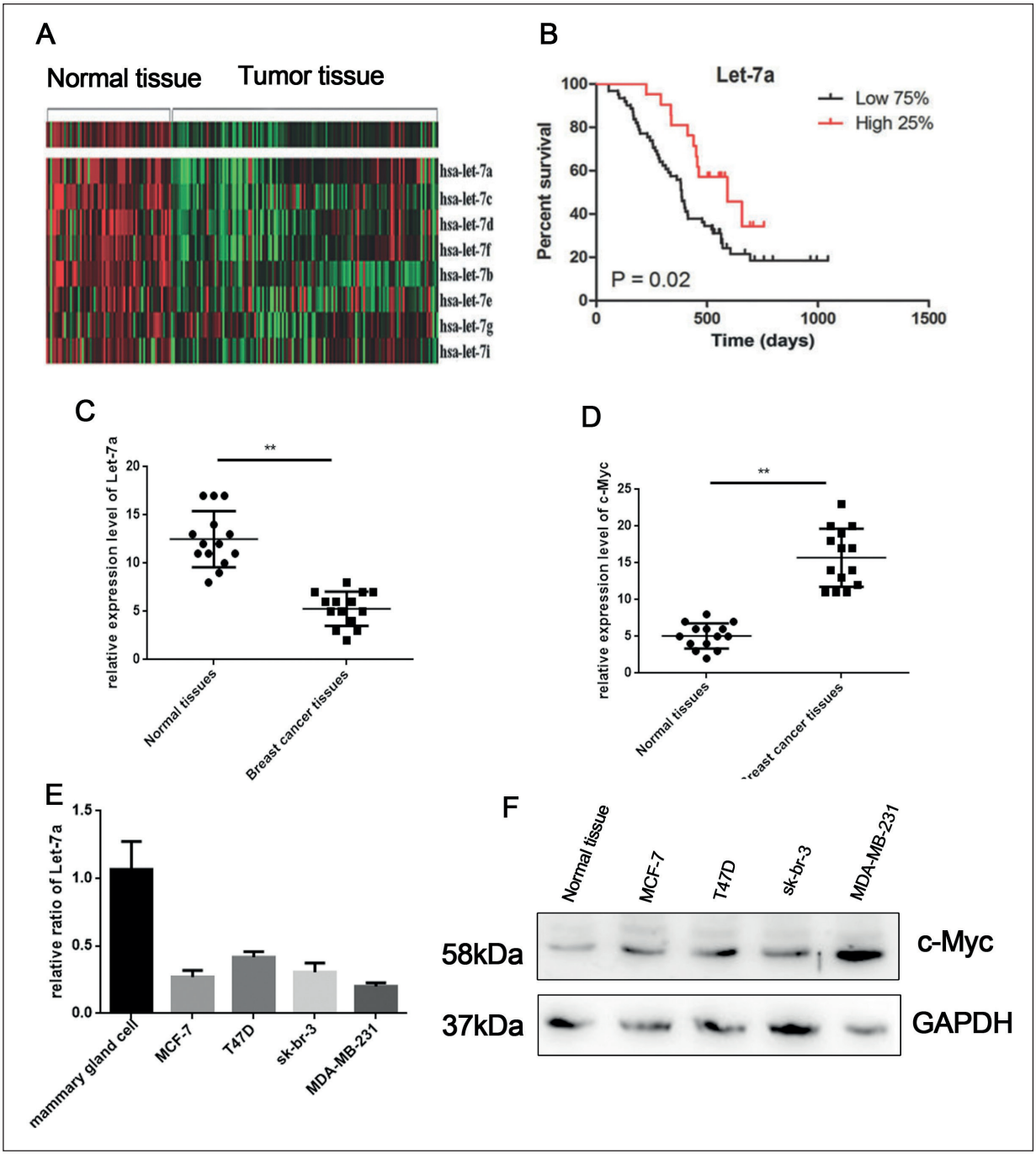

Figure 1. Expression of c-Myc and Let-7a in triple negative breast cancer. **A,** Tissue microarray detection of Let-7a in triple negative breast cancer. **B,** Correlation between Let-7a and survival rates in triple negative breast cancer patients. **C,** qPCR results of Let-7a in triple negative breast cancer tissues. **D,** qPCR results of c-Myc in triple negative breast cancer tissues. **E,** Expression of Let-7a in triple negative breast cancer cell lines. **F,** Expression of c-Myc protein in triple negative breast cancer cell lines (n=3, bar value indicates standard deviation, $^{**}p<0.01$).

c-Myc Promotes the Proliferation, Migration, and Invasion of Breast Cancer Cells

A knockout model of c-Myc in MDA-MB-231 cell was constructed by Crisper/cas-9, and the expression of c-Myc in the knockout cells was determined by qPCR and Western blot analysis (Figure 2 A, B). The results showed that in the knockout cells c-Myc was barely expressed, indicating that the c-Myc gene in MDA-MB-231

