ORIGINAL ARTICLE

MicroRNA-132-3p inhibits tumor malignant progression by regulating lysosomal-associated protein transmembrane 4 beta in breast cancer

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Abstract

Lysosomal-associated protein transmembrane 4 beta (LAPTM4B), a proto-oncogene, has been shown to be a positive modulator in cancer progression. However, the mechanism of LAPTM4B regulation is not fully elucidated. Aberrant microRNAs (miRNAs) can regulate gene expression by interfering with target transcripts and/or translation to exert tumor-suppressive or oncogenic effects in breast cancer. In the present study, miR-132-3p, which was predicted by relevant software, was confirmed to directly bind to the 3′ untranslated region (3′UTR) of LAPTM4B and negatively regulate its expression in luciferase reporter and western blot assays. Subsequently, we validated that miR-132-3p was downregulated in breast cancer tissues. Receiver-operating characteristic curve analysis indicated that miR-132-3p had accurate diagnostic value, and a Kaplan-Meier and Cox regression model showed that miR-132-3p was a potential prognostic marker for recurrence, showing low levels in breast cancer patients. In addition, we showed that miR-132-3p was inversely correlated with LAPTM4B expression in the above samples. Functionally, miR-132-3p suppressed the migration and invasion of breast carcinoma cells through LAPTM4B by mediating epithelial-mesenchymal transition signals, and partially reversed the carcinogenic effects of LAPTM4B by inhibiting the PI3K-AKT-mTOR signaling pathway. Taken together, these findings provide the first comprehensive analysis of miR-132-3p as a direct LAPTM4B-targeted miRNA, and shed light on miR-132-3p/LAPTM4B as a significant functional axis involved in the oncogenesis and metastasis of breast cancer.

KEYWORDS
breast cancer, LAPTM4B, metastasis, miR-132-3p, regulation

1 | INTRODUCTION

Breast cancer is one of the most common malignancies in women, and its incidence has risen annually worldwide.[1] Early detection and timely treatment usually leads to a good prognosis; however, recurrence or metastasis is the leading cause of death from breast cancer.[2] Thus, understanding the molecular mechanisms involved in breast oncogenesis and the acquisition of malignancy is essential.

Lysosomal-associated protein transmembrane 4 beta (LAPTM4B) overexpression, which occurs in a variety of human cancers, can promote tumor proliferation, invasion, metastasis, resistance to apoptosis, autophagy and multidrug resistance through the activation of

the PI3K-AKT and Ras-MAPK signaling pathways.[3-6] Additionally, the protein encoded by the LAPTM4B gene, namely, LAPTM4B-35, is a poor prognostic factor for many solid tumor types.[7-12] In previous studies, we identified transcription factors that acted on the promoter region and showed that SP1,[13] CREB1[14] and AP4[15,16] increased LAPTM4B gene transcription; however, the role of LAPTM4B in the function of the protein and polymorphic region and the underlying regulatory mechanism remain to be explored.

MicroRNAs (miRNAs) can inhibit the transcription or translation process of target genes to regulate related genes by specifically binding to the 3′UTR of target mRNAs. Aberrant miRNAs can function as either oncogenes or tumor suppressors in various types of carcinomas.[17] Overexpression of miR-196b upregulates the expression levels of VIM and MMP-2 and downregulates the expression of CDH1, thereby promoting the metastasis of non-small cell lung cancer by inducing epithelial-mesenchymal transition (EMT) signaling.[18] In addition, miR-19a, miR-24, miR-155, and miR-181b can be used to assess the degree of cancer risk in breast cancer patients.[19] Elucidation of the molecular mechanism underlying LAPTM4B regulation by tumor-suppressive miRNAs will provide new insights into breast carcinogenesis and metastasis.

In the present study, we determined that miR-132-3p directly bound to the 3′UTR of the LAPTM4B gene and functioned at the post-transcriptional level. Moreover, miR-132-3p expression and the prognostic value in breast cancer were demonstrated, and a correlation analysis was conducted between the expression levels of miR-132-3p and LAPTM4B in breast cancer tissues. Importantly, miR-132-3p partially had an impact on LAPTM4B by the PI3K-AKT-mTOR signaling pathway. These findings suggest that a complex regulatory network, namely, miR-132-3p/LAPTM4B/AKT/mTOR, exists in breast cancer and that this network may comprise clinical tools for targeting tumorigenesis and progression.

2 | MATERIALS AND METHODS

2.1 | Cells and cell culture

Human breast cells were cultured in appropriate media supplemented with essential materials. ZR-75-1, MCF7 and MCF-10A cells were purchased from Cellcook Biotech Co. (Guangzhou, China). MDA-MB-231 and T47D cells were kindly provided by Dr SHOU Cheng-chao (Department of Biochemistry and Molecular Biology, Peking University Cancer Hospital & Institute).

2.2 | Patients and clinical samples

From January 2008 to January 2013, 131 tumor samples and their corresponding 78 non-tumor tissues were obtained from breast carcinoma patients at Beijing Cancer Hospital (Beijing, China). All specimens were histopathologically confirmed. We excluded patients if they had received any previous chemoradiotherapy or immunotherapy treatment, and follow up was carried out until December 2016. Consent from each patient and approval by the ethics committees of Beijing Cancer Hospital were obtained.

2.3 | Oligonucleotide and plasmid

The siRNA sequences targeting LAPTM4B (Table S1), miR-132-3p mimics, miR-139-5p mimics, miR-582-5p mimics, miR-625-5p mimics, miR-132-3p and miR-139-5p inhibitors, and the corresponding negative controls used in this study were synthesized by RiboBio. We blocked endogenous miRNA activity in cells by transfection of individual miRNA inhibitors. To enhance miRNA activity, cells were transfected with miRNA mimics. Negative-control mimics and inhibitors were used. Expression vector pcDNA3.1 carrying the full-length LAPTM4B gene-coding sequence was purchased from GeneChem.

2.4 | Luciferase reporter assay

To construct a pmiR-LAPTM4B-3′UTR plasmid, a 1539-bp sequence was amplified and inserted into the XhoI and NotI sites of the pmiR-RB-REPORT Dual-Luciferase vector (RiboBio). Mutant-type genes were inserted downstream of the luciferase gene in the pmiR-RB-REPORT Dual-Luciferase vector. Cells were cotransfected with oligonucleotide and the luciferase reporter construct using the Lipofectamine 3000 reagent. After transfection for 48 hours, luciferase activity was measured using a dual-luciferase reporter assay system (Promega).

