miR-720 inhibits tumor invasion and migration in breast cancer by targeting TWIST1

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Breast cancer is the leading cause of cancer death among females, with tumor metastasis being primarily responsible for breast cancer-associated mortality. Current literatures have shown that microRNAs (miRNAs) are implicated in tumor metastasis. In this study, we found that the expression of miR-720 was significantly downregulated in primary breast cancer, with greater downregulation in metastatic tumors. Statistical analysis of 105 cases of primary human breast cancer demonstrated that decreased expression of miR-720 was correlated with lymph node metastasis. Furthermore, reexpression of miR-720 in breast cancer cells remarkably inhibited cell invasiveness and migration both in vitro and in vivo. Mechanistically, downregulation of TWIST1, a promoter of metastasis that was identified as a direct functional target of miR-720, was attributed to the inhibition of miR-720. Consistent with the reduced TWIST1 levels in breast cancer, reexpression of miR-720 upregulated epithelial markers (E-cadherin and β-catenin) and downregulated mesenchymal markers (N-cadherin, fibronectin, vimentin and matrix metalloproteinase-2). Expression of miR-720 was inversely associated with TWIST1 in human breast cancer tissue. Knockdown of TWIST1 expression by small interfering RNA exhibited similar effects to reintroduction of miR-720, whereas overexpression of TWIST1 (without the 3′-untranslated region) abrogated miR-720-mediated metastasis inhibition. Collectively, our data indicate that miR-720 is frequently decreased in breast cancer and manifests antimetastatic activity by downregulating TWIST1, presenting a novel mechanism of miRNA-mediated regulation of tumor metastasis.

Introduction

Breast cancer is the most frequent malignant disease and the leading cause of cancer death in females worldwide (1). Tumor metastasis, which accounts for 90% of the associated mortality, is a key event in the progression of breast cancer (2). Despite major efforts in metastasis research, the precise mechanisms governing metastatic dissemination remain largely unknown. Thus, it is of immense importance and clinical significance to understand the molecular circuitry of the invasion–metastasis cascade.

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs that have been recognized as highly efficient cell process regulators since their discovery in 1993 (3,4). Through base pairing with the 3′-untranslated region (3′ UTR) of the target messenger RNA (mRNA), miRNAs can suppress protein expression of their target genes (5). Increasing evidence indicates that dysfunction of miRNAs is involved in the development of cancer, suggesting that miRNAs can function as classical oncogenes or tumor suppressor genes (6,7). In addition, because of their pleiotropic nature of regulating major genes, recent studies implicate endogenous human miRNAs in tumor metastasis. miR-31 can inhibit breast cancer metastasis via coordinate repression of a cohort of metastasis-promoting genes (8). Furthermore, upregulation of miR-143 expression in hepatitis B virus-related hepatocellular carcinoma promotes cancer cell invasion/migration and tumor metastasis (9), miR-374a can promote breast cancer metastasis by activating Wnt/β-catenin (10), whereas ectopic expression of miR-124 in breast cancer cell lines suppresses metastasis-related traits including the formation of spindle-like morphology, migratory capacity, adhesion to fibronectin and anoikis (11). miR-720 has been associated with epithelial development under physiological conditions in adult mouse keratinocytes (12); however, research on the biological and clinical significance of miR-720 is predominantly limited. Although it has been reported that miR-720 can inhibit cell proliferation via targeting p63 in esophageal squamous cell carcinoma cells (13), the role that miR-720 plays in tumorigenesis and cancer progression remains largely unknown.

TWIST1, a highly conserved basic helix-loop-helix transcription factor, plays an essential role in many biological processes, including mesoderm development (14,15) and differentiation (16). It is upregulated and may function as an oncogene in many types of tumors, such as cancers from breast (17), liver (18), prostate (19), stomach (20) and pancreas (21). The role of TWIST1 in tumor invasion and metastasis has been attracting increasingly greater interest. Knockdown of TWIST1 in metastatic mammary carcinoma cells leads to the inhibition of the ability of cells to metastasize from the mammary gland to the lung, and reexpression of TWIST1 results in the induction of cell motility in epithelial cell lines (22). Depletion of TWIST1 can suppress the migration rate of prostate carcinoma cells (23). Moreover, TWIST1 can also enhance cell migration and invasion in gastric cancer both in vitro and in vivo (24).

In this study, we demonstrate that miR-720 is significantly downregulated and is associated with aggressive clinicopathological features in human primary breast cancer. Reexpression of miR-720 resulted in the inhibition of cell migration and invasion in vitro and in vivo via direct targeting of TWIST1. The newly identified miR-720/TWIST1 axis elucidates a molecular mechanism for the metastasis of breast cancer and provides a novel strategy for the treatment of patients with this lethal disease.