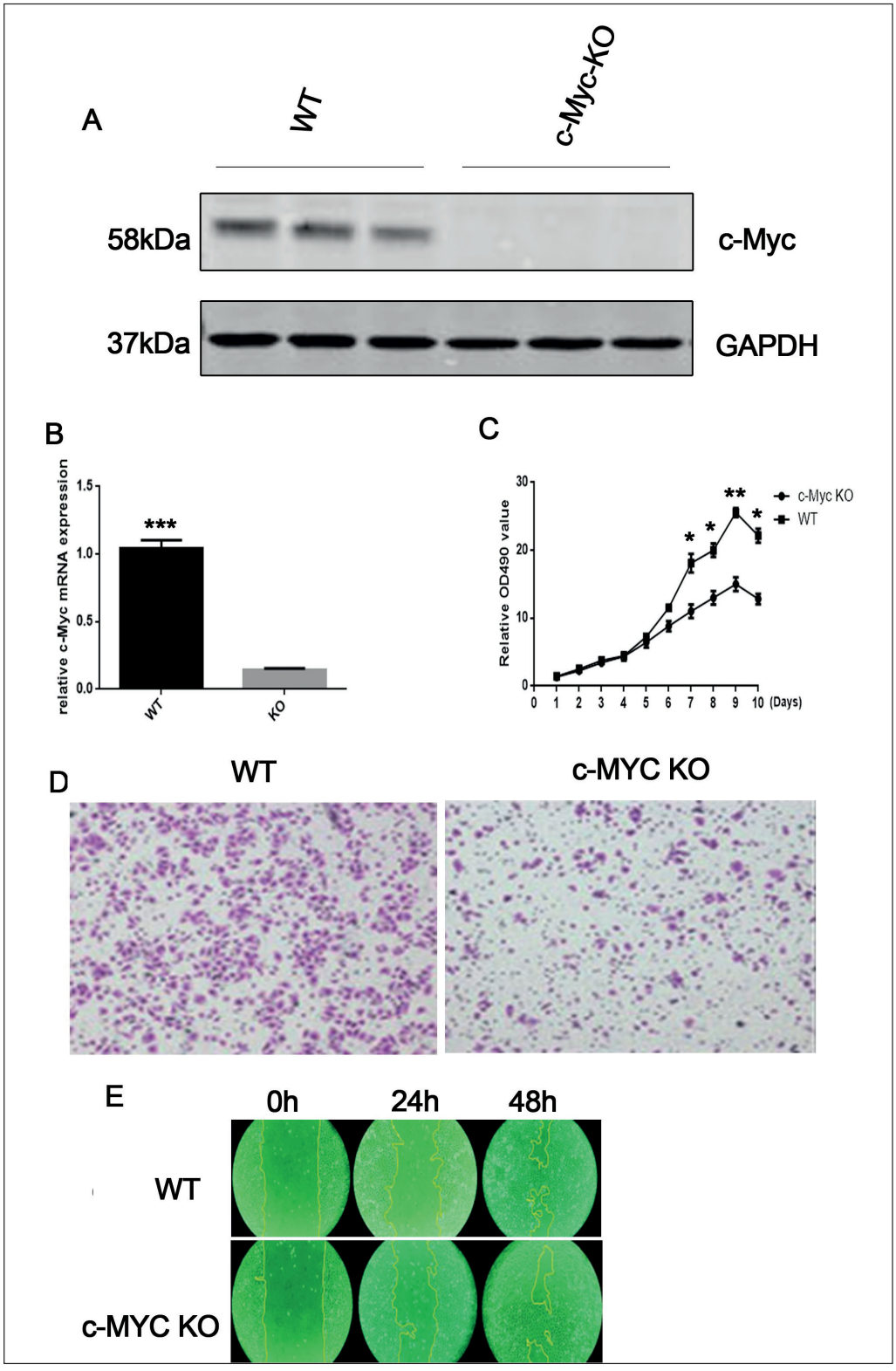

Figure 2. Effects of c-Myc on the biological function of triple negative breast cancer cells. **A,** Expression of c-Myc in knockout cells by Western blot analysis. **B,** Content of the c-Myc gene in knockout cells determined by qPCR. **C,** Detection of c-Myc on the proliferation of triple negative breast cancer cells by MTT assay. **D,** Matrigel chamber assay to test the effect of c-Myc on the invasion ability of triple negative breast cancer cells. **E,** Scratch assay to evaluate the effect of c-Myc on the migration ability of triple negative breast cancer cells (n=3, bar value indicates standard deviation, *p<0.05, **p<0.01, ***p<0.001).

cells was successfully knocked out. We next studied whether the biological characteristics of MDA-MB-231 KO cells would be different from that of wild-type MDA-MB-231 cells. To demonstrate the effect of c-Myc protein on cell proliferation, a growth curve of MDA-MB-231 KO cells was created based on the MTT assay. The results showed that knocking down c-Myc significantly inhibited the proliferation of MDA-MB-231 cells (Figure 2 C) in contrast with that of normal MDA-MB-231 cells. In addition, the invasion ability of MDA-MB-231 cells was evaluated by transwell invasion assay. Our data indicated that the invasion rate of MDA-MB-231 cells (89.33±4.91) was higher compared to that of MDA-MB-231 KO cells (50.00±3.06, $p<0.001$, Figure 2 D). In addition, the migration ability of cells was determined by scratch assay. The results showed that MDA-MB-231 cells were more likely to heal from scratch compared to MDA-MB-231 KO cells at both 24h and 48h (Figure 2 E). The above findings proved that c-Myc promoted the proliferation, migration, and invasion of breast cancer cells.