2.5 | RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissues and cells using the miRNAprep Pure FFPE Kit (cat. no. DP502; TIANGEN Biotech) and Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription and quantitative PCR measurements for miR-132-3p and internal control U6 from tissue samples were carried out with the miRcute Plus miRNA First-Strand cDNA Synthesis Kit (cat. no. KR211; TIANGEN Biotech) and miRcute Plus miRNA qPCR Detection Kit (cat. no. FP411; TIANGEN Biotech). Quantitative real-time PCR (qRT-PCR) assays for miR-132-3p and miR-139-5p from cells were carried out with the Bulge-Loop miRNA qRT-PCR Starter Kit (cat. no. R11067.2; RiboBio). cDNA for LAPTM4B and the internal control GAPDH from tissue samples were synthesized using FastKing gDNA Dispelling RT SuperMix (cat. no. KR118; TIANGEN Biotech) according to the manufacturer's instructions, and transcripts were quantified by TaqMan quantitative real-time PCR (cat. no. FP206; TIANGEN Biotech). qRT-PCR was carried out to detect mRNA expression in cells using the FastQuant RT kit (cat. no. KR106; TIANGEN Biotech) and the FastFire qPCR PreMix SYBR Green kit (cat. no. FP207; TIANGEN Biotech). Primers and probes for the Bulge-Loop, polyA and TaqMan methods were designed (RiboBio and TIANGEN Biotech), and other primers used for qRT-PCR are listed in Table S1.

2.6 | Immunohistochemistry

Immunohistochemistry analysis of LAPTM4B (dilution 1:200, bs-6542R; Bioss Inc.) was carried out in breast cancer tissues as previously described.[15]

2.7 | Antibodies and western blot

Antibodies are shown in Table S2, and western blot assay was carried out according to a previous description.[15]

2.8 | Cell proliferation analysis

Cell Counting Kit-8 (CCK-8; Dojindo) was used to evaluate cell viability. Experiments were carried out three times.

2.9 | Colony formation assay

Cells were seeded in small culture plates or six-well plates (500 cells/well). After incubation for 2 weeks at 37°C, cells were fixed in 4% paraformaldehyde and stained with crystal violet. Finally, positive colony formation (>50 cells/colony) was evaluated by counting the number of colonies. All experiments were carried out in triplicate wells.

2.10 | Cell migration and invasion assay

Cells were seeded to the upper portion of a 24-well chamber and allowed to traverse across a 0.8-µmol/L polycarbonate insert (Costar) or Matrigel (BD) toward a chemoattractant (10% FBS) for 24 hours or 48 hours. Cells traversing the insert or Matrigel were fixed, stained and counted under a microscope.

2.11 | Statistical analysis

SPSS 18.0 (IBM) was used for all statistical analyses, and a *P*-value <.05 was considered to be statistically significant. Spearman test was used to evaluate the relationship between LAPTM4B and miR-132-3p expression. The multivariate Cox regression model was used to analyze predictive variety, and survival analysis was done using the Kaplan-Meier method. Differences in cell growth curves were confirmed with repeated-measures ANOVA. Two-tailed Student's *t* test was used for comparisons of two independent groups.

3 | RESULTS

3.1 | Prediction and identification of miRNAs targeting LAPTM4B

To predict upstream miRNAs targeting LAPTM4B, we screened out the intersection of miRNAs predicted by prediction software packages such as TargetScan, miRanda, miRGator and TarBase 7.0 (Figure 1A). As a result, hsa-miR-132-3p was obtained from the common prediction of four databases, and seven miRNAs were predicted from three databases. According to available studies, miR-188-5p suppresses LAPTM4B expression by binding to its 3′UTR area, acting as a tumor suppressor in prostate cancer.[20] Moreover,

the expression levels of miR-27a-3p, miR-196a-5p and miR-501-5p can be increased in tumors.[21-23] Considering the above observations, hsa-miR-132-3p, has-miR-139-5p, has-miR-582-5p and has-miR-625-5p were selected for screening and identification.

The dual-luciferase reporter assay is a classical approach used to verify the target genes regulated by miRNAs. The miRNA sequences that bind to the wild-type region of the 3′UTR (3′UTRwt) are shown in Figure 1B. Cotransfection of 3′UTRwt and miR-Ctrl in HeLa cells, a cervical cancer cell line, showed a high transfection efficiency and was selected as the control group. Figure 1C shows model charts of the site mutations of LAPTM4B 3′UTR (3′UTRmut) that bound to miR-132-3p and miR-139-5p. Cells that were cotransfected with 3′UTRwt + miR-132-3p or 3′UTRwt + miR-139-5p showed the lowest luciferase activity levels (Figure 1D-F). These results show that miR-132-3p and miR-139-5p may be potential miRNAs targeting LAPTM4B.

3.2 | miR-132-3p negatively regulates LAPTM4B expression

To elucidate the influence of miR-132-3p and miR-139-5p on LAPTM4B regulation, LAPTM4B expression was evaluated at the mRNA and protein levels in the setting of the upregulation and downregulation of miR-132-3p or miR-139-5p. We first observed that the expression levels of miR-132-3p (Figure 2A) and miR-139-5p (Figure S1A) in breast cancer cells were lower than those in immortalized breast epithelial MCF-10A cells. MDA-MB-231 and ZR-75-1 showed lower expression, whereas MCF7 and T47D showed higher expression. Then, with the overexpression or inhibition of miR-132-3p and miR-139-5p in MDA-MB-231 or MCF7, the expression of LAPTM4B did not significantly change, as shown in Figure S1B-I. These results indicated that miR-132-3p and miR-139-5p may only inhibit the translation process without the degradation of mRNA. Accordingly, western blot analysis showed that the overexpression or inhibition of miR-132-3p could decrease or increase LAPTM4B expression, respectively, in breast cancer cells, whereas the corresponding analysis for miR-139-5p showed that LAPTM4B expression in cells showed no significant change (Figure 2B).

