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Original Article

miR-124 suppresses multiple steps of breast cancer metastasis by targeting a cohort of pro-metastatic genes *in vitro*

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Abstract

Metastasis is a multistep process involving modification of morphology to suit migration, reduction of tumor cell adhesion to the extracellular matrix, increase of cell mobility, tumor cell resistance to anoikis, and other steps. MicroRNAs are well-suited to regulate tumor metastasis due to their capacity to repress numerous target genes in a coordinated manner, thereby enabling their intervention at multiple steps of the invasion-metastasis cascade. In this study, we identified a microRNA exemplifying these attributes, *miR-124*, whose expression was reduced in aggressive MDA-MB-231 and SK-3rd breast cancer cells. Down-regulation of *miR-124* expression in highly aggressive breast cancer cells contributed in part to DNA hypermethylation around the promoters of the three genes encoding *miR-124*. Ectopic expression of *miR-124* in MDA-MB-231 cells suppressed metastasis-related traits including formation of spindle-like morphology, migratory capacity, adhesion to fibronectin, and anoikis. These findings indicate that *miR-124* suppresses multiple steps of metastasis by diverse mechanisms in breast cancer cells and suggest a potential application of *miR-124* in breast cancer treatment.

Key words miR-124, breast cancer, metastasis, pro-metastasis gene

Metastasis is responsible for >90% of cancer-related deaths[1]. Metastasis is a multistep process involving cell detachment from the primary tumor, extravasation, and, finally, invasion to the secondary site. Many researches have been focused on identifying the critical regulators of the metastatic process. These regulatory molecules include both proteins and microRNAs (miRNAs)[2,3].

miRNAs are small non-coding RNA molecules that suppress gene expression by interacting with the 3'-untranslated regions (UTRs) of target mRNAs. These interactions may result in either inhibited translation or degradation of targeted mRNAs[4]. An individual miRNA can regulate dozens of distinct mRNAs, involving different steps for regulating the metastasis of breast cancer cells[5], indicating the important role and multiple functions of miRNAs in control of tumor progression.

The regulatory mechanism of miRNA expression has been documented at the transcriptional and post-transcriptional processing levels. In cancer, transcriptional silencing due to epigenetic pathways is a significant alteration[6]. Hypermethylation of gene promoters is a frequent mechanism of miRNA silencing at the transcriptional level[7]. The facts that miRNA expression occurs in a tissue-specific or developmental stage-specific manner and that some miRNAs are imprinted[8] support the hypothesis that DNA methylation may regulate miRNA expression, as indicated in

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recent studies[9-11]. Therefore, it is conceivable that DNA methylation regulates miRNA expression during tumor progression.

miR-124, which is highly expressed in the central nervous system, was found to be epigenetically silenced in a variety of tumor cells [9-12] and modulate the proliferation of tumor cells by targeting cyclin-dependent kinase 6 (CDK6) [13-15]. More recently, Hunt *et al.*[16] reported that *miR-124* could also suppress the motility of oral squamous cell carcinoma by targeting integrin-β1 (ITGB1). Additionally, Zheng *et al.*[17] found that *miR-124* modulated hepatocellular carcinoma cell aggressiveness by repressing the expression of Rho-associated coiled-coil containing protein kinase 2 (*ROCK2*) and enhancer of zeste homolog 2 (*EZH2*). These data suggest a potential tumor suppressive function for *miR-124*. To date, however, the role of *miR-124* in breast cancer and the molecular mechanisms by which *miR-124* expression is regulated remain largely unknown.

In this study, we report that dysregulation of *miR-124* was correlated with metastatic potential. Ectopic expression of *miR-124* in breast cancer cells suppressed multiple steps of metastasis. More specifically, we provide evidence that *miR-124* directly suppressed multiple pro-metastasis targets, including connective tissue growth factor (*CTGF*), ras homolog gene family member G (*RhoG*), *ITGB1*, and *ROCK1*. In addition, DNA hypermethylation of CpG islands in the promoters of *MIR124-1*, *MIR124-2*, and *MIR124-3* might contribute in part to the down-regulated expression of *miR-124* in highly aggressive breast cancer cell lines. Collectively, our results show that *miR-124*, a pleiotropically acting miRNA, suppressed multiple steps of breast cancer metastasis and suggest that *miR-124* may be a new target for breast cancer therapy.

Materials and Methods

Cell lines and culture

The breast cancer cell lines BT474, MDA-MB-231, MCF-7, and MDA-MB-436 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). SK-3rd was previously established by consecutively passaging breast cancer cell line SKBR3 in NOD/SCID mice under the pressure of chemotherapy[18]. BT474, MDA-MB-231, MDA-MB-436, and SK-3rd cells were maintained in DMEM (Gibco, USA) complete medium (90% DMEM and 10% FBS). For MCF-7 cells, additional insulin was added to the DMEM complete medium. For demethylation, cells cultured in DMEM complete medium were treated with 5'-aza-2- deoxycytidine (AZA) (Sigma, USA) at a concentration of 2 μmol/L or DMSO for 72 h.

