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miR-124 regulates EMT based on ZEB2 target to inhibit invasion and metastasis in triple-negative breast cancer

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ABSTRACT

Background: Triple-negative breast cancer (TNBC) is highly invasive and aggressive and lacks specific molecular targets to improve the prognosis. MicroRNAs (miRNAs) serve a role in promoting and suppressing tumors in various types of malignant cancer, including TNBC. However, the regulatory mechanism of miR-124 in TNBC has still remains unclear.

Methods: Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to detect the expression of miR-124. Cell viability was analyzed with CCK-8 assay. Cell colony formation ability was detected with colony formation assay. Cell invasion was measured with transwell assay. Dual luciferase reporter assay was conducted to verify whether ZEB2 is a target gene of miR-124. The mRNA and protein expression levels of ZEB2 and EMT markers were detected by quantitative real time PCR and western blot, respectively.

Results: Our results showed that miR-124 was down-regulated in TNBC tissues and cells. Overexpression of miR-124 inhibited the proliferation, metastasis and epithelial-mesenchymal transition (EMT) of TNBC cells. Furthermore, ZEB2 3'UTR was considered to be a direct target of miR-124 with luciferase reporter assay. Rescue experiments confirmed that EMT was regulated by miR-124 via suppression of ZEB2.

Conclusion: miR-124 suppresses EMT and metastasis via ZEB2. Therefore, miR-124 may represent a potential therapeutic target for TNBC.

1. Introduction

Breast cancer (BC) is one of the major cancers causing death of women, ranking the first in malignant neoplasm among women in China [1],with nearly 1.7 million new cases annually diagnosed in the world [2]. With regard to the expression status of hormone receptors and human epidermal growth factor receptor-2 (HER2), BC consists of luminal A-like, luminal B-like, HER2-positive and triple-negative molecular subtypes, respectively [3]. Triple-negative breast cancer (TNBC), accounting for 10–20% of patients with breast cancer, is negative for estrogen receptor (ER), progesterone receptor (PR), and HER2 [4]. Compared with other types of breast cancer, TNBC showed more aggressive and higher mortality risk [5]. Due to lack of specific molecular markers and therapeutic targets, despite response to chemotherapy, TNBC is more prone to early recurrence and metastasis [6]. There is an increasing evidence that abnormal activation and dysfunction of key genes can lead to the progression of TNBC. Therefore, investigation of molecular markers of TNBC plays an important role in improving its therapeutic strategy and prognosis.

Epithelial-mesenchymal transition (EMT), also contributes to cancer progression with alterations in cellular morphology, cellular architecture, adhesion, and migration capability [7]. Previous studies have indicated that ZEB is an important factor in the EMT process and promotes the occurrence of the EMT process [8]. We have previously focused on ZEB2, which is a potent repressor of E-cadherin expression, through binding to CACCT(G) motif in the E-cadherin promoter, and is considered to be a key factor for EMT [9,10]. Besides, ZEB2 plays an important role in the ocuurence and development of cancers, including breast cancer [11].

MicroRNA regulated multiple target mRNAs, leading to mRNA degradation or the inhibition of protein translation, generally through perfect or imperfect binding to the 3′-untranslated region (UTR) of the mRNA [12–15]. Recently, Some studies showed that various miRNAs are aberrantly expressed in TNBC, indicating that these miRNAs play

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important roles in carcinogenesis [16,17]. In the present study, we compared the expression patterns of miR-124-3p in TNBC tissues and luminal BC, and explored the function of miR-124-3p in vitro. Here, ZEB2 was confirmed to be the direct target of miR-124-3p. which indicates that miR-124-3p may be able to assist as a potential therapeutic target for human TNBC.

2. Materials and methods

2.1. Tissue samples and cell lines

Primary breast cancer (30 TNBCs and 30 non-TNBCs) and adjacent normal tissues were chosen from the Department of Thyroid and Breast Surgery, The Second Hospital of Hebei Medical University (Shijiazhuang, Hebei province, China). All specimens were confirmed by pathology and quickly stored at −80 ℃. Patients did not accept radiotherapy and chemotherapy before surge. They all signed informed consent. The research was approved by the Institutional Ethics Committee of The Second Hospital of Hebei Medical University (Shijiazhuang, Hebei province, China).

Several non-TNBC (MDA-MB-453) and TNBC cell lines (MDA-MB-231 and BT-549) were purchased from the ATCC (Invitrogen, Carlsbad, CA, USA). They were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA), added 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin-streptomycin. (Sigma-Aldrich, St. Louis, MO, USA).