These above results indicated that miR-132-3p was the target miRNA for the regulation of LAPTM4B in breast cancer. Subsequently, western blot analysis was conducted to verify the concentration of miR-132-3p mimics or inhibitors in cells (Figure 2C). The optimal concentration for the upregulation and downregulation of miR-132-3p was selected to determine whether miR-132-3p could negatively regulate LAPTM4B in breast cancer (Figure 2D). Figure 2E shows that the luciferase activity in MDA-MB-231 cotransfected with 3′UTRwt + miR-132-3p is significantly reduced, whereas that of MCF7 cells cotransfected with 3′UTRwt + miR-132-3p inhibitors is stronger than the luciferase activity of other cells (Figure 2F). These results confirmed that miR-132-3p was the target miRNA for the negative regulation of LAPTM4B in breast cancer.
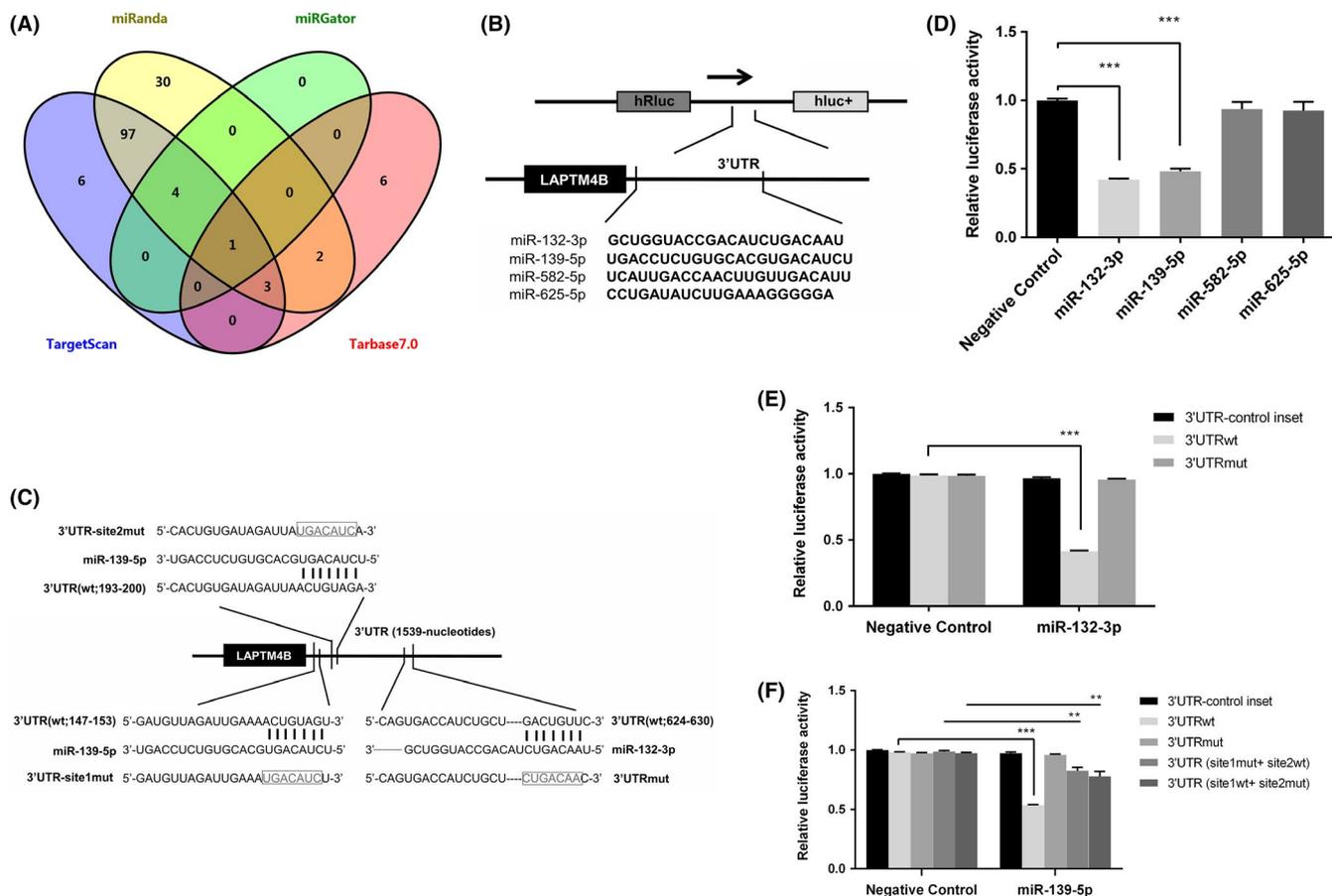
FIGURE 1 MicroRNA (miR)-132-3p and miR-139-5p are potential miRNAs targeting lysosomal-associated protein transmembrane 4 beta (LAPTM4B). A, miRNAs targeting LAPTM4B by bioinformatics prediction. B, Schematic diagram of the LAPTM4B 3′UTR and sequence representation of four miRNAs. C, Luciferase activity in cotransfected cells. D, Sequences containing miR-132-3p and miR-139-5p binding sites and their corresponding mutated forms of LAPTM4B 3′UTR are shown. E-F, miR-132-3p and miR-139-5p could bind to the wild-type region of LAPTM4B 3′UTR. All **P < .01, ***P < .001

3.3 | Expression of miR-132-3p in breast cancer and correlation with prognosis

To determine the role of miR-132-3p, we first examined miR-132-3p expression at the mRNA level in breast cancer. As shown in Figure 3A-C, the relative expression of miR-132-3p in breast cancer tissues (0.275, 0.073-0.894, expressed in terms of the median and quartile) was significantly lower than that in matched adjacent normal tissues (1.506, 0.422-4.139). These results showed that miR-132-3p expression was downregulated in breast cancer.

We investigated the relative expression of miR-132-3p in 131 breast cancer tissues and 78 adjacent normal tissues, and the receiver operating characteristic (ROC) curve of miR-132-3p showed an area under the curve (AUC) value of 0.748 (Figure 3D). Additionally, the point of the highest sensitivity and specificity was set as the cut-off value (0.313) to distinguish miR-132-3p high- and low-expression groups and, as a consequence, there were 54 cases in the high-expression group and 77 cases in the low-expression group.

Furthermore, we conducted correlation analyses between the expression of miR-132-3p and clinicopathological features and prognosis. As a result, miR-132-3p expression was significantly associated with TNM stage, lymph node metastasis (LNM), vascular tumor thrombus and recurrence (Table S3). miR-132-3p expression in patients with stage II/III was lower than that in patients with stage I, and patients with LNM, vascular tumor thrombus and recurrence showed significantly lower miR-132-3p expression (Figure 3E,F). Meanwhile, survival analysis showed that patients with low miR-132-3p expression had a poor outcome in terms of disease-free survival (DFS), which could be identified as an independent predictor for breast cancer recurrence (Table S4 and Figure 3G).