Let-7a Inhibits the Proliferation, Migration, and Invasion of Breast Cancer Cells

MDA-MB-231 cells that over-expressed Let-7a were constructed by lentivirus transfection. Moreover, the expression of Let-7a was measured by qPCR after lentivirus transfection. qPCR results showed that the expression of Let-7a was significantly increased in transfected cells when compared with wild-type MDA-MB-231 cells (Figure 3 A). To study the biological characteristic changes of cells that overexpress Let-7a, the MTT assay was employed to analyze the growth curve of cells with Let-7a overexpression as well as in wild-type cells (Figure 3 B). The results of the MTT assay showed that overexpression of Let-7a significantly inhibited the proliferation of MDA-MB-231 cells. The cell migration and invasion ability were measured by scratch assay and transwell invasion assays (Figure 3 C, D). The results showed that overexpression of Let-7a significantly inhibited the migration and invasion of MDA-MB-231 cells. In conclusion, our data showed that overexpression of Let-7a inhibited proliferation, migration, and invasion of breast cancer cells.

Determination of c-Myc as a Target of Let-7a

To understand whether c-Myc is a direct target gene of Let-7a, we cloned the 3' untranslated region of c-Myc mRNA (3'UTR-WT) into the pmirGLO vector and predicted the Let-7a binding site (3'UTR-MUT) by site-directed mutagenesis, as shown in Figure 4A. Next, the mixed plasmid was transfected into the cells. As predicted, overexpression of Let-7a significantly inhibited the activity of 3'UTR-WT-mediated luciferase, and the inhibition was reversed by a mutation in the Let-7a binding site (Figure 4 B). Subsequently, we tested the interaction between c-Myc and Let-7a through Western blot analysis. In cells that overexpressed Let-7a, the c-Myc expression was remarkably lower when compared to that of wild-type cells (Figure 4 C). Furthermore, when cells that overexpressed Let-7a were treated with antibodies directed against Let-7a, the cellular expression levels of c-Myc increased (Figure 4 D). Together, the above results suggested that Let-7a silenced expression of the c-Myc gene by binding to the 3'UTR region and, therefore, reduced the expression of c-Myc protein.

Let-7a Affects Cellular Biological Functions Through Exosomes

In recent studies, it was reported that miRNAs are commonly found in exosomes (EXO), mainly in the form of precursors. Although free miRNAs are present in the cellular circulation, it is believed that miRNAs in EXO are more bioactive than free miRNAs. Therefore, we extracted exosomes from wild-type cells and Let-7a overexpressing MDA-MB-231 cells and identified the extracted exosomes by electron microscopy and Western blot analysis. Our studies showed that exosomes extracted from wild-type and Let-7a overexpressing MDA-MB-231 cells presented a typical exosome structure using transmission electron microscopy, with a diameter between 40-100 nm and had a bilayer lipid membrane, cup-shaped vesicular-like structure (Figure 5 A). Western blot analysis showed that exosomes isolated from both types of cells expressed exosomal surface markers, CD9, and CD81. Both at the morphological and molecular level, it was detected that the extracted vesicles were exosomes (Figure 5 B). In the two groups of exosomes, Let-7a was quantified by qPCR. The level of Let-7a in the exosomes extracted from Let-7a overexpressing cells was much higher when compared to that in wild type cells (Figure 5 C), suggesting that Let-7a was