2.2. RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) based on the product protocol. Reverse transcription process was performed with the TaqMan MiRNA Reverse Transcript Kit (Applied Biosystems, Foster City, CA, USA). PCR reactions were carried out on an Applied Biosystems Prism7500 Fast Sequence Detection System with TaqMan miRNA primers (Applied Biosystems, Foster City, CA, USA). The following primers were used for qPCR: cDNA was synthesized with RT-qPCR using the following primers: miR-124-3p, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCATT-3′; and U6, 5′-AAAATATGGAACGCTTCACGAATTTG-3′. PCR was conducted using the primers as Table1. The RT-qPCR was carried out under the following conditions: 94 ˚C for 4 min followed with 94 ˚C for 1 min, 56 ˚C for 1 min, and 72 ˚C for 1 min. Relative expression of the genes were evaluated with the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primers of Real-time PCR.

Gene		Primer
miR-124-3p	Sense	5′-GCTAAGGCACGCGGTG-3′
	Antisense	5′-GTGCAGGGTCCGAGGT-3′
U6	Sense	5′-CTCGCTTCGGCAGCACATATACT-3′
	Antisense	5′-ACGCTTCACGAATTTGCGTGTC-3′
ZEB2	Sense	5′-GGAGACGAGTCCAGCTAGTGT-3′
	Antisense	5′-CCACTCCACCCTCCCTTATTTC-3′
E-caherin	Sense	5′-AATAGTGCCTAAAGTGCTGC-3′
	Antisense	5′-AGACCCACCTCAATCATCCT-3′
N-cadherin	Sense	5′-ATCCTACTGGACGGTTCG-3′
	Antisense	5′-TTGGCTAATGGCACTTGA-3′
Vimentin	Sense	5′-GAACGCCAGATGCGTGAAATG-3′
	Antisense	5′-CCAGAGGGAGTGAATCCAGATTA-3′
GAPDH :	Sense	5′-CGGAGTCAACGGATTTGGTCGTAT-3′
	Antisense	5′-AGCCTTCTCCATGGTGGTGAAGAC-3′

2.3. Cell transfection

miR-124 mimics (miR-124) and negative control were purchased from Genechem Company (Shanghai, China). ZEB2 plasmid vector was acquired from GeneChem (Montreal, Quebec, Canada). Cell transfection used the Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) in Opti-RPMI 1640 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Twenty-four hours later, the cells were collected, and in vitro assays were performed.

2.4. Western blot analysis

The total protein was extracted using RIPA Lysis & Extraction Buffer, supplemented with phenylmethanesulfonyl fluoride (PMSF). Cell lysates were mixed with loading buffer, and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by being transferred onto PVDF membranes. Then they were blocked with 5% skimmed milk, followed by respectively incubation with antibodies against E-cadherin, N-cadherin, Vimentin and ZEB2 (Abcam, Cambridge, UK) at 4 ℃ overnight. Then, the membranes were washed with TBST and incubated with the horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) for 1 h at 37 ℃. ECL Chemiluminescence kit (UltraSignal, Beijing, China) was utilized to visualize the proteins. β-actin was used as control as well.

2.5. Cell proliferation assay

Cell Counting Kit-8 (CCK-8) (Beyotime Institute of Biotechnology, Shanghai, China) was used to examine the cell proliferation ability. There were 2000 cells seeded into 96-well plate with complete medium. At the appointed time, the medium was exchanged by 110 μl DMEM with CCK-8 (100 μl DMEM and 10 μl CCK-8), then the cells were incubated for about two hours. Then, we measured the OD450 absorbance with auto-microplate reader.

2.6. Colony formation assay

Cell colony formation ability was detected with colony formation assay. A total of 500 cells were cultured in the 6-well plate for 2 weeks until colony was evidently formed. Then the cells were washed and stained with crystal violet. The number of colonies was calculated. The experiment was repeated three times independently

2.7. Transwell invasion assay

Transwell invasion assay was performed to evaluate cell invasion ability. The cells (2×10^5 cells suspended in 200 μl of serum-free medium) were added to the upper chamber of a transwell plate precoated with Matrigel (BD Biosciences), the lower chamber was filled with complete medium. After 24 h incubation, cells were fixed with paraformaldehyde and stained with crystal violet. The invaded cells were counted under the microscopy.