3.4 | Correlation between LAPTM4B and miR-132-3p expression in breast cancer

We tested LAPTM4B and miR-132-3p mRNA levels in breast cell lines by qPCR. Compared to MCF-10A, MDA-MB-231 showed the lowest miR-132-3p expression level, and T47D showed the highest levels (Figure 2A); however, LAPTM4B expression was highest in MDA-MB-231 and lowest in T47D (Figure 4A), and the LAPTM4B protein level corresponded to its expression at the mRNA level in these breast cancer cells.[24] In breast cancer tissues and matched adjacent normal tissues, LAPTM4B mRNA level was detected by
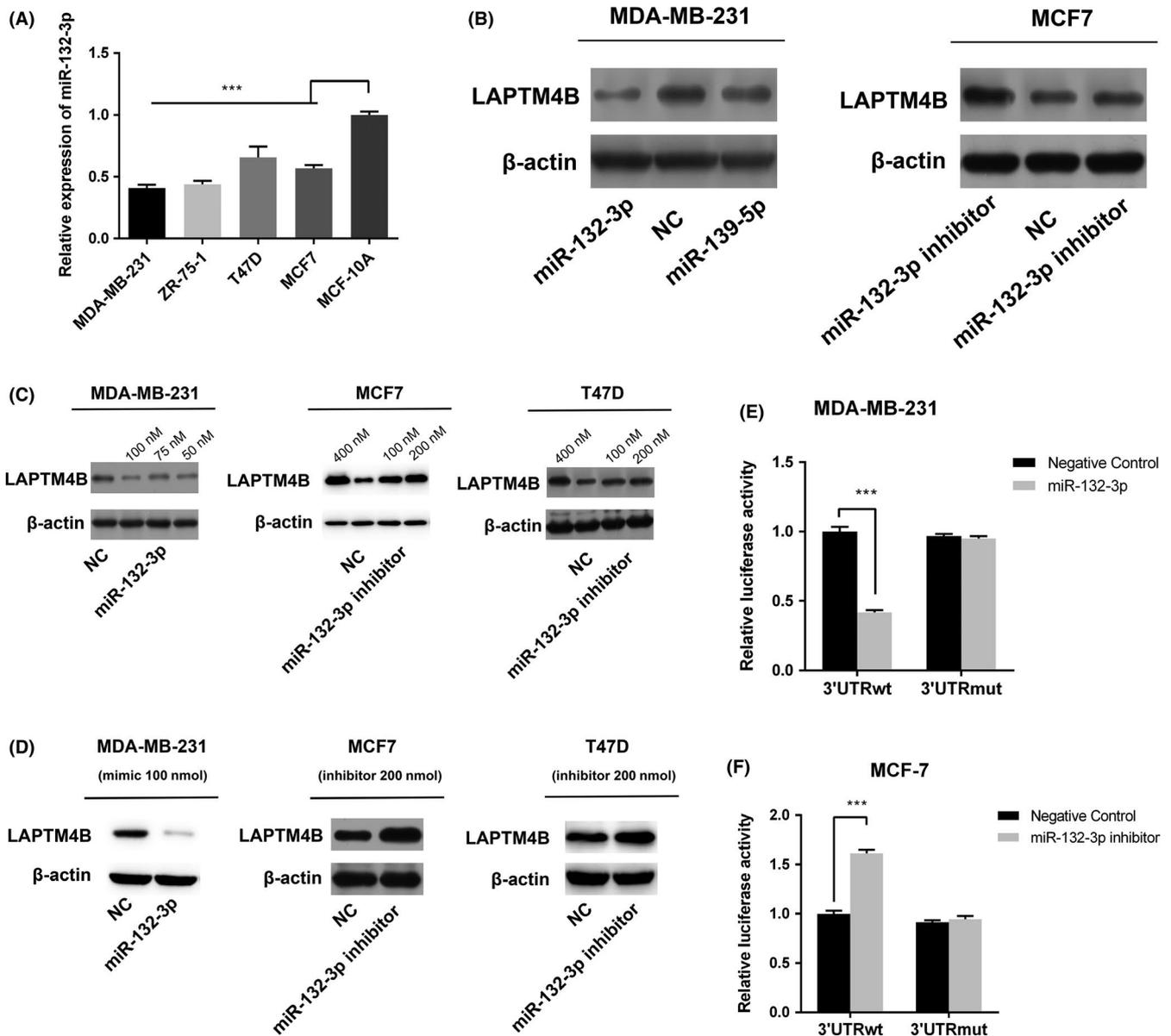
FIGURE 2 MicroRNA (miR)-132-3p is the target miRNA for the negative regulation of lysosomal-associated protein transmembrane 4 beta (LAPTM4B) in breast cancer. A, Expression of miR-132-3p in cells was detected by quantitative PCR. B, LAPTM4B protein level was reduced in MDA-MB-231 cells transfected with miR-132-3p mimics, but the inverse occurred in MCF7 cells transfected with miR-132-3p inhibitors. C-D, miR-132-3p mimics at 100 nM could reduce LAPTM4B expression in MDA-MB-231, whereas miR-132-3p inhibitors at 200 nM could increase the expression of LAPTM4B in MCF7 and T47D. E-F, Relative luciferase activities were measured when MDA-MB-231 or MCF7 cells were cotransfected with 3'UTRwt or 3'UTRmut and miR-132-3p mimics or inhibitors. All ***$P < .001$

TaqMan quantitative real-time PCR. We found that LAPTM4B expression was significantly elevated in breast cancer specimens (8.790, 3.135-14.267, expressed as the median and quartile) (Figure S1J).

Furthermore, The Cancer Genome Atlas (TCGA) database not only showed that LAPTM4B in the mRNA level was upregulated in breast cancer (Figure 4B), but also suggested a negative correlation between LAPTM4B and miR-132-3p mRNA levels in 1081 breast cancer patients (Figure 4C). Our data showed that miR-132-3p expression was significantly higher in breast cancer tissues with low LAPTM4B expression than that in tissues with high LAPTM4B

expression, indicating a negative correlation between LAPTM4B and miR-132-3p in breast cancer (Figure 4D,E).

3.5 | miR-132-3p inhibits the proliferation, invasion and metastasis of breast cancer cells

To investigate the effects of miR-132-3p on breast cancer, cells were transfected with mimics or inhibitors targeting miR-132-3p. miR-132-3p expression in MDA-MB-231 was 4753-fold higher in the mimic group than in the control group (Figure S1B,C). Moreover, the miR-132-3p expression levels in MCF7 and T47D decreased by
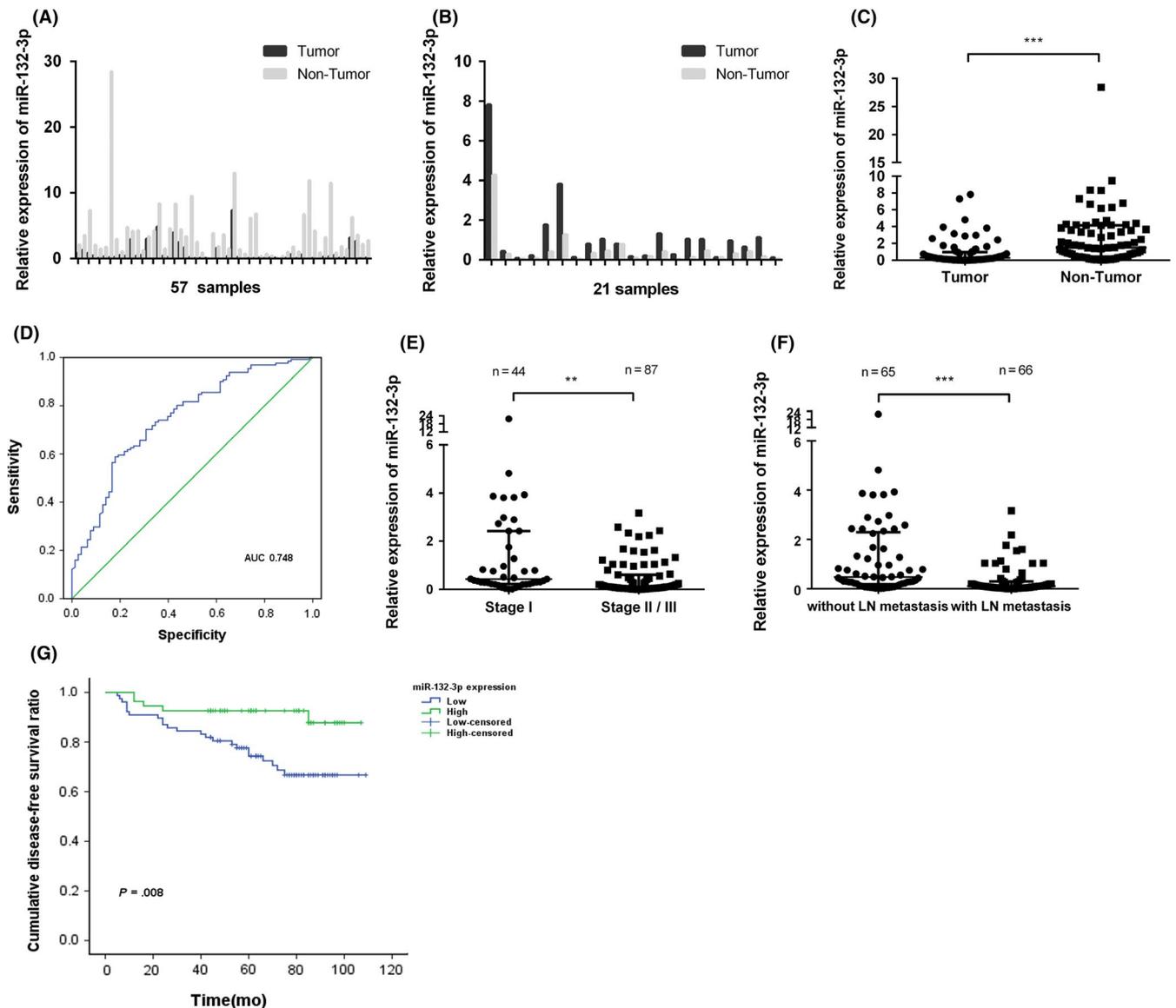
FIGURE 3 MicroRNA (miR)-132-3p is downregulated in breast cancer tissues and is a marker of poor prognosis. A, Relative to miR-132-3p expression in normal adjacent tissue, miR-132-3p expression was low in the cancer tissues of 57 patients with breast cancer. B, Relative to miR-132-3p expression in normal adjacent tissue, miR-132-3p expression was high in the cancer tissues of 21 patients with breast cancer. C, Average relative expression of miR-132-3p in 78 cases of breast cancer tissues and paired normal, adjacent tissues. D, Receiver operating characteristic curve of miR-132-3p expression. E-F, Breast cancer patients with stage II/III or lymph node metastasis had lower miR-132-3p expression than patients with stage I or without lymph node metastasis. G, Patients with low miR-132-3p expression had a higher risk of recurrence than patients with high miR-132-3p expression. All **$P < .01$, ***$P < .001$