Materials and methods

Cell culture

MCF-7, MDA-MB-453, SKBR-3, MDA-MB-468, MDA-MB-231 and MDA-MB-435 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, MCF-10A was maintained in keratinocyte/serum-free medium supplemented with growth factors (Gibco, Grand Island, NY). All cells were incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Tissue samples

and July 2011 were collected from Sun Yat-sen University Cancer Center, Guangzhou, China. None of the patients had received radiotherapy or chemotherapy before surgery. The use of tissues for this study was approved by the Institute Research Medical Ethics Committee of Sun Yat-sen University Cancer Center. No informed consent (written or verbal) was obtained for the use of retrospective tissue samples from the patients with breast cancer; most of whom were deceased, because this was not deemed necessary by the Ethics Committee, who waived the need for consent. All samples were anonymous.

Plasmid construction and lentivirus production

The firefly luciferase-expressing vector psiCHECK-2 was used in the luciferase reporter assay. To construct the psiCHECK2-TWIST1-3′-UTR wt plasmid, a wild-type 3′ UTR segment of TWIST1 mRNA that contains the putative miR-720 binding sites was amplified and cloned into the XhoI and NotI sites downstream of the luciferase reporter gene in psiCHECK-2. psiCHECK2-TWIST1-3′-UTR-mut carries a mutated sequence in the miR-720 binding sites. Lentivirus with miR-720 expression vector (PLV-720) and control vector (PLV3) were from GenePharma. Briefly, oligonucleotides encoding short hairpin RNA with mature miR-720 sequences (Supplementary Table S2, available at Carcinogenesis Online) were subcloned into the restrictive sites BamHI and EcoRI of pGLV3/H1/GFP+ Puro Vector (GenePharma Co., Ltd) and verified by DNA sequencing. Virus particles were harvested 48 h after cotransfection of PLV-720 or PLV3 with the lentivirus packaging vector into HEK-293T cells. TWIST1 complementary DNA lacking its 3′ UTR or carrying a wild-type 3′ UTR expression cassette with the miR-720 response element were cloned into pcDNA 3.1 for ‘rescue’ experiments.

Oligonucleotide and plasmid transfection and real-time RT–PCR

miR-720 mimic and non-specific negative control oligos were synthesized by Invitrogen (Camarillo, CA). In total, 100 nmol/l of miR-720 mimic was used for transfection experiments. Oligonucleotide and plasmid transfection were performed using RNAmax and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Total RNA was extracted with Bioopure reagent (Bioo Scientific). Regular and real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR) were used to detect the expression of primary transcript of TWIST1. Briefly, 1 μg of the total RNA was reversely transcribed using oligo-dt primer (Takara). Real-time PCR was conducted using SYBR Premix Ex Taq (Takara) with the Stratagene Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA). The reactions were incubated in a 96-well plate at 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 55°C for 30 s and 72°C for 1 min. The fold changes of mRNA expression were calculated using the 2−ΔΔCt method. Sequences of small interfering RNAs (siRNAs) and primers for transfection and PCR are provided in Supplementary Table S2, available at Carcinogenesis Online. 18s ribosomal RNA was used as internal controls for mRNA quantification.

Quantitative stem-loop RT–PCR

Quantitative stem-loop RT-PCR for mature miR-720 was performed. Reverse transcription (RT) reactions contained purified total RNA, 50 nM miR-720-specific stem-loop RT primer, 50 nM U6 RT buffer (Promega), 0.25 mM deoxyriboonucleotide triphosphates, 3.33 U/μl M-MLV (Promega), 0.25 U/μl RNase inhibitor (Promega). The reactions were incubated at 16°C for 30 min, 37°C for 30 min, 70°C for 10 min and then held at 4°C. Real-time PCR conducted using SYBR Premix Ex Taq (Takara) with the Stratagene Mx3000P Real-Time PCR system (Agilent Technologies). The reactions were incubated in a 96-well plate at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 62°C for 30 s. The fold changes of miRNA expression were calculated using the 2−ΔΔCt method. Sequences of primers for PCR are provided in Supplementary Table S2, available at Carcinogenesis Online. snRNA U6 was used as internal controls for miRNA quantification.

Cell proliferation assays

Cells were seeded at 1500 cells per well in 96-well plates and cultured for 1–5 days after transfection. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (AMRESCO, Solon, OH) was added and plates were incubated for 3 h at 37°C. Supernatants were then removed and formazan crystals were dissolved in 150 μl dimethyl sulfoxide. The absorbance at 490 nm of each sample was measured using a multilabel plate reader (PerkinElmer).