2.8. Luciferase reporter assays

The pMIR plasmid was applied to construct the ZEB2 3′UTR luciferase reporter gene plasmid. The wild-type or mutant 3'UTRs were cloned into the downstream sites of the pMIR vector and were defined as ZEB2-wt or ZEB2-mut, respectively. This experiment was performed in 96-well plates with Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA). Cells were co-transfected with miR-124 mimics and 10 μg of ZEB2-wt or ZEB2-mut with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, luciferase activity was measured with Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA), under the manufacturer's instruction. The results were analyzed with

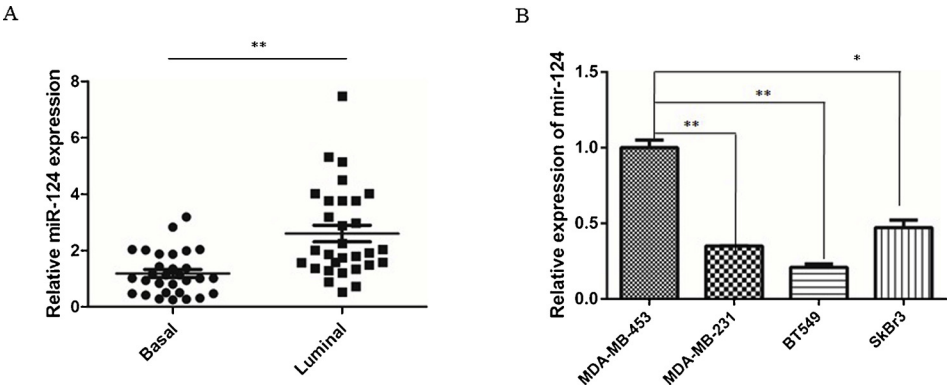

Fig. 1. Down-regulated expression of miR-124 in TNBC tissues and TNBC cells. (A) RT-qPCR analysis tested the expression of miR-124 in TNBC tissues and luminal tissues. (B) RT-qPCR analysis assessed the relative expression of miR-124 in TNBC cells and luminal cells. Each experiment was performed in triplicate. **P < 0.01.

Spectra Max M5 instrument software (Molecular Devices, San Jose, CA, USA). The experiment was repeated three times.

2.9. Statistical analysis

The Student's *t*-test or one-way analysis of variance (ANOVA) were performed to analyze the quantitative data, and they were expressed as means ± standard error (SE). The Mann-Whitney U test was applied to analyze the differences of miR-124 expression between adjacent normal tissues and breast cancer tissues, TNBCs and non-TNBCs. $P < 0.01$ was considered to be statistically significant.

3. Results

3.1. Down-regulated expression of miR-124 in TNBC tissues and TNBC cells

To investigate the biological function of miR-124, we first performed RT-qPCR assay to investigate the expression of miR-124 in TNBC tissues and cells. The results of RT-qPCR assay indicated that miR-124 expression was lower in TNBC tissues and cells than that in luminal tissues and cells (Fig. 1A, B). This indicated that miR-124 might function as a suppressor to hinder the tumorigenesis of TNBC.

3.2. MiR-124 suppressed invasion and metastasis in TNBC

To further define the potential biological significance of miR-124 in TNBC, we used miR-124 mimics to perform the gain-of-function analysis. The expression level of miR-124 was significantly upregulated in the miR-124 mimic group compared to the miR-NC group (Fig. 2A), CCK8 assay showed that up-regulation of miR-124 diminished TNBC cells proliferation (Fig. 2B). Meanwhile, overexpression of miR-124 significantly reduced the number of colonies of MDA-MB-231 and BT-549 cells, suggesting that miR-124 depressed the colony-forming ability in TNBC (Fig. 2C). Furthermore, the effects of miR-124 on invasion of TNBC cells were further tested. Transwell assay showed that ectogenic miR-124 repressed the invasive ability of TNBC cells (Fig. 2D). These results suggest that miR-124 may effectively suppress the growth and invasion of TNBC cells in vitro.

3.3. Overexpression of miR-124 suppressed the expression of EMT in TNBC cells

To figure out the underlying mechanism that miR-124 suppresses cell invasion and metastasis, RT-qPCR assay was performed. We found that miR-124 mimics could enhance the expression of epithelial markers (E-cadherin), remarkably retard the expression of mesenchymal markers (N-cadherin and Vimentin) (Fig. 3A). We further tested the protein levels of E-cadherin, N-cadherin, and Vimentin. The epithelial marker E-cadherin was significantly increased in MDA-MB-231 and BT549 after transfected with miR-124 mimics. By contrast, the mesenchymal markers N-cadherin and Vimentin were decreased in miR-124 over-expressed MDA-MB-231 and BT549 cells compared to the control group (Fig. 3B). Meanwhile, cell morphology was transformed from fusiform mesenchyme to polygonal epithelial after over-expression of miR-124 in MDA-MB-231 and BT549 (Fig. 3C). Above-mentioned results indicated that the epithelial cell polarity of MDA-MB-231 and BT549 cells were partly restored after the transfection of miR-124 mimics.