Genomic DNA isolation and bisulfite DNA sequencing PCR (BSP) analysis

Genomic DNAs were isolated from cells using the DNeasy Tissue Kit (Qiagen, USA). DNA samples were treated with sodium bisulfite to convert cytosine to uracil using the Methyl Detector™ Bisulfite Modification Kit (Active Motif, North America) according to the manufacturer's instructions.

For BSP, a 1 μL aliquot of sodium bisulfite-treated DNA was amplified by PCR with commonly used primers for methylated and unmethylated DNA sequences. The PCR products were cloned into the pGEM-T Easy vector (Promega, USA), and 10 clones from each sample were sequenced to determine the methylation status of each CpG site. BSP primers for *MIR-124-1*, *MIR-124-2*, and *MIR-124-3* were designed according to previously validated oligonucleotides[10] and synthesized commercially (Invitrogen, USA).

Oligonucleotide transfection

miR-124 mimics and negative control were purchased from GenePharma (Shanghai, China). Small interfering RNAs (siRNAs) targeting human *CTGF*[19], *ITGB1*[20,21], *RhoG*[22], and *ROCK1*[23,24] were designed according to previously validated oligonucleotides and synthesized by GenePharma. Oligonucleotide transfection was performed with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized with MLV transcriptase kit (Invitrogen, USA). Real-time PCR was performed with LightCycler480 System using SYBR Premix Ex Taq kit (TaKaRa, Japan). The silencing effects on *CTGF*, *ITGB1*, *RhoG*, and *ROCK1* were evaluated using qRT-PCR. The sequences of qRT-PCR primers for *CTGF*[19], *ITGB1*[25], *RhoG*[26], and *ROCK1*[27] were validated previously and synthesized by Invitrogen. The mature form of miRNAs was detected using the miRNA qPCR Quantitation Assay according to the manufacturer's instructions (GenePharma, China). The U6 small nuclear RNA purchased from GenePharma was used as an internal control.

Western blotting

Western blot analysis was performed as described previously[28]. Briefly, cells were lysed in MCLB [50

mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 5 mmol/L EDTA, 0.5% Nonidet P-40, 2 mmol/L dithiothreitol (DTT), and 2 mmol/L phenylmethylsulfonyl fluoride (PMSF)] and clarified lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. GAPDH antibody (PeproTech, USA) and ROCK1 antibody (Santa Cruz Biotechnology, USA) were used in accordance with the manufacturer's instructions.

Luciferase reporter assay

Putative *miR-124*–binding sites in the 3'-UTR of the *ROCK1*, *CTGF*, *RhoG*, and *ITGB1* mRNAs were cloned into pMIR-REPORT vector (Ambion, USA). Mutations were detected using QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) according to the manufacturer's instructions. The primers used for construction of luciferase reporters and mutations of *miR-124*–binding sites are listed in Table 1.

The firefly luciferase constructs were co-transfected with a control *Renilla* luciferase vector pRL-TK (Promega, USA) into MBA-MD-231 cells in the presence of either *miR-124* mimics or negative control. A dual luciferase assay (Promega, USA) was performed 24 h after transfection. The experiments were performed independently in triplicate.

Wound healing and Transwell assays

Cell motility was assessed by measuring the movement of cells into a scraped, acellular area created by a 200 μL pipette tip. The spread of wound closure was observed after 24 h and 48 h and photographed under a microscope.

Migration assays were carried out in modified Boyden chambers with 8 μm pore filter inserts in 24-well plates (BD Transduction, USA). Briefly, 1×10^5 cells suspended in serum-free DMEM were added to the upper chamber of the insert in each well of a 24-well culture plate. FBS was added to the lower chamber as a chemoattractant. After 8 h, the non-filitered cells were gently removed with a cotton swab. Filtered cells located on the lower side of the chamber were stained with crystal violet, air dried, and photographed.

Anoikis assay

MBA-MD-231 cells were suspended in growth medium at a density of 5×10^5 cells/mL and plated on ultra-low attached anoikis plates for 72 h. Cells were then washed with 1× PBS and stained using the Annexin V-FITC Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's instructions. Briefly, FITC-labeled annexin V and propidium iodide were added to the cells, which were incubated in the dark at room temperature for 15 min and then analyzed using flow cytometry. Each experiment was carried out at least three times.