98.60% and 98.80%, respectively, in the inhibitor groups compared to the control groups (Figure S1D,E,K,L). In addition, miR-132-3p negatively affected LAPTM4B expression at the protein level (Figure 2D), thereby proving the success of the transfection.

We investigated the effect of miR-132-3p on breast cancer cell growth using cell viability (Figure 5A-C), colony formation assays (Figure 5D-F), migration (Figure 5G-I) and invasion (Figure 5J-L). Results showed that overexpressing miR-132-3p in MDA-MB-231 significantly inhibited cell proliferation and metastasis and that the individual inhibitor treatment of miR-132-3p significantly enhanced the viability, clonality and metastasis of MCF7 and T47D cells compared to the control groups. Taken together, our functional in vitro

assays indicate that miR-132-3p significantly represses cell viability, colony formation, migration and invasion of breast cancer cells.

3.6 | Effect of LAPTM4B regulation by miR-132-3p on the biological behavior of breast cancer cells

We speculated that miR-132-3p was a key regulator of the LAPTM4B translation process, affecting LAPTM4B function in tumors. Thus, we designed rescue experiments to explore the effect of LAPTM4B regulation by miR-132-3p on cell proliferation, migration and invasion.

Initially, LAPTM4B overexpression was more remarkable in MDA-MB-231, when the concentration was 4 μg (Figure 6A). Three siRNAs
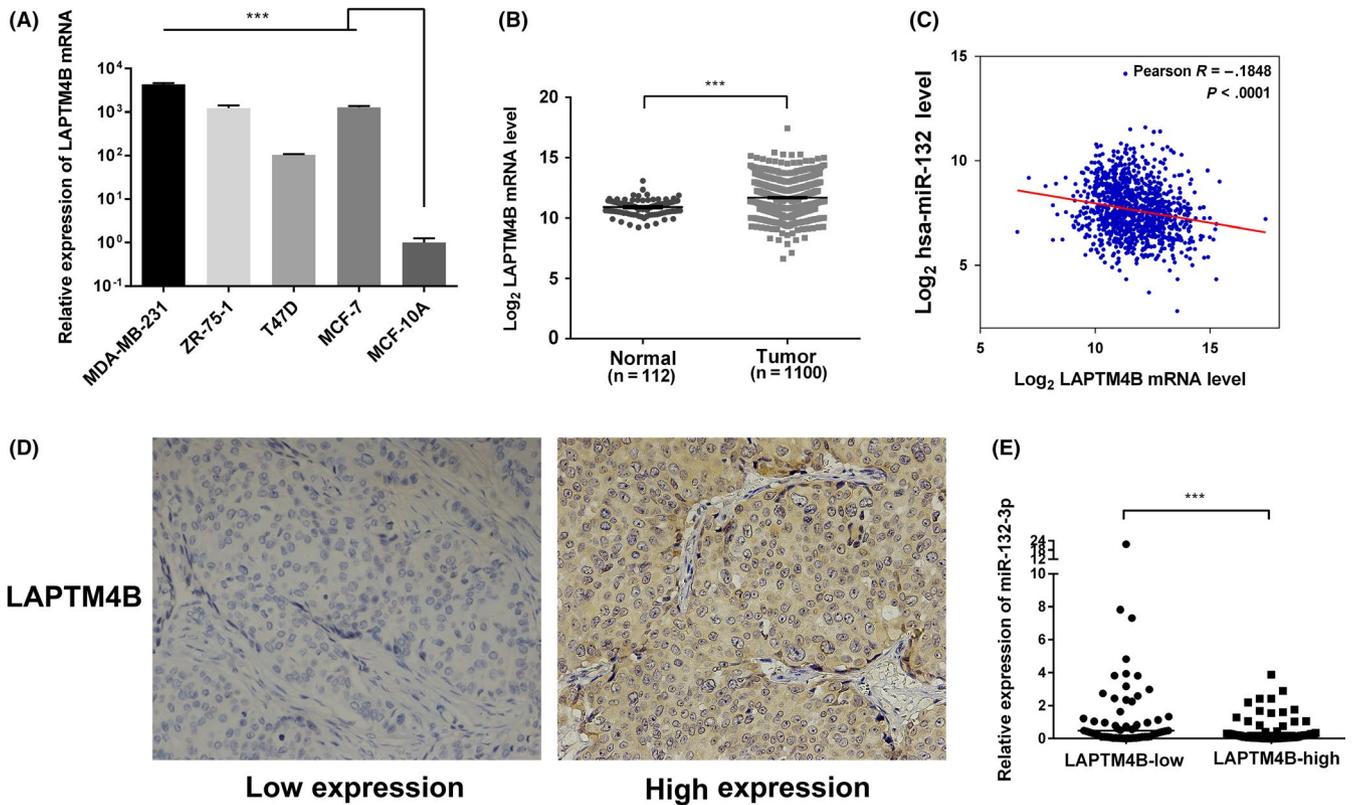
FIGURE 4 Correlation of microRNA (miR)-132-3p and lysosomal-associated protein transmembrane 4 beta (LAPTM4B) in breast cancer and with prognosis. A, Expression levels of LAPTM4B in breast cell lines were determined at the mRNA level. B, LAPTM4B expression in 1100 breast cancer tissues and 112 adjacent tissues was analyzed with The Cancer Genome Atlas (TCGA) database. C, Correlation analysis between miR-132-3p and LAPTM4B in breast cancer was analyzed with TCGA database. D, Representative results of LAPTM4B staining by immunohistochemistry (magnification ×200) are shown. E, miR-132-3p expression in patients with low LAPTM4B expression was higher than that in patients with high LAPTM4B expression. All ***$P < .001$