3.4. ZEB2 is a direct target of miR-124 in TNBC

TargetScan analysis revealed that there was a miR-124 binding site in the 3′-UTR region of ZEB2 (Fig. 4A). In order to eliminate the individual differences caused by transfection efficiency issues, the present study used HEK293 T cells for the Luciferase reporter assay. The luciferase reporter assay demonstrated that luciferase activity was inhibited by co-transfection of the wild type 3′-UTR of ZEB2 and miR-124 mimic, and the deletion of the miR-124 target site inhibited this decrease in luciferase activity (Fig. 4B).

In addition, RT-qPCR and Western blotting results showed that both the mRNA and the protein expression level of ZEB2 in MDA-MB-231 and BT549 cells were obviously decreased after transfected with miR-124 mimics (Fig. 4C and D).

3.5. miR-124 overexpression attenuates TNBC cell invasion and EMT by targeting ZEB2

To examine whether the inhibitory effects of miR-124 on TNBC cell invasion and EMT which were mediated by targeting of ZEB2, we rescued ZEB2 expression in TNBC cells transfected with an miR-124 mimic using a ZEB2 overexpression plasmid. Transfection of this vector successfully mitigated miR-124 overexpression-induced restriction of ZEB2 expression and EMT activities (Fig. 5A). In addition, overexpression of ZEB2 rescued the invasion (Fig. 5B) of cells from suppression with miR-124 overexpression. These findings suggest that overexpression of miR-124 attenuates TNBC cell metastasis and EMT by targeting ZEB2, at least in part.

4. Discussion

Breast cancer is the most common cancer in women worldwide. TNBC, as a special type of breast cancer, is characterized with highly aggressive, early metastasis, and poor clinical prognosis. EMT is considered as the initial stage of tumor metastasis. Therefore, EMT-associated functions have been considered as a potential novel therapeutic tool for cancers including TNBC.