Cell adhesion assay

MDA-MB-231 cells (2×10^5 cells/100 μL) were transfected with *miR-124* mimics, negative control, or mock and were seeded to 24-well plates coated with fibronectin at 20 μg/mL and incubated for 1 h. The cells were fixed with 4% paraformaldehyde. Separated cells were washed off with PBS. Cells that adhered to the substrate were stained with crystal violet and observed

Table 1. Primers for *CTGF*, *RhoG*, *ITGB1*, and *ROCK1* luciferase reporter constructures

Gene	Forward primer sequence	Reverse primer sequence
CTGF	5′-CGACTAGTGCCAGAGAGTGAGAGACATTAAC-3′	5′-TGAACGATCAGACAAGCTTTAC-3′
RhoG	5′-TTACTAGTCCCTGGCACTTGGCTTGGA-3′	5′-TAGAAGCTTGAGTCAGTCAGCAAATGCGT-3′
ITGB1	5′-TAGAGCTCCCGTGCAAATCCCACAACA-3′	5′-ATACGCGTTACATCAGAGTCAAGACATCCG-3′
ROCK1	5′-TTGAGCTCGTGCCCTGTGGAATCGTG-3′	5′-TCACGCGTTTATGTTGGTGCAACCTTCTA-3′
CTGF mutant	5′-TTAAAGTTGTTTCTCCGTCTTTATTTTTG-3′	5′-CAAAAATAAAGACGGAGAAACAACTTTAA-3′
RhoG mutant		
Site-1	5′-CCCACCAGTTATACATAGGTGCCTTGTCC-3′	5′-GGACAAGGCACCTATGTATAACTGGTGGG-3′
Site-2	5′-TCCGCCTCAGCTATACATAAAGGACTAATTC-3′	5′-GAATTAGTCCTTTATGTATAGCTGAGGCGGA-3′
Site-3	5′-CTTTTTCTCTGAATACATATTTCTCCTTAAG-3′	5′-CTTAAGGAGAAATATGTATTCAGAGAAAAAG-3′
ITGB1 mutant		
Site-1	5′-TAAGGTCACATTCTCCGTCTTTGACCTTTTC-3′	5′-GAAAAGGTCAAAGACGGAGAATGTGACCTTA-3′
Site-2	5′-ACATTCTTGTTTTAACTCCGTCTAGTTTTAACAG-3′	5′-CTGTTAAAACTAGACGGAGTTAAAACAAGAATGT-3′
ROCK1 mutant	5′-ATTGTCCTTTTACTCCGTCAATTTGAGAT-3′	5′-ATCTCAAATTGACGGAGTAAAAGGACAAT-3′

CTGF, connective tissue growth factor; RhoG, ras homolog gene family member G; ITGB1, integrin-β1; ROCK1, Rho-associated coiled-coil containing protein kinase 1.

under a microscope.

Results

miR-124 expression is attenuated in metastatic breast cancer cell lines

To explore the potential role of *miR-124* in breast cancer, we examined the expression level of *miR-124* in a panel of breast cancer cell lines with distinct metastatic capacity. As shown in Figure 1, the expression level of *miR-124* was attenuated in aggressive human breast cancer cells, MDA-MB-231, and SK-3rd, compared to non-metastatic BT474 cells. The decreased expression level suggests an inhibitory effect of *miR-124* on metastasis of breast cancer cells.

miR-124 expression suppresses metastasis-relevant traits *in vitro*

Given the inverse correlation between *miR-124* level and aggressive phenotype, we assessed the potential anti-metastatic role of *miR-124* in breast cancer cells. We transiently transfected MDA-MB-231 cells with *miR-124* mimics, negative control, or mock (untreated cells) and examined metastasis-relevant traits *in vitro*. Overexpression of *miR-124* mimics in MDA-MB-231 cells was detected by qRT-PCR (Figure 2A). Ectopic *miR-124* expression resulted in a switch in cellular shape from spindle-shaped to round (Figure 2B), a phenotype that presumably indicated less invasiveness.

To determine whether *miR-124* expression suppresses the migratory capacity of MDA-MB-231 cells, a wound-healing assay was used. Duplicate experiments consistently showed decreased migratory capacity of MDA-MB-231 cells expressing *miR-124* mimics compared to MDA-MB-231 control cells and mock cells at 24 and 48 h (Figure 2C). A migration chamber assay was also used to further assess the migratory capacity of MDA-MB-231 cells overexpressing *miR-124*. Ectopic expression of *miR-124* reduced migrated cells by 4 folds compared to negative control and mock (Figure 2D).

Because adhesive capacity is one of the important traits contributing to metastasis, we examined the adhesion of MDA-MB-231 cells overexpressing *miR-124* to fibronectin. As expected, *miR-124* expression dramatically suppressed the adhesive capacity of MDA-MB-231 cells (Figure 2E).

In addition, we studied whether *miR-124* expression reduced the resistance of MDA-MB-231 cells to anoikis, another important process contributing to metastasis of cancer cells. As shown in Figure 2F, MDA-MB-231 cells overexpressing *miR-124* were more sensitive to anoikis than control and mock cells. Together, these results indicate that *miR-124* suppresses multiple steps of metastasis of breast cancer cells.

miR-1 24 directly regulates a cohort of pro-metastatic genes

To explore the targets directly regulated by *miR-124*, we used two algorithms that predict the mRNA targets of a miRNA–PicTar[29] and TargetScan[30]. Based on the representation of *miR-124* sites in their 3'-UTRs, >1000 mRNAs were predicted to be regulated by *miR-124*. Gene Ontology revealed that these targets included a