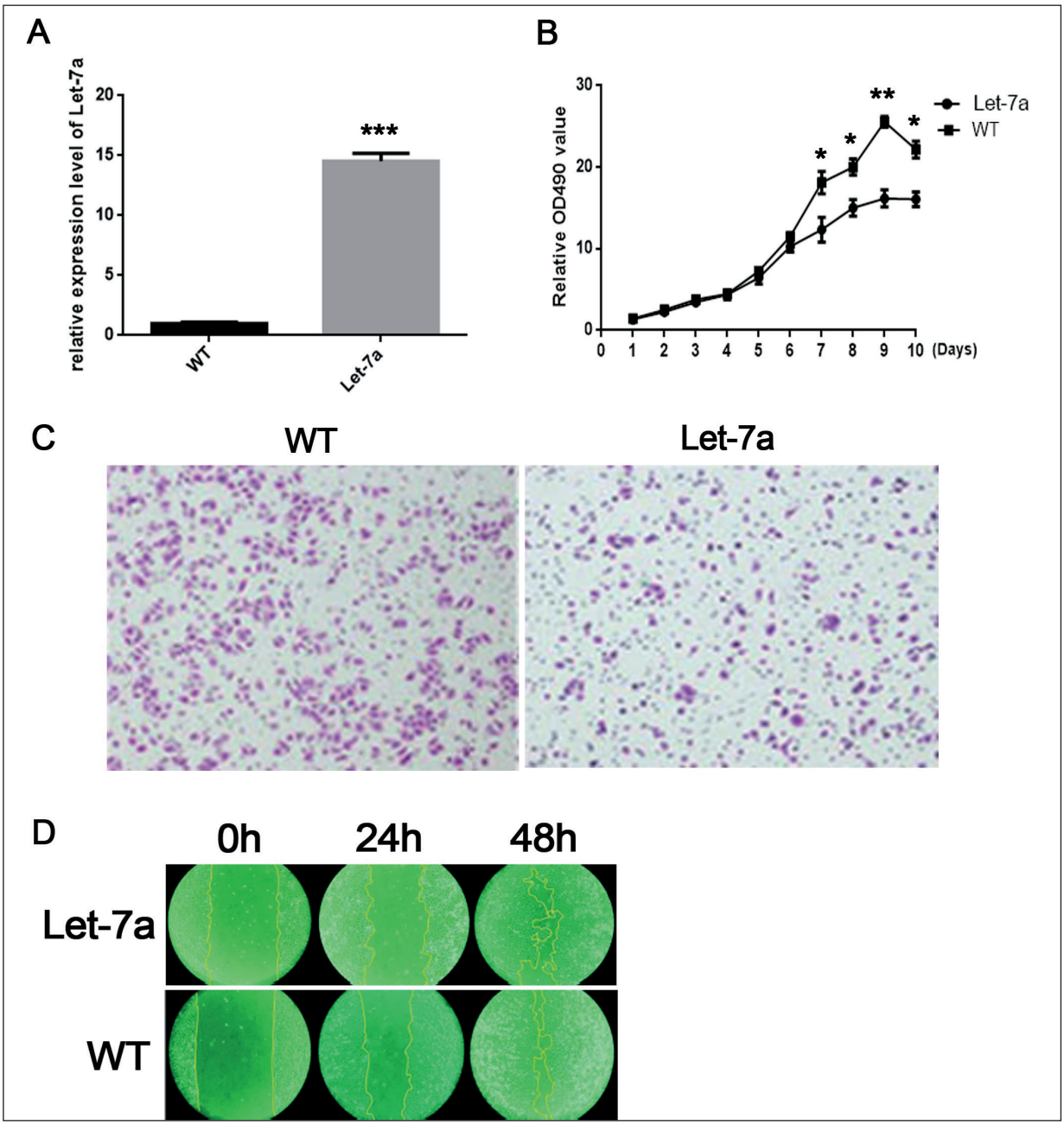

Figure 3. Effects of Let-7a on the biological function of triple negative breast cancer cells. **A,** The content of the Let-7a gene in overexpressing cells determined by qPCR. **B,** MTT assay results of Let-7a on the proliferation of triple negative breast cancer cells. **C,** Matrigel chamber assay to test the effect of Let-7a on the invasion ability of triple negative breast cancer cells. **D,** Scratch assay to evaluate the effect of Let-7a on the migration ability of triple negative breast cancer cells (n=3, bar value indicates standard deviation, *p<0.05, **p<0.01, ***p<0.001).

wrapped in exosomes. To understand whether exosomes would affect protein expression of normal cells, we co-incubated the two types of extracted exosomes simultaneously with wild type MDA-MB-231 cells and measured the expression of c-Myc by Western blot analysis (Figure 5 D). We found that Exo-Let-7a significantly inhibited the expression of c-Myc in wild type MDA-MB-231 cells. At the same time, to investigate the effect of Let-7a transported by exosomes on the regulation of biological processes of MDA-MB-231 cells, such as proliferation, migration,