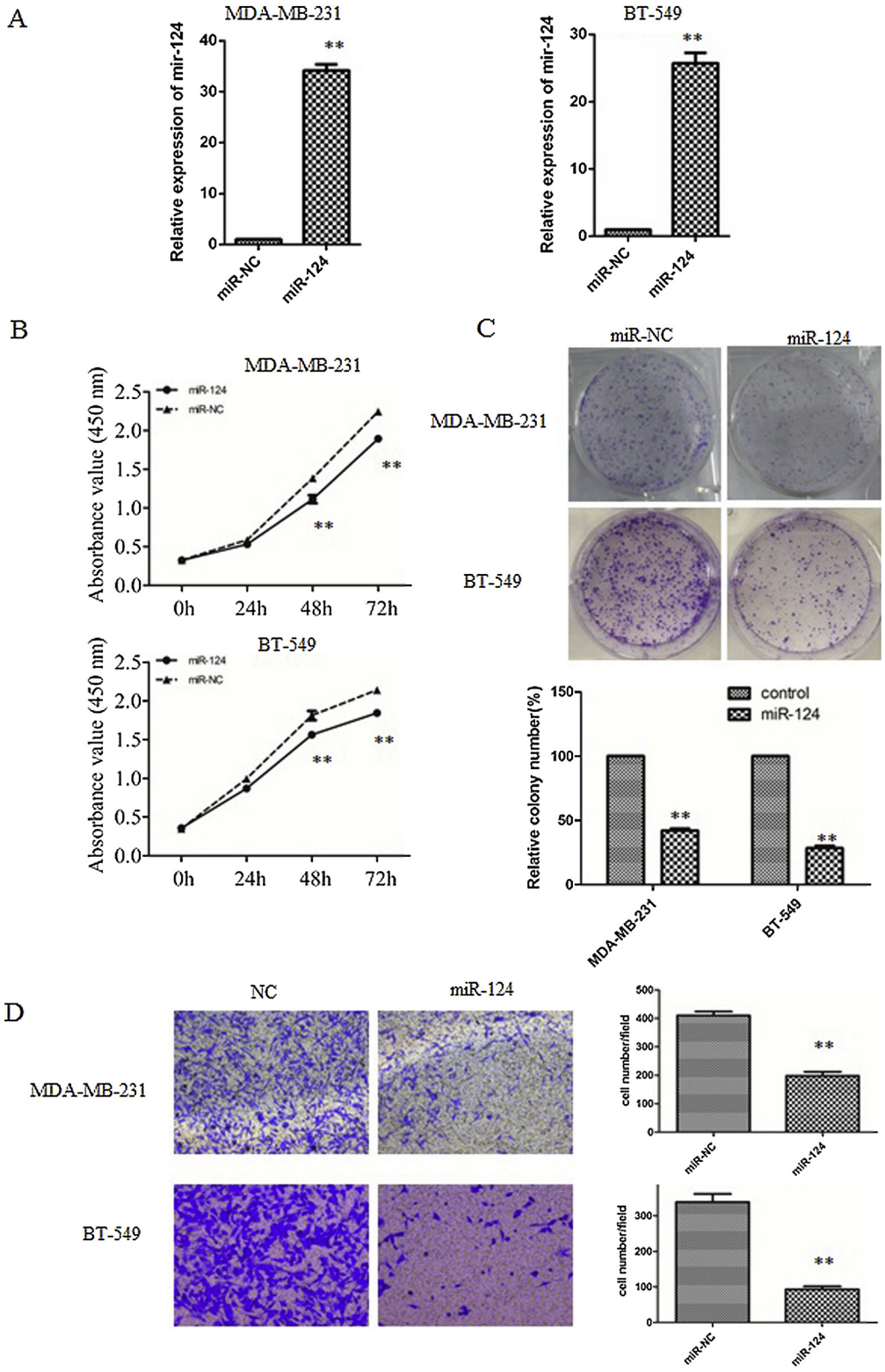

Fig. 2. Effects of miR-124 on TNBC cell proliferation, colony formation, and invasion. (A) Relative miR-124 levels were assessed in MDA-MB-231 and BT-549 cells after transfection with miR-124 or miR-NC. (B) CCK8 assay was performed to assay cell proliferation ability. (C) Cell colony formation was determined in MDA-MB-231 and BT-549 cells expressing miR-NC or miR-124. (D) Transwell invasion assay was used to observe of invasion ability of TNBC cells after transfected with miR-NC and miR-124. Cells were observed in five random fields at 100x magnification and counted. **P < 0.01.

Some studies have shown that ZEB play important role in EMT process and promotes the occurrence of EMT process [8]. ZEB2 can inhibit the transcription of E-cadherin by binding to the promoter of E-gene and induce the occurrence of EMT in tumor cells, thereby enhancing tumor invasion and metastasis [18,19]. It is also believed that high expression of ZEB2 can promote the expression of interstitial markers, such as matrix metalloproteinase (MMP) family, inducing the occurrence of EMT, thereby promoting the invasion and metastasis of