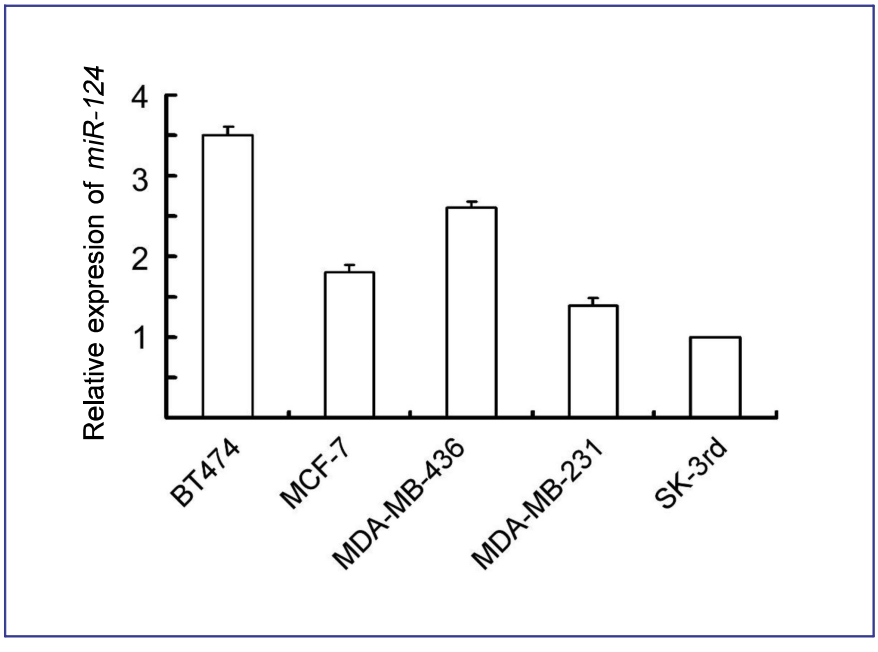

Figure 1. ***miR-124* expression in breast cancer cell lines.** Expression levels of *miR-124* were examined by real-time polymerase chain reaction (PCR) in 5 breast cancer cell lines. *Columns*, mean of three independent experiments; *bars*, standard deviation (SD).