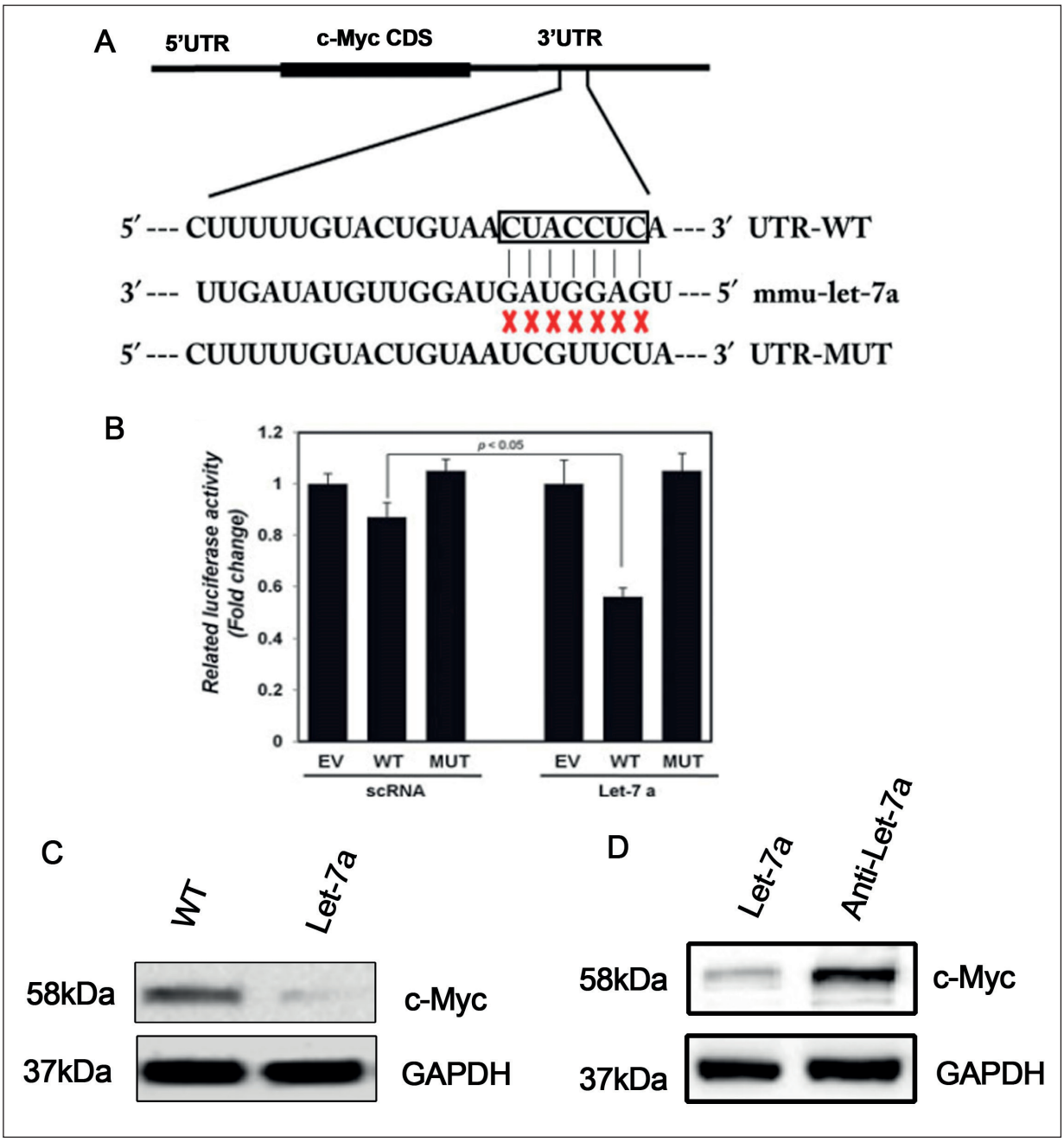

Figure 4. Interaction between Let-7a and c-Myc. **A,** Mutated sequence of the 3' UTR region of c-Myc gene. **B,** HEK293T cells co-transfected with Let-7a mimics or control mimics (scRNA) and pmirGLO vector containing c-Myc 3'UTR-WT or -MUT or empty vector (EV). Luciferase activity was measured at 48 h after transfection. **C,** The expression of c-Myc in Let-7a overexpressing MDA-MB-231 cells measured by Western blot analysis. **D,** Expression of c-Myc in Let-7a overexpressing MDA-MB-231 cells after Let-7a antibody treatment.

and invasion, extracted exosomes were incubated with MDA-MB-231 cells and the growth curve of MDA-MB-231 cells was created by MTT assay (Figure 5 E). The results showed that Exo-Let-7a significantly inhibited the proliferation of MDA-MB-231 cells. Furthermore, through the transwell invasion assay, it was demonstrated that exosomes extracted from Let-7a overexpressing cells significantly inhibited the invasion ability of MDA-MB-231 cells (Figure 5 F). In addition, the effects of exosomes on the migration ability of MDA-MB-231 cells were evaluated by scratch