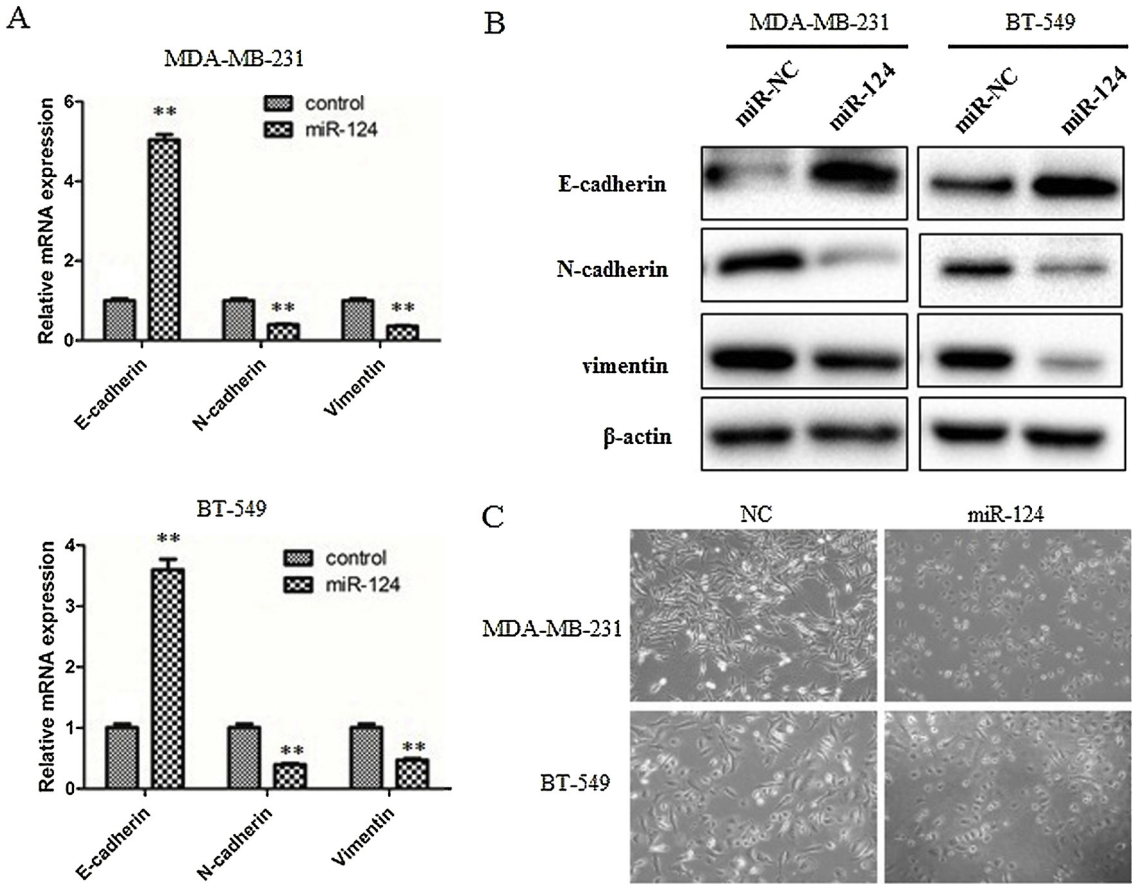

Fig. 3. Overexpression of miR-124 suppressed epithelial-to-mesenchymal transition (EMT) in TNBC cells. (A) RT-qPCR analyzed the relative expression of epithelial markers (E-cadherin) and mesenchymal markers (N-cadherin and Vimentin) in TNBC cells. (B) Western blot tested the relative expression of epithelial markers (E-cadherin) and mesenchymal markers (N-cadherin and Vimentin) in TNBC cells. β-actin served as the internal control. **P < 0.01. (C) The representative pictures of MDA-MB-231 and BT549 cells in the presence or absence of the exogenously expressed miR-124.

tumor cells [20]. At present, numerous studies have confirmed that ZEB2 can promote the invasion and metastasis of tumor cells in many malignant tumor tissues, such as colorectal cancer [21], gastric cancer [22], breast cancer [23], etc. It was revealed that ZEB2 was expressed in breast cancer cells, and the expression level of ZEB2 was negatively correlated with E-cadherin expression [24]. Si et al. pointed out that ZEB2 can inhibit nuclear transcription through the cyclic feedback effect of GATA3 and further induce the occurrence of EMT and promote the metastasis of breast cancer [25]. Gregory et al. showed that miR-205 was in relatively low expression or lack of expression in high invasive breast cancer cells with interstitial cell phenotypes [26]. The overexpression of miR-205 in breast cancer cell MDA-MB-231 was able to reduce the protein expression of the two proteins by directly targeting the 3 '-UTRs of the ZEB1 and ZEB2 genes. On the contrary, in breast cancer cells with epithelial phenotype MCF–7, inhibition of miR-205 can promote the occurrence of EMT and improve the ability of invasion and metastasis of cancer cells by increase expression of ZEB1 and ZEB2 genes, thereby increasing the number of cancer cells in nude mice in vivo [24]. Wang et al. reported that MALAT1 can induce cell metastasis and EMT phenotype through miR-204/ZEB2 axis in BC [27]. A recent study revealed that miR-29b affects breast cancer proliferation and metastasis via targeting gene TET1, which regulates EMT-related gene ZEB2 by binding to its promoter and demethylating CpG islands [27]. However, the function of ZEB2 in TNBC has not been fully illuminated.

Recent studies have shown that microRNAs (miRNAs) are involved in the malignant progression of breast cancer [28–30]. Also, miRNAs can modulate a wide variety of targets, which endowed a supposition that a single miRNA might regulate cancer progression in multiple steps by targeting numerous genes [31]. Besides, miRNAs are a class of post-transcriptional regulators composed of short non-coding RNAs that bind to the complementary sequences in the 3′-UTR of multiple mRNA transcripts, which results in the silencing of target genes [32,33]. Recent studies have shown that miRNAs act as tumor suppressors or oncogenes in cancer [34,35]. It has been confirmed that miR-21, miR-10b, miR-373, miR-155, miR-372, miR-17-5p, and miR-520c can promote the invasion and metastasis of breast cancer [36–43], while miR-31, let-7 family miRNAs, miR-339-5p, miR-17/20, miR-126, miR-146, and miR-205 can inhibit the invasion and metastasis of breast cancer cells [44–46]. One of the most conserved miRNAs is miR-124, which is abundantly and specifically expressed in the nervous system. Recent reports have further demonstrated that deregulation of miR-124 is related to carcinogenesis [47]. The expression level of miR-124 is significantly decreased in glioma, medulloblastoma, oral squamous cell carcinoma (OSCC), hepatocellular carcinoma (HCC), and bladder cancer, suggesting a potential tumor suppressive function of miR-124 [48–53]. Feng et al. found that miR-124 inhibited proliferation of breast cancer cells by regulating CDK4 expression, and patients with high-level of miR-124 expression showed longer overall survival [47]. Wang et al. pointed out that miR-124-3p functions as a tumor suppressor in breast cancer by targeting CBL [54]. However, the function of miR-124 in breast cancer cell, especially its role in human breast cancer patients, has not been completely elucidated. Further, the molecular mechanisms utilized by miR-124 to modulate the malignant phenotype of breast cancer cells were not fully understood.