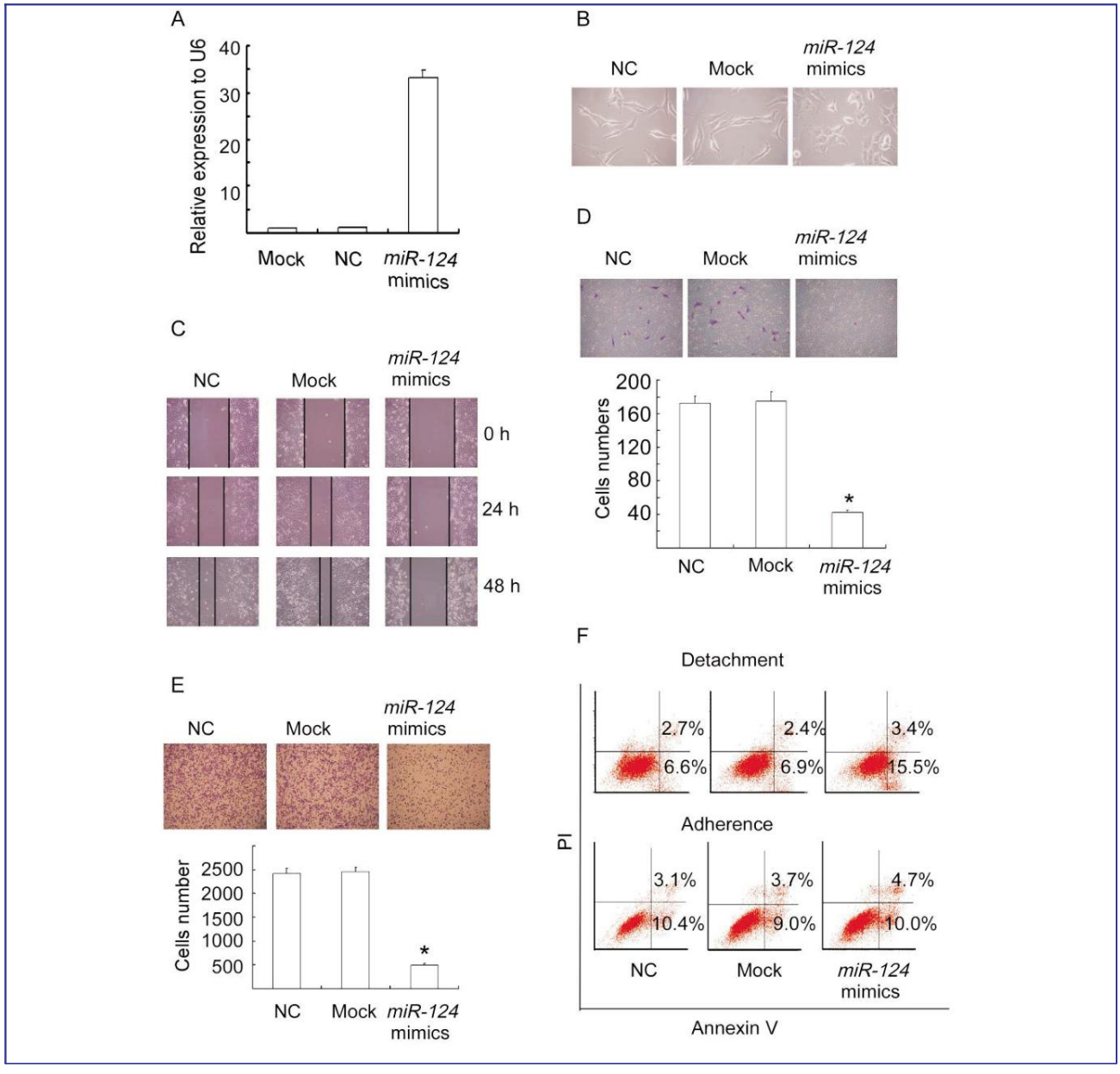

Figure 2. ***miR-124* expression suppresses metastasis-relevant traits *in vitro*.** A, overexpression of *miR-124* in MDA-MB-231 cells was detected by quantitative real-time PCR (qRT-PCR). MDA-MB-231 cells were transfected with *miR-124* mimics, negative control (NC), or mock for 48 h and the RNAs were extracted. B, the morphology of MDA-MB-231 cells changed from spindle-shaped to round as observed under a microscope after transfection with *miR-124* mimics, negative control, or mock for 48 h. C, the wound-healing assay shows decreased cell motilities in *miR-124* ectopically expressed MDA-MB-231 cells. MDA-MB-231 cells transfected with *miR-124* mimics, negative control, or mock were cultured with serum-free medium for 24 h and scraped to created acellular area. The spread of wound closure was observed 24 h and 48 h after scrape and photographed under a microscope. D, ectopic expression of *miR-124* suppresses the migratory capacity of MDA-MB-231 cells. MDA-MB-231 cells transfected with *miR-124* mimics, negative control, or mock for 48 h were placed in serum-free medium and added to the upper chamber of transwell plates. Medium containing 10% serum was added to the lower chamber as a chemoattractant. The migratory capacity was assessed by calculating the filtered cells. *Columns*, mean of three independent experiments; *bars*, SD. $*P < 0.01$, vs. negative control and mock cells. E, ectopic expression of *miR-124* reduces the adhesion of MDA-MB-231 cells to fibronectin. MDA-MB-231 cells transfected with *miR-124* mimics, negative control, or mock for 48 h were detached from culture dishes with trypsin and suspended in serum-free medium. The suspended cells were seeded to 24-well plates coated with fibronectin for 30 min and then observed under a microscope. *Columns*, mean of three independent experiments; *bars*, SD. $*P < 0.01$, vs. control and mock cells. F, ectopic expression of *miR-124* increases the sensitivity of MDA-MB-231 cells to anoikis. MDA-MB-231 cells transfected with *miR-124* mimics, negative control, or mock for 24 h were detached from culture dishes with trypsin and seeded to anoikis plates for another 72 h. Apoptotic cells were evaluated by staining with FITC-annexin V and propidium iodide (PI) and analyzed with FACS.

large number of genes encoding proteins with roles in motility-related processes, such as cell adhesion, cytoskeletal remodeling, and cell polarity (data not shown).

Guided by this Gene Ontology analysis, we cloned the 3'-UTRs of 4 putative *miR-124* targets from these overrepresented categories, including *CTGF*, *RhoG*, *ROCK1*, and *ITGB1* (a well-validated target in oral squamous cell carcinoma) into a luciferase plasmid (Figure 3A). The fluorescence activities of 3'-UTR reporters of *IPTG1*, *RhoG*, and *CTGF* were suppressed by *miR-124* mimics by more than 2 folds as compared to negative control, whereas that of ROCK1 was reduced to 55%; mutations of the putative miR-124–binding sites in the four 3'-UTRs abrogated response to *miR-124* mimics (Figure 3B). To further confirm that *miR-124* directly targets these genes, we examined whether *miR-124* expression reduced the endogenous protein level of ROCK1, the target least suppressed by *miR-124* in the luciferase reporter assay. As shown in Figure 3C, *miR-124* expression obviously decreased the protein level of ROCK1 compared to negative control and mock.

To assess the functional contributions of these *miR-124* targets to aggressive phenotypes, we examined if their inhibition affected the migration of MDA-MB-231 cells using transwell assays. Transfection with siRNAs potently reduced mRNA levels of *CTGF*, *ITGB1*, *RhoG*, and *ROCK1* (Figure 3D). As expected, siRNAs against *CTGF*, *ITGB1*, *RhoG*, and *ROCK1* reduced filtered MDA-MB-231 cells markedly (Figure 3E).

Collectively, these observations demonstrated that *miR-124* directly regulates a cohort of pro-metastatic genes including *CTGF*, *ITGB1*, *RhoG*, and *ROCK1*.