were designed to knock down LAPTM4B, and 1-siRNA and 3-siRNA dramatically reduced LAPTM4B mRNA and protein levels in MCF7 (Figure 6B). Meanwhile, the knockdown effect for 3-siRNA was more noticeable at 50 nmol/L and 100 nmol/L. In view of the high frequency of off-target effects at the high concentration, 50 nmol/L was more suitable for si-LAPTM4B and the control group (Figure 6C).

LAPTM4B expression was forced, and miR-132-3p was simultaneously overexpressed by transfection with mimics in MDA-MB-231; however, there were no differences between the expression levels in the LAPTM4B- and miR-132-3p-overexpressing cells versus the control cells (Figure 6D). Additionally, we knocked down LAPTM4B and blocked endogenous miR-132-3p activity in MCF7 (through the transfection of inhibitors), but the rescued group with the LAPTM4B knockdown and miR-132-3p inhibitors expressed LAPTM4B protein at levels that were no different from those of the control (Figure 6E). These results further confirmed that miR-132-3p could negatively regulate LAPTM4B expression.

To explore the effect of LAPTM4B on cell proliferation, migration and invasion modulated by miR-132-3p, functional experiments were designed. CCK-8, clone formation and Transwell assays showed that LAPTM4B overexpression in MDA-MB-231 noticeably facilitated cell viability, clonality and metastasis. Nevertheless, miR-132-3p mimics markedly arrested the cell viability (Figure 6F),

clonality (Figure 6G) and metastasis (Figure 6H,I) promotion observed with the forced expression of LAPTM4B. Moreover, LAPTM4B knockdown obviously inhibited cell viability (Figure 6F), clonality (Figure 6G) and metastasis (Figure 6H,I) in MCF7; however, the effect was rescued by the inhibitor treatment of miR-132-3p. Thus, these results suggest that miR-132-3p may partially reverse the stimulating effect of LAPTM4B on cell proliferation.

We investigated key regulators involved in EMT activation and found that LAPTM4B overexpression in MDA-MB-231 upregulated Vimentin expression (a mesenchymal cell marker) but downregulated E-cadherin expression(an epithelial cell marker); however, miR-132-3p overexpression reversed the expression of these regulators. Reciprocally, the changes in Vimentin and E-cadherin induced by LAPTM4B knockdown were significantly restored by miR-132-3p inhibitors. Therefore, LAPTM4B could regulate the EMT process and promote the migration and invasion of breast cancer cells, which was partly reversed by upregulating miR-132-3p (Figure 6J).

Importantly, to explore the underlying mechanism of how miR-132-3p/LAPTM4B modulated biological behaviors in breast cancer, we evaluated the levels of mediators of the PI3K/AKT signaling pathway. As shown in Figure 6K, LAPTM4B overexpression increased the levels of p-AKT and p-mTOR, but miR-132-3p mimics suppressed the phosphorylation levels of the mediators of the AKT pathway in
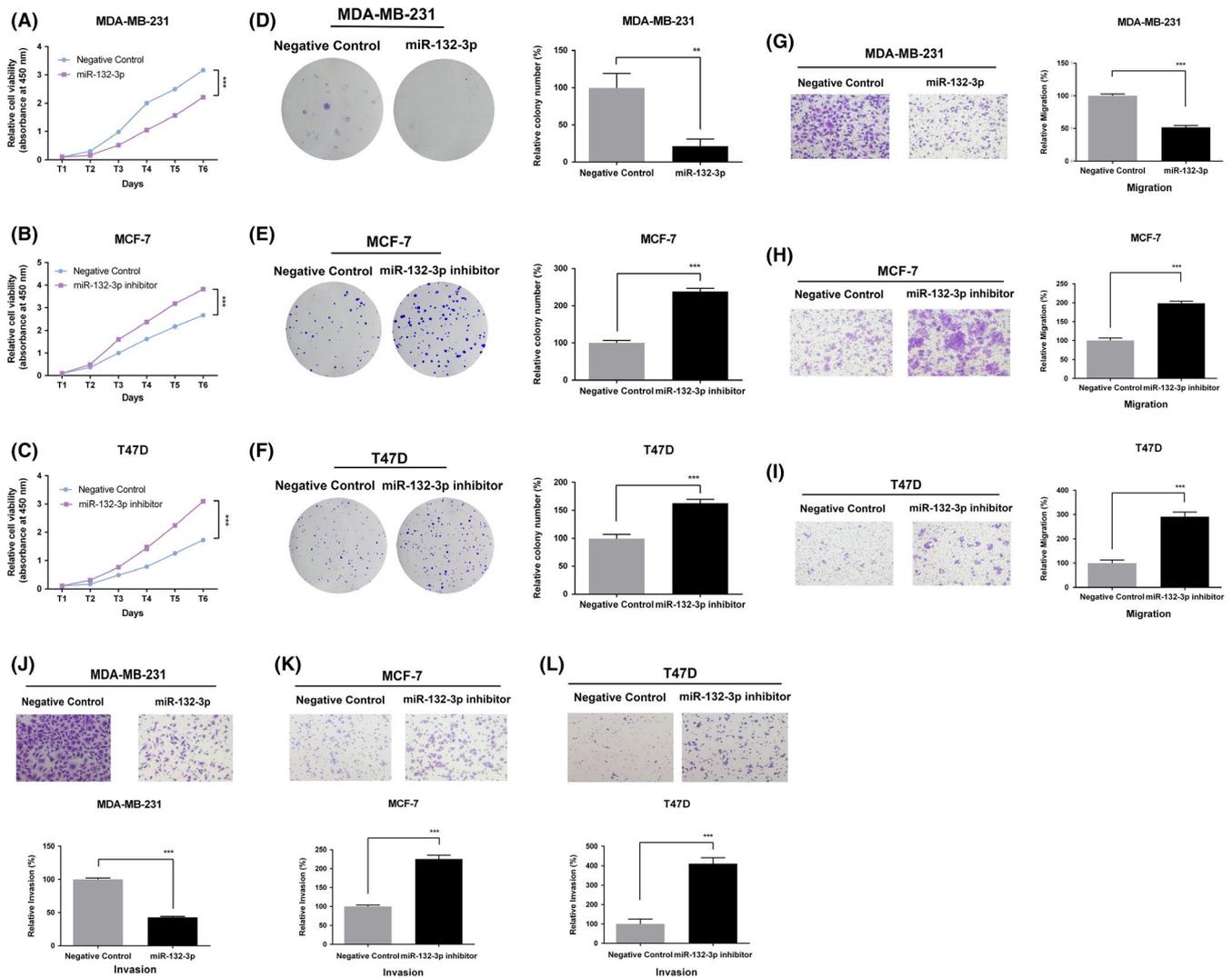
FIGURE 5 MicroRNA (miR)-132-3p inhibits cell proliferation, invasion and metastasis in vitro. A-C, Ectopic expression of miR-132-3p in MDA-MB-231 cells inhibits cell viability, but miR-132-3p downregulation in MCF7 and T47D cells promotes cell viability. D-F, Colony formation assays confirmed the inhibitory effect of miR-132-3p mimics on MDA-MB-231 cells and the stimulatory effects of miR-132-3p inhibitors on MCF7 and T47D cells. G-L, In Transwell assays, miR-132-3p mimics attenuated the migration and invasion of MDA-MB-231 cells compared with the control group; miR-132-3p inhibitors in MCF7 and T47D cells promoted migration and invasion in Transwell assays. All **$P < .01$, ***$P < .001$