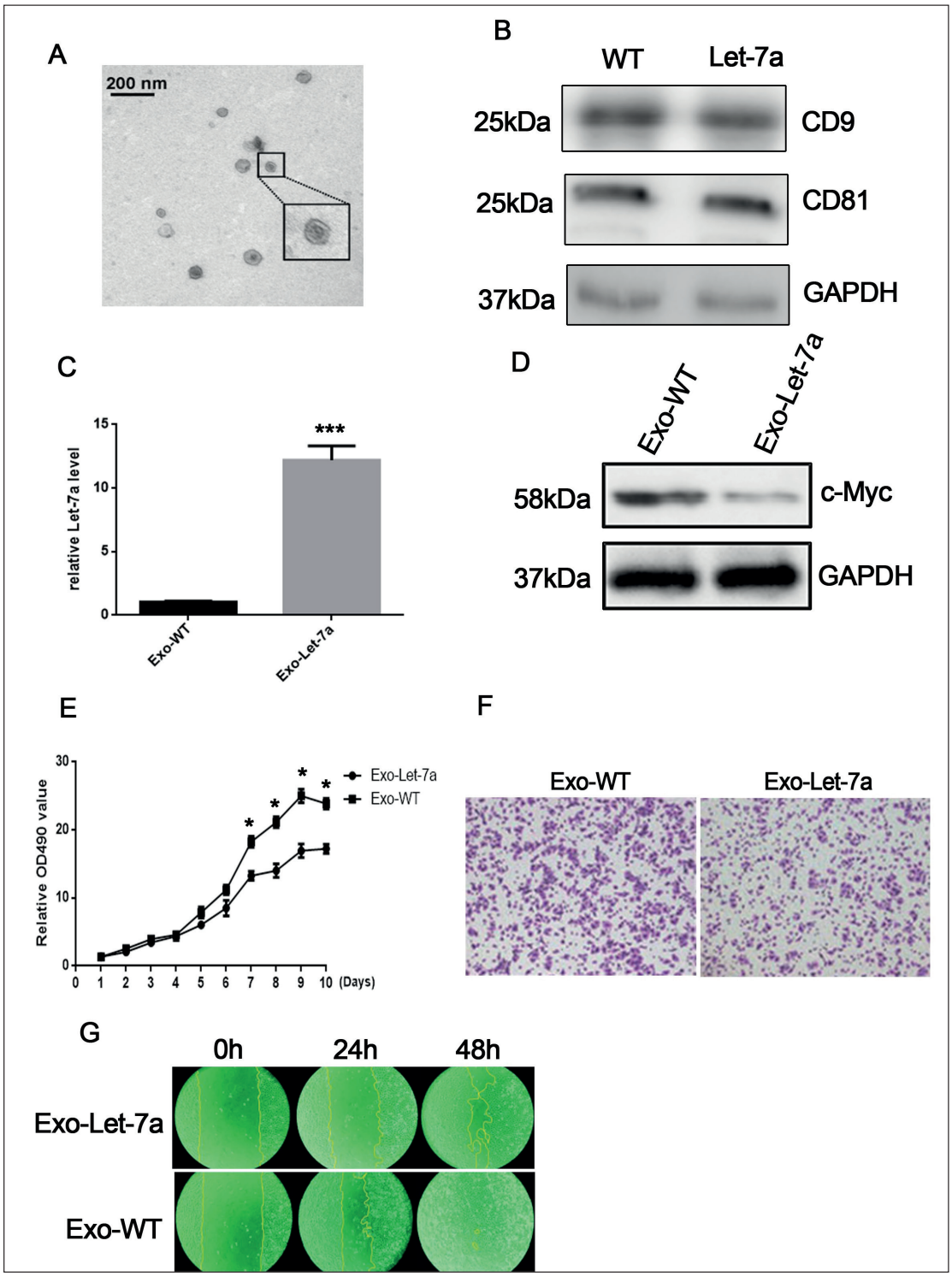

Figure 5. Effects of exosome-mediated Let-7a on the biological function of triple negative breast cancer cells. **A,** Electron micrographs of the isolated exosomes. Scale bar, 200 nm. **B,** Western blot analysis was used to determine the expression of exosome markers CD9 and CD81. **C,** qPCR was used to measure the quantity of Let-7a in exosomes. **D,** Western blot analysis was used to measure the content of c-Myc in wild-type MDA-MB-231 cells, which were co-incubated with exosomes. **E,** Matrigel chamber assay results to present the effect of exosomes on the invasion ability of triple negative breast cancer cells. **F,** MTT assay evaluating the effect of exosomes on the proliferation ability of triple negative breast cancer cells. **G,** Scratch assay to examine the effect of exosomes on the migration ability of triple negative breast cancer cells (n=3, bar value indicates standard deviation, *p<0.05, ***p<0.001).

assay. The results suggested that exosomes extracted from let-7 overexpressing cells significantly inhibited the migration of MDA-MB-231 cells (Figure 5 G). In summary, we found that Let-7a could be transported into tumor cells by exosomes, and inhibited the proliferation, migration, and invasion of MDA-MB-231 cells.