Transcriptional down-regulation of *miR-124* in aggressive breast cancer cells contributes in part to DNA hypermethylation in the promoters of the genes encoding *miR-124*

miR-124 was reported to be down-regulated by DNA hypermethylation in its promoters in several types of cancer[9]. We hypothesized that DNA hypermethylation resulted in down-regulated *miR-124* level in aggressive breast cancer cells. Thus, we analyzed the methylation status of the putative promoters of its three genes (*MIR-124-1*, *MIR-124-2*, and *MIR-124-3*) in MDA-MB-231 and BT474 cells, which are highly aggressive and non-metastatic breast cancer cells, respectively, by bisulfite sequencing PCR (BSP) sequence assay. As shown in Figure 4A, the promoters of *MIR-124-1*, *MIR-124-2,* and *MIR-124-3* were heavily methylated in aggressive MDA-MB-231 cells but only methylated at low levels in non-metastatic BT474 cells. More importantly, treatment of MDA-MB-231 cells with the DNA demethylating agent AZA partially restored *miR-124* expression (Figure 4B). These results indicate that DNA hypermethylation contributes at least in part to the decreased *miR-124* expression in aggressive MDA-MB-231 cells.

Discussion

miRNAs can modulate a wide variety of targets, which endowed the supposition that a single miRNA might regulate cancer progression in multiple steps by targeting numerous genes. In this study, we demonstrate that a single human miRNA, *miR-124*, can concomitantly repress multiple pro-metastatic targets and thereby to inhibit several distinct steps of the invasion-metastasis cascade in breast cancer cells. In addition, low-level expression of *miR-124* and high-level methylation of *MIR-124* promoters were observed in highly metastatic breast cancer cells. Moreover, treatment of MDA-MB-231 cells with demethylation agent AZA partially restored *miR-124* expression, indicating that DNA hypermethylation plays an important role in down-regulation of *miR-124* expression in highly aggressive breast cancer cells.

Metastasis is a multistep process. Therefore, identifying factors that modulate several steps of metastasis and understanding the underlying molecular mechanisms involved in tumor metastasis progression are critical[5]. Tumor cells undergoing mesenchymal-to-epithelial transition (MET) have been reported to exhibit a switch from a spindle-like to a round morphology and to have reduced metastatic capacity. Our results show that MDA-MB-231 cells with ectopic expression of *miR-124* became rounder than control cells. In addition, *miR-124* expression in MDA-MB-231 cells resulted in down-regulation of the protein level of vimentin, a mesenchymal marker. However, the protein level of epithelial marker E-cadherin was not visibly changed upon *miR-124* expression (data not shown). These observations suggest that ectopic expression of *miR-124* might regulate MET in part in breast cancer cells. In addition, cell motility, migratory and adhesive ability, and resistance to anoikis are important factors contributing to cancer cell metastasis to distant sites. In the current study, we evaluated the effects of *miR-124* expression on these crucial steps of metastasis and found that reintroduction of *miR-124* into MDA-MB-231 cells reduced cell mobility and migration, repressed cell adhesion to the extracellular matrix, and increased cell sensitivity to anoikis. These observations indicate that *miR-124* is a powerful metastasis suppressor.

Subsequently, we assessed *CTGF*, *ITGB*, *RhoG*, and *ROCK1* as potential functional targets of *miR-124*. Our results show that *miR-124* bound the complementary sites in the 3'-UTRs of the *CTGF*, *ITGB1*, *RhoG*, and *ROCK1* genes. Luciferase reporter assay indicated that