MDA-MB-231. Cotransfection of LAPTM4B with miR-132-3p partially reversed the changes in p-AKT and p-mTOR induced by LAPTM4B overexpression. Additionally, these results showed that the lower phosphorylation levels of AKT and mTOR appeared in MCF7 cells with LAPTM4B knocked down compared with the control cells and that the alterations in these molecules could be partly rescued by miR-132-3p inhibitors. Based on these findings, we concluded that miR-132-3p may affect the biological function of breast cancer via LAPTM4B through the inhibition of the PI3K-AKT-mTOR signaling pathway.

4 | DISCUSSION

Lysosomal-associated protein transmembrane 4 beta overexpression contributes to cellular transformation, tumorigenesis, and metastatic progression and is associated with poor prognosis in several human cancers, including breast cancer.[25] Previous studies showed that LAPTM4B allele*2 was a susceptibility factor for breast cancer,[26] and CREB1[14] and AP4[15,16] could bind to the LAPTM4B polymorphism region and function as positive transcriptional regulators. However, the molecular mechanism underlying miRNA regulation of LAPTM4B in breast cancer has not been fully elucidated.

Mature miRNAs can degrade target mRNAs and/or inhibit the expression of target genes at the post-transcriptional level by incomplete or complete complementarity with the 3′UTR regions of target mRNAs, thus negatively regulating target genes. In the present study, prediction software analysis showed that multiple miRNAs may regulate LAPTM4B, and common predictive results were evident in at least three databases for hsa-miR-132-3p, hsa-miR-139-5p, hsa-miR-582-5p and hsa-miR-625-5p.[27-31] Moreover, luciferase
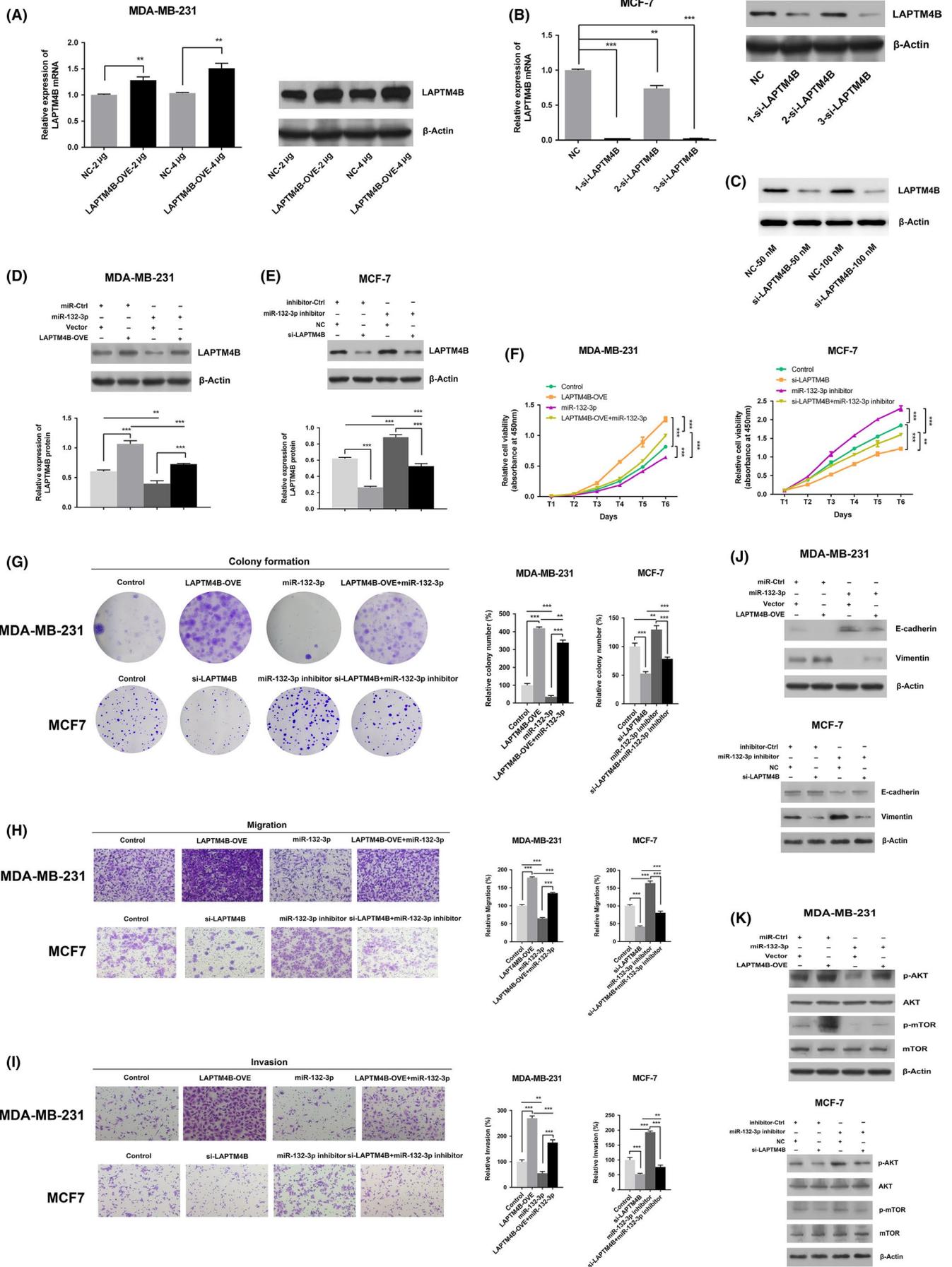
FIGURE 6 MicroRNA (miR)-132-3p can reverse the partial promoting functions of lysosomal-associated protein transmembrane 4 beta (LAPTM4B). A, Verification of the concentration of LAPTM4B overexpression at the mRNA and protein levels. B-C, Appropriate concentration for the effective siRNA-LAPTM4B (si-LAPTM4B) was selected by quantitative PCR and western blot. D-E, LAPTM4B was measured by western blot in MDA-MB-231 cells after cotransfection with miR-Ctrl or miR-132-3p mimics with negative control (NC) or LAPTM4B overexpression (LAPTM4B-OVE) and in MCF7 cells after cotransfection with inhibitor-Ctrl or miR-132-3p inhibitors with NC or si-LAPTM4B. F-G, Cell viability was determined with CCK-8 assays, and clonality was detected in MDA-MB-231 and MCF7 cells. H-I, Effect of miR-132-3p via LAPTM4B on cell migration and invasion was determined with Transwell assays in MDA-MB-231 and MCF7 cells. J, Several important epithelial-mesenchymal transition markers were detected by western blot. K, Protein levels of p-AKT, p-mTOR, AKT and mTOR in MDA-MB-231 and MCF7 cells. All **$P < .01$, ***$P < .001$