Although ZEB2 and miR-124 are both associated with the

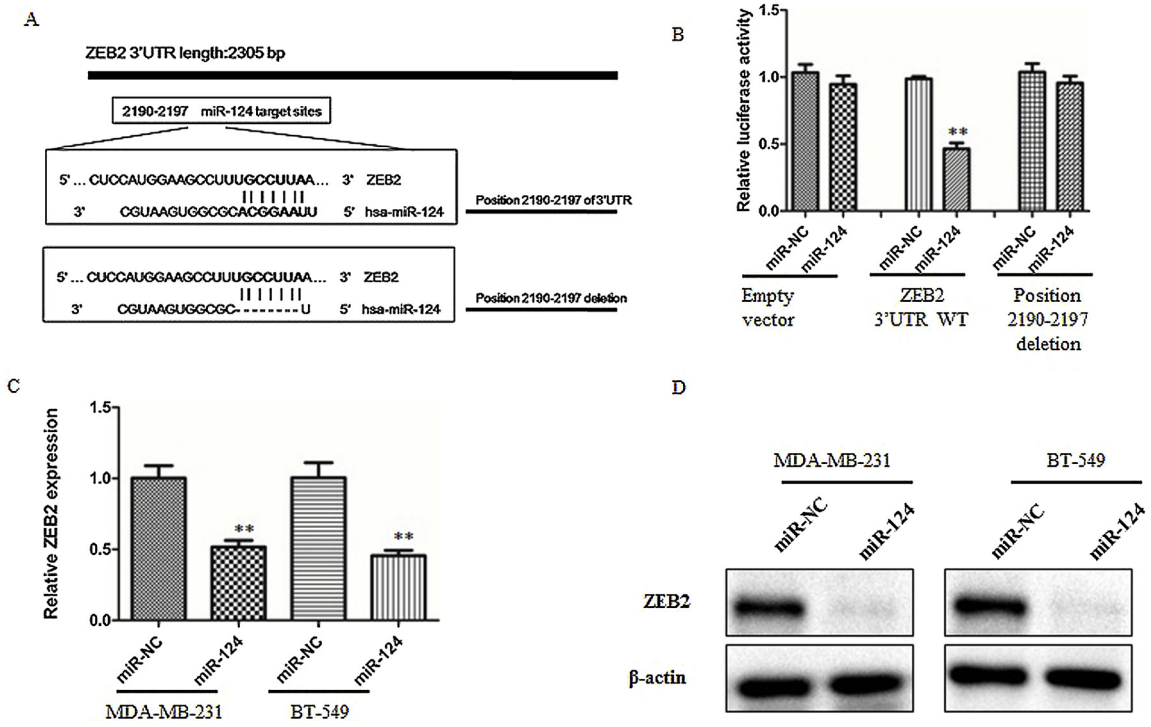

Fig. 4. Prediction and validation of ZEB2 as the target of miR-124. A. Predicted miR-124 binding sites in the 3'-untranslated region (3'UTR) of the ZEB2 mRNA sequence, and the mutations that were induced at these sites. B. Relative luciferase activity was determined using HEK293 T cells co-transfected with a wild-type or mutant ZEB2 3'UTR reporter plasmid and miR-124 mimic or miR-NC. WT: wild-type; MUT: mutant. C. ZEB2 mRNA expression in MDA-MB-231 and BT-549 cells transfected with an miR-124 mimic or miR-NC, as determined by RT-qPCR. D. Levels of ZEB2 proteins in MDA-MB-231 and BT-549 cells transfected with an miR-124 mimic or miR-NC, as determined by Western blotting using β-actin as a loading control. **P < 0.01.