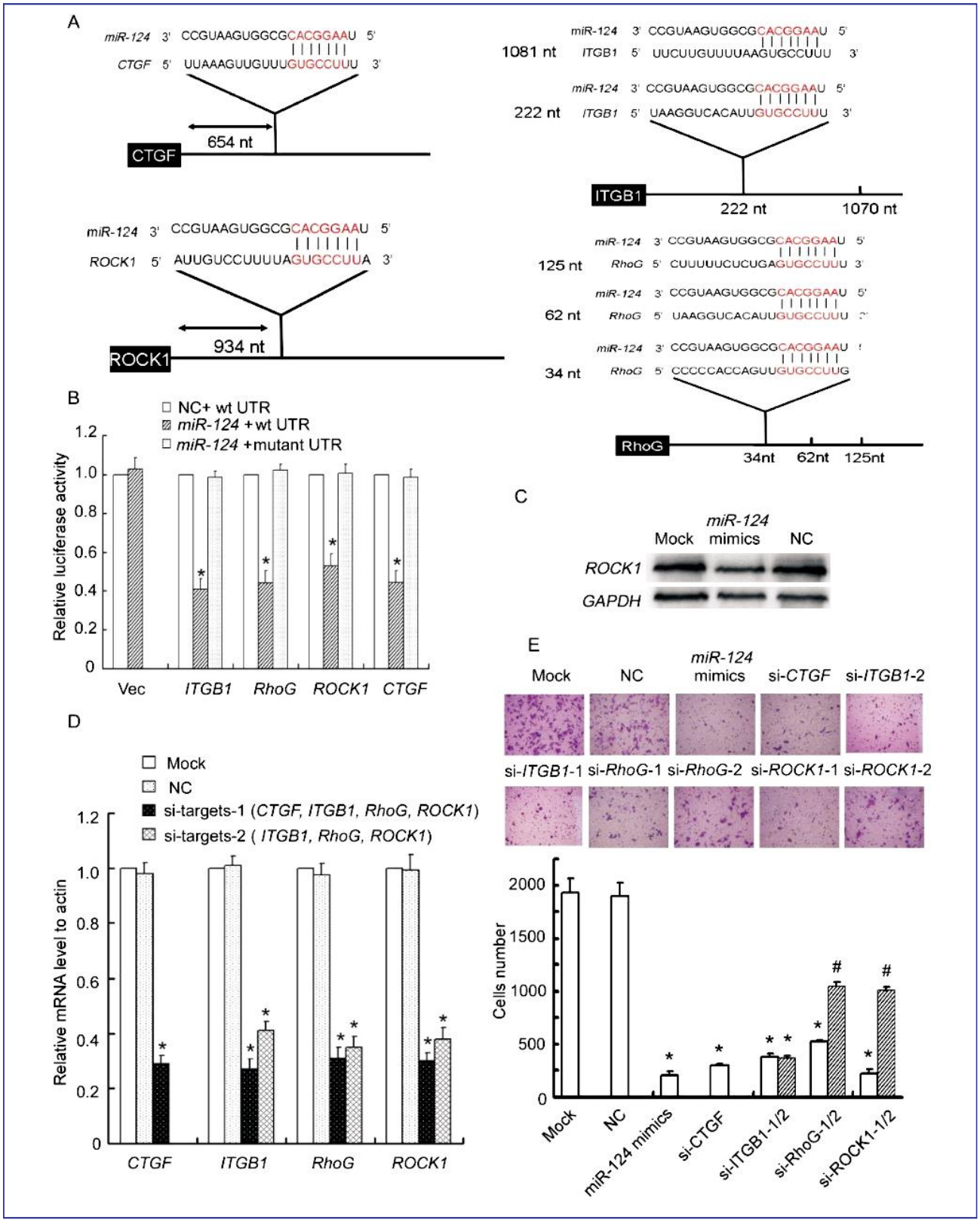

Figure 3. ***CTGF, RhoG, ITGB1,* and *ROCK1* are targets of *miR-124*.** A, schematic illustration of the predicted *miR-124*–binding sites in the 3′-UTRs of *CTGF*, *RhoG*, *ITGB1*, and *ROCK1*. B, *miR-124* significantly reduces the luciferase activities of *CTGF*, *RhoG*, *ITGB1*, or *ROCK1*. miR

reporter constructs containing wild-type and mutated 3′-UTRs of the 4 putative target genes were co-transfected with *miR-124* mimics or negative control into MDA-MB-231 cells and incubated for 24 h. Relative repression of firefly luciferase expression was standardized to a transfection control. *Columns*, mean of three independent experiments; *bars*, SD; **P* < 0.01, vs. negative control. C, ectopic expression of *miR-124* decreases endogenous levels of ROCK1. MDA-MB-231 cells were transfected with *miR-124* mimics, negative control, or mock for 72 h. ROCK1 expression was assessed by Western blotting. D, siRNA transfection potently reduces target mRNA levels. MDA-MB-231 cells were transfected with siRNAs against *CTGF*, *ITGB1*, *RhoG*, *ROCK1*, negative control, or mock for 48 h. The target mRNA levels were evaluated by real-time quantitative PCR. E, siRNAs against *CTGF*, *ITGB1*, *RhoG*, or *ROCK1* reduce the migratory ability of MDA-MB-231 cells. The migration of siRNA-transfected MDA-MB-231 cells was assessed using transwell assays. *Columns*, mean of three independent experiments; *bars*, SD. **P* < 0.001, # *P* < 0.01, vs. negative control and mock cells.