reporter and western blot assays showed that miR-132-3p targeted LAPTM4B and regulated its expression. A few studies have reported the expression and possible role of miR-132-3p in breast cancer. Tahiri et al[32] found miRNAs that were downregulated in breast cancer tissues and acted as tumor suppressors, and these miRNAs included miR-193b, miR-126, miR-134, miR-132, miR-486-5p and miR-195 in 29 breast cancer tissues, 29 adjacent normal tissues and 21 benign breast cancer tissues.

To determine the key role of miR-132-3p in the regulation of LAPTM4B, we first examined the expression of miR-132-3p in breast cancer and found that low miR-132-3p expression was closely related to TNM staging, LNM, tumor thrombus, and recurrence, suggesting that miR-132-3p might play a tumor-suppressive role in the malignant transformation of breast cancer. Additionally, Cox regression analysis suggested that miR-132-3p could serve as an independent risk factor for predicting the recurrence of breast cancer. Interestingly, Damavandi et al[33] found that there was no significant correlation between miR-132 expression and the histological grade or TNM staging in 36 breast cancer tumors and matched adjacent non-tumor tissues analyzed by quantitative PCR. Sample size, different detection methods and different study designs may underlie the different conclusions. Therefore, samples from multicenter research, with a larger sample size and uniform detection methods could lead to more definitive results. Furthermore, LAPTM4B expression in breast cancer was negatively correlated with the expression of miR-132-3p analyzed by TCGA database and our results, which further showed that miR-132-3p was a potential regulator of LAPTM4B.

Multiple studies have proven that differentially expressed miRNAs could widely modulate the proliferation, invasion and metastasis of breast cancer. miR-99a inhibited the activation process of the phosphorylation of mTOR and its downstream molecules 4E-BP1 and S6K1, eventually suppressing the proliferation of MCF-7 and MDA-MB-231.[34] We next found that miR-132-3p could inhibit cell proliferation, migration and invasion, showing that miR-132-3p had tumor-suppressive function in breast cancer. Consistent with these results, Li et al[35] confirmed that miR-132 could arrest the BT-549 cell cycle in S/G2.

To investigate the effect of miR-132-3p on LAPTM4B function in breast cancer, cell functional processes were first examined. LAPTM4B has been shown to boost cell proliferation and metastasis in breast cancer. However, our results suggested that miR-132-3p inhibited breast cancer cell proliferation, invasion and migration by negative regulation of the LAPTM4B oncogene. Previous studies have indicated that the interactions between LAPTM4B and SH3 domain-containing proteins may be involved in migration and invasion.[36] To determine the mechanism of the effect of miR-132-3p via LAPTM4B on migration and invasion, we examined the expression levels of EMT-related proteins, and the results were consistent with those of the Transwell assays.

Several studies have shown that LAPTM4B can activate three important signaling pathways that are correlated with cell proliferation, metastasis, the cell cycle and other biological activities, including PI3K-AKT, ECM-Integrin-FAK-Ras-ERK/MAPK and GF-RTK-Ras-ERK/MAPK.[8,36-38] Meanwhile, some studies have explored the role of miR-132 in neuronal function,[39] inflammation,[40] angiogenesis[41] and tumors.[42,43] Shukla et al[44] found that miR-132/212
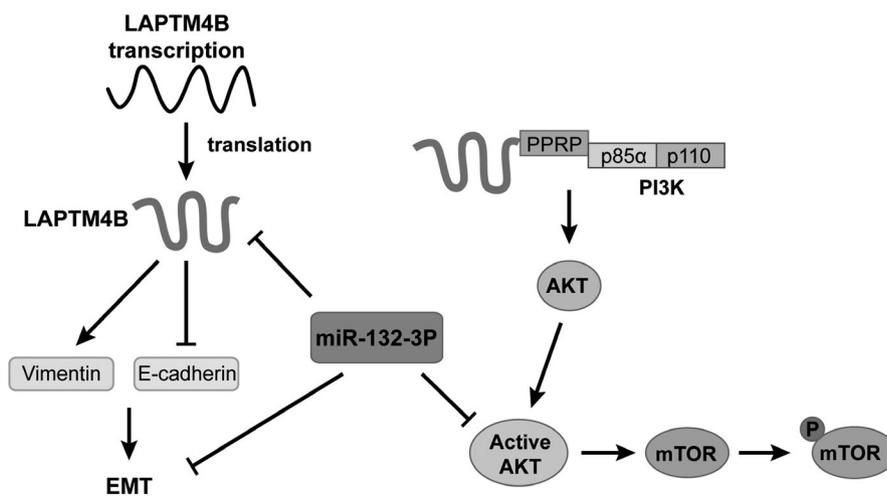
FIGURE 7 Schematic representation of microRNA (miR)-132-3p/lysosomal-associated protein transmembrane 4 beta (LAPTM4B) actions in breast cancer. EMT, epithelial-mesenchymal transition

targeting of CYP2E1 regulated the expression of miR-132/212 and miR122/181a in rat hepatocytes through the PI3K-AKT-mTOR signaling pathway. In this research, to explore the mechanism of the miR-132-3p targeting of LAPTM4B in breast cancer, we evaluated the phosphorylation of AKT and mTOR. Collectively, these results suggest that the regulation of LAPTM4B by miR-132-3p might decrease proliferation, migration and invasion by suppressing the PI3K-AKT-mTOR signaling pathway (Figure 7).

In conclusion, our study is the first to confirm that miR-132-3p inhibits the cell proliferation, migration and invasion of breast cancer by negatively regulating target gene LAPTM4B, indicating that miR-132-3p/LAPTM4B/PI3K/AKT might play a pivotal role in breast cancer progression. In future studies, we will focus on drug resistance and functional coupling between miR-132-3p and LAPTM4B in vivo.

DISCLOSURE

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.