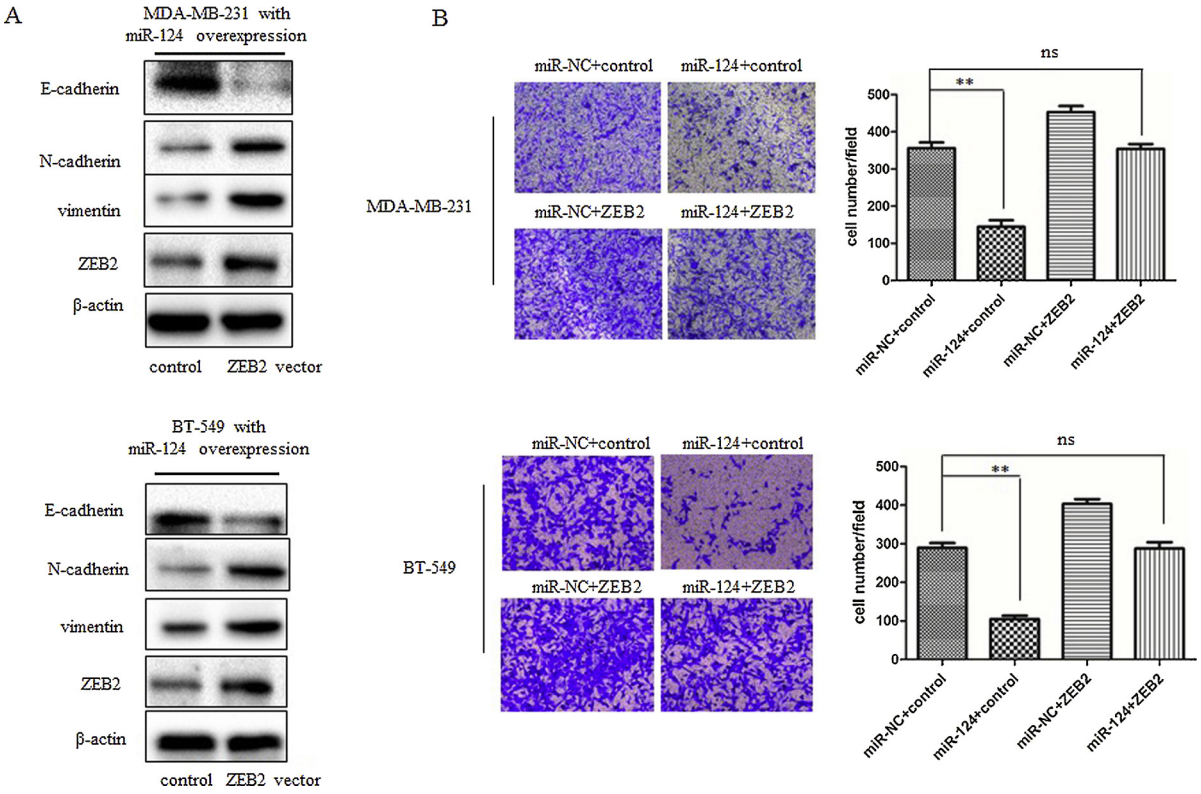

Fig. 5. miR-124 attenuates TNBC cell invasion and EMT by targeting ZEB2. A. Western blot analysis of ZEB2, E-cadherin, N-cadherin, and Vimentin protein levels in MDA-MB-231 and BT-549 cells transfected with an miR-124 mimic with or without a ZEB2 overexpression plasmid. β-actin was used as an internal control. B. MDA-MB-231 and BT-549 cells following transfection with an miR-124 mimic with or without a ZEB2 overexpression plasmid. **P < 0.01.

occurrence and progression of breast cancer, the interrelationship between ZEB2 and miR-124 in TNBC has remained uncertain. In our study, the expression levels of miR-124 in TNBC tissues and TNBC cells were analyzed, and we found that miR-124 was significantly downregulated in TNBC tissues and cells compared with luminal tissues and cells. Next, we proved that MiR-124 can inhibit proliferation, invasion, and metastasis in TNBC cells. Then, ZEB2 was considered to be the direct downstream target of miR-124. Furthermore, we found that miR-124 could inhibit epithelial mesenchymal transformation through ZEB2, as well as further inhibiting the invasion and metastasis of TNBC cells.

In short, our study demonstrate that miR-124 may decrease the ratio of invasion and metastasis of TNBC cells by targeting ZEB2 and inhibit the EMT process as well. The present data provide novel insights into the mechanism responsible for the development of human TNBC. Therefore, miR-124 might be a novel target therapy for TNBC treatment.

Financial disclosure

None.

Ethics statement

This study was approved by the Institutional Medical Ethics Committee of the 4th Hospital of Hebei Medical University.

Declarations of interest

None.

Acknowledgment

Not applicable.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2018.12.039.

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