Exosome-Mediated Let-7a Inhibited Tumor Proliferation and Invasion In Vivo

To study the potential effects of exosome-mediated Let-7a on tumor growth, a mouse subcutaneous tumor model was employed. Mice were randomly divided into 3 groups. After subcutaneous injection of MDA-MB-231 cells, exosomes were administrated through tail vein injection every 3 days with 6 injections in total. Tumor growth in mice was monitored (Figure 6 A). *In vivo* imaging was performed on days 7 and 42 after tumor formation (Figure 6 B). The data showed that the tumor growth rate of mice in the Exo-Let-7a infusion group was significantly lower when compared to that of the Exo-WT infusion group. At the end of the experiment, Hematoxylin and eosin staining of mouse tumor tissue showed that tumors treated with Exo-Let-7a had distinct edges, and the migration degree of the tumor cells into the surrounding tissue was significantly decreased, indicating that the tumor invasion ability was significantly weakened (Figure 6 C). In conclusion, *in vivo* experiments using infusion exosomes in mice showed that exosome-mediated Let-7a significantly inhibited the proliferation and invasion of MDA-MB-231 cells *in vivo*. Thus, our findings provide novel ideas for targeted therapy for triple negative breast cancer.

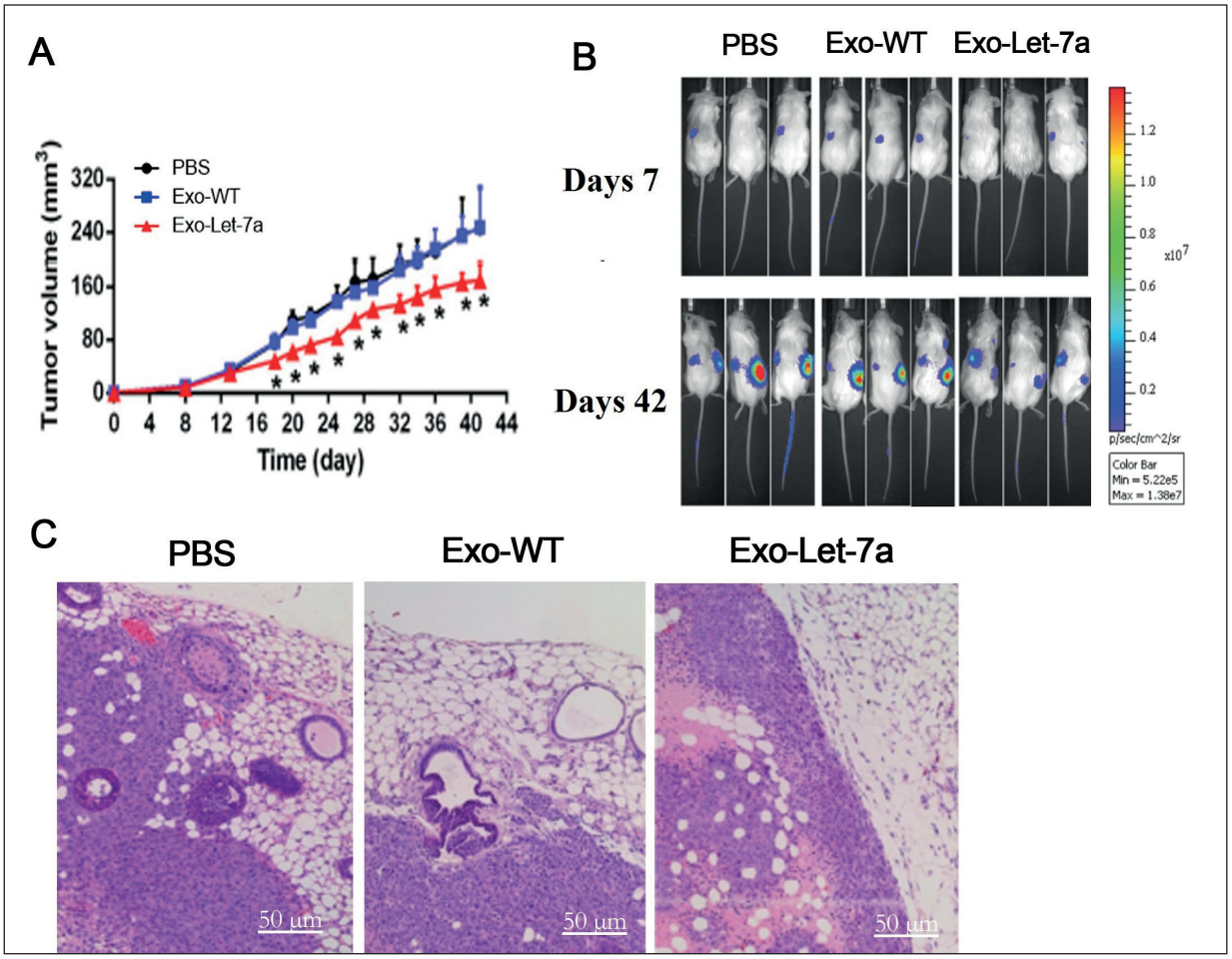

Figure 6. The inhibition of Let-7a mediated by exosomes *in vivo* on proliferation, metastasis, and invasion of MDA-MB-231 cells. **A,** Changes in tumor size in 3 groups of mice. **B,** *In vivo* imaging of mice on days 7 and 42 after tumor formation. **C,** Hematoxylin and eosin staining at the border of the tumor characterized local invasiveness (n=3, bar value indicates standard deviation, **p*<0.05).