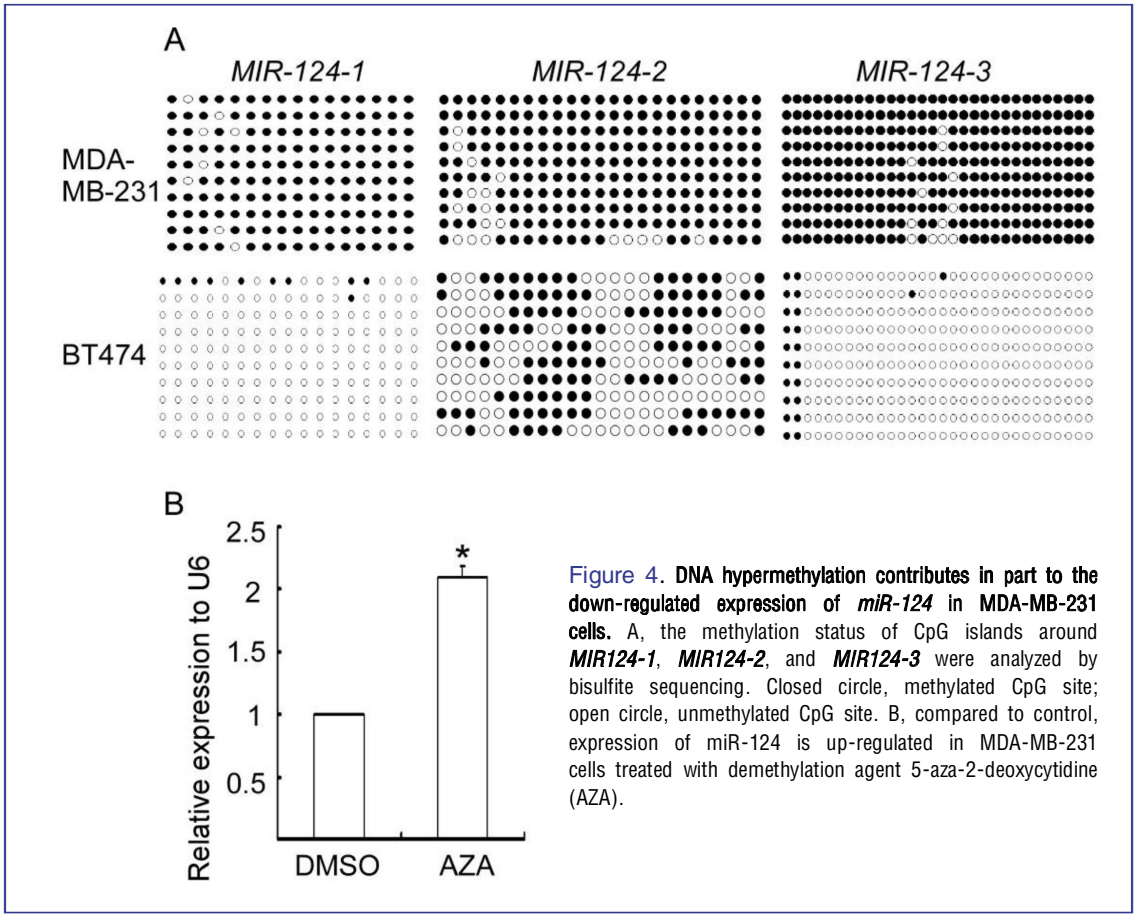

Figure 4. **DNA hypermethylation contributes in part to the down-regulated expression of *miR-124* in MDA-MB-231 cells.** A, the methylation status of CpG islands around ***MIR124-1***, ***MIR124-2***, and ***MIR124-3*** were analyzed by bisulfite sequencing. Closed circle, methylated CpG site; open circle, unmethylated CpG site. B, compared to control, expression of miR-124 is up-regulated in MDA-MB-231 cells treated with demethylation agent 5-aza-2-deoxycytidine (AZA).

miR-124 suppressed the reporter fluorescence activity delivered 3'-UTRs of these genes. In addition, we analyzed the effects of *miR-124* expression on the mRNA levels of these genes in the GEO Profiles Database (http://www.ncbi.nlm.nih.gov/geoprofiles/). The microarray data showed that the mRNA levels of the four genes were dramatically down-regulated in HepG2 hepatocellular carcinoma cells with ectopic expression of *miR-124* compared to negative control. These observations provide the first evidence, to our knowledge, that *miR-124* mechanistically acts via the regulation of *CTGF*, *ITGB1*, *RhoG*, and *ROCK1*. *CTGF*, *RhoG*, *ITGB1*, and/or *ROCK1* are reportedly up-regulated in several types of human cancer, including breast cancer, and overexpression of these proteins is positively correlated with tumor metastasis and/or poor prognosis[31-34]. This is consistent with our findings that down-regulation of *miR-124* is correlated with highly aggressive breast cancer. Furthermore, *miR-124* has been found to modulate hepatocellular carcinoma cell aggressiveness by repressing *ROCK2* and *EZH2*[17]. Thus, our findings, together with the work of other groups, demonstrate that *miR-124* may target multiple proteins that function spatiotemporally or in cooperation with different cellular processes.

Previous studies have described effects of specific miRNAs on local invasion, an early stage of the invasion-metastasis cascade. The present work

demonstrates that miRNAs can also influence later steps of metastasis and that an individual miRNA can intervene at multiple distinct stages of the invasion-metastasis cascade. *miR-124* regulates the motility, adhesion to ECM, and the intraluminal survival of breast cancer cells.

Collectively, the findings in the present study carry significant implications regarding our understanding of the pathogenesis of high-grade breast cancer. Our data suggest that down-regulation of *miR-124* expression is correlated with aggressiveness in breast cancer cell lines. Ectopic expression of *miR-124* suppresses multiple distinct steps of the invasion-metastasis cascade *in vitro*. As distant metastases are responsible for patient mortality, *miR-124*, with the ability to impede metastasis, may prove to be clinically useful.

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