Discussion

In many cell-signaling pathways, c-Myc is an important target molecule that is critical for the development of breast cancer. Abnormal expression of c-Myc may activate oncogenes and promote the development of tumors. Recent studies have shown that c-Myc was highly expressed in triple negative breast cancer when compared with other breast cancer subtypes[16,17]. Moreover, it has been reported that due to its functional location at the end of the signal cascade, c-Myc could activate multiple signaling pathways and may play a key role in the development of cancer[18]. In our study, the high expression level of c-Myc in both triple negative breast cancer tissues and cells was proven. In addition, c-Myc knockout MDA-MB-231 cells were constructed to reveal that c-Myc significantly promoted proliferation, migration, and invasion of MDA-MB-231 cells.

miRNAs are a class of naturally existing, non-coding, and single-stranded small RNAs of 18-26 nucleotides in length. miRNA could lead to the degradation of target mRNA or the inhibition of post-transcriptional translation, and therefore regulate the expression of target genes by binding to the 3'-UTR region of target mRNA in a fully or partially complementary manner[19]. MiRNA's can regulate multiple target genes and play an important regulatory role in physiological activities, including growth and development, cell proliferation, cell apoptosis, and differentiation. So far, miRNA's have mainly shown two applications in breast cancer treatments: the targeted treatment with miRNA as the target itself; a personalized therapeutic plan according to the molecular classifications of breast cancer. Let-7a is one of the most applied miRNAs and regulates the cell cycle and cell differentiation by regulating the expression of oncogenes and cell cycle-related genes[20]. Liu et al[21] demonstrated that the expression of Let-7a was low in breast cancer tissue when compared to normal breast tissue. In addition, studies also reported that the expression of Let-7a was significantly lower in breast cancer tissue when measuring by gene chips[22]. However, only few studies have focused on the relationship between Let-7a and triple negative breast cancer. We first employed bioinformatics to determine that low expression of Let-7a was associated with poor prognosis of triple negative breast cancer patients. Next, the low expression levels of Let-7a in both triple negative breast cancer tissue and cell samples were measured and proven. By constructing a Let-7a overexpressing MDA-MB-231 cell line, it was found that the low expression of Let-7a significantly promoted the proliferation, migration, and invasion of MDA-MB-231 cells.

c-Myc, as an important tumor-associated gene, is regulated post-transcriptionally by miRNAs with tumor suppression activity[23]. Mackedenski et al[24] reported that mature Let-7a could degrade target mRNA or inhibit its translation process via binding to the 3' untranslated region (3'UTR) of target mRNA in a complete or incomplete form. In this work, the regulatory interaction between Let-7a and c-Myc was analyzed by a fluorescent reporter assay, which indicated that Let-7a performed its function by binding to the 3'UTR region of the c-Myc gene.

Exosomes can effectively protect miRNAs, stabilize, and transport miRNAs into recipient cells to regulate cellular signaling pathways by inhibiting the expression of certain genes[25]. In previous investigations, it has been shown that non-coding RNAs in exosomes secreted by tumor cells mainly included inactivated anti-oncogene and activated tumor suppressor genes, which could regulate tumorigenesis after the entrance into recipient cells. Ohno et al[26] used genetic engineering to insert the epidermal growth factor receptor (EGFR)-binding fragment GE11 into the membrane of exosomes and using the modified exosomes to specifically transfer Let-7a into EGFR-expressing breast cancer cells. Let-7a inhibited tumor cell proliferation by inhibiting the expression of HMGA2 or RAS families. Therefore, we investigated whether the regulation of Let-7a and c-Myc on triple negative breast cancer was mediated by exosomes. Through *in vitro* and *in vivo* studies, it was found that Let-7a inhibited the proliferation, migration, and invasion of MDA-MB-231 cells by mediating exosomes.

Conclusions

We demonstrated that Let-7a, which is mediated by exosomes, inhibited proliferation, migration, and invasion of MDA-MB-231 cells by binding on the 3'UTR region of the c-Myc gene and silencing of the c-Myc expression. However, the reason for the low expression of the Let-7a gene in MDA-MB-231 cells is not known. In previous studies, it was shown that c-Myc could regulate the expression of miRNA through transcriptional activation or suppression, and further promoted the development of tumors through target miR-

NAs[27]. miRNAs that are inhibited by c-Myc transcriptional regulation are all direct targets of c-Myc-related miRNAs, whose expression in tumor cells is usually suppressed[28]. Therefore, in the future, the inhibitory effect of c-Myc against c-Myc in triple negative breast cancer will be further investigated. In conclusion, to some extent, we revealed the role of c-Myc, Let-7a, and exosomes in the development of triple negative breast cancer, thereby providing novel insights into treatments.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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