Migration and invasion assays

Briefly, 3 × 104 cells were suspended in 200 μl serum-free Dulbecco’s modified Eagle’s medium and seeded in the upper compartment of a Transwell chamber (Corning, Lowell, MA). For the cell invasion assay, Matrigel (BD Biosciences, Sparks, MD) was coated on the upper surface of the transwell chamber before use. After 24–48 h incubation, the cells that migrated or invaded through the membrane were fixed with methanol and stained with crystal violet. Images of five randomly selected fields of cells were captured and the cells were counted.

Wound-healing assays

Cells were seeded into 6-well plates and allowed to grow to ~95% confluence. A vertical wound was created using a 200 μl pipette tip, and the old medium was replaced with serum-free medium. Images were captured at 0 and 48 h under the microscope to assess the rate of gap closure.

Luciferase reporter assays

HEK-293T cells were cotransfected with 20 μM of either miR-720 mimic or the negative control and 500 ng of psiCHECK2-TWIST1-3′-UTR-wt or psiCHECK2-TWIST1-3′-UTR-mut. Cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega, San Luis Obispo, CA). Luciferase activity was detected using a GloMax fluorescence reader (Promega, San Luis Obispo, CA). A psiCHECK-2 vector that provides constitutive expression of Renilla luciferase was cotransfected as an internal control.

Western blotting

Whole cell proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and blotted onto nitrocellulose membranes. The filters were hybridized with polyclonal anti-TWIST1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-E-Cadherin, anti-β-Catenin, anti-N-Cadherin, anti-Vimentin, anti-Fibronectin and anti-matrix metalloproteinase-2 (MMP-2) (Epitomics, Burlingame, CA) at 4°C overnight, followed by incubation with secondary anti-rabbit or anti-mouse antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) was used as a loading control.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as described (25). Briefly, after blocking, the sections were incubated with primary antibodies overnight (anti-TWIST1, 1:50; Abcam), followed by incubation with secondary antibodies and further incubation with the Streptavidin–Biotin complex (Dakopatts, Glostrup, Denmark). Reactivity was developed in chromogen diaminobenzidine solution. After contrast staining, the sections were dehydrated and mounted. A brown particle in a cell was considered as positive labeling.

Animal studies

MDA-MB-231 cells infected with pLV3 or pLV3-720 were resuspended in phosphate-buffered saline. Five-week-old female nude BALB/c athymic mice were injected with 5 × 105 cells via the tail vein. Six weeks later, mice were killed. Lungs of the mice were fixed and stained with hematoxylin and eosin. Lung metastasis was quantified by counting the number of tumor foci in 10 randomly selected high-power fields.

Statistical analysis

Data from at least three separate experiments are presented as means ± SD. The Student’s t-test was used for comparisons between groups unless otherwise noted. Differences were considered significant for P values <0.05.

Results

miR-720 is frequently downregulated in human breast cancer

To evaluate the expression of miR-720 in breast cancer, 48 pairs of primary breast cancer tissues and their corresponding adjacent non-tumor tissues were subjected to qRT–PCR. Results showed that miR-720 expression in primary breast cancer tissues was significantly lower than that in pair-matched adjacent non-tumor tissues (P < 0.01; Figure 1A). In 77.1% (37 of 48) of the primary breast cancer samples, miR-720 expression was reduced by at least 4-fold as compared with the matched non-tumorous sample (Figure 1B).

We next examined miR-720 expression in a series of human mammary tumor cell lines. miR-720 expression was reduced in several breast cancer cell lines compared with the immortalized mammary cell line MCF-10A (Figure 1C). Interestingly, MDA-MB-231 and MDA-MB-435, which are of high metastatic capacity, expressed the lowest levels of miR-720 (Figure 1C). Furthermore, on average, miR-720 expression was reduced in metastatic tumors compared with primary tumors for 31 patients with metastatic breast cancer (P < 0.05; Figure 1D).
miR-720 expression correlates inversely with metastasis in human breast cancer

Statistical analysis of 105 cases of breast cancer patients revealed that downregulation of miR-720 was significantly associated with aggressive clinicopathological features, including lymph node metastases (P = 0.003), positive expression of human epidermal growth factor receptor-2 (HER2) (P = 0.030) and negative expression of vascular endothelial growth factor (VEGF) (P = 0.008). There was no statistical correlation between miR-720 expression and the other clinicopathological parameters, such as age, tumor size, clinical stage, estrogen receptor, progesterone receptor, Ki67 and p53 (P > 0.05; Supplementary Table S1, available at Carcinogenesis Online). These data indicate that miR-720 expression may be inversely associated with tumor metastasis.

To understand the prognostic significance of miR-720 in breast cancer, the correlation between the expression of miR-720 and patient prognosis was analyzed. Results showed no statistical significant connection between low miR-720 expression and patient survival (P = 0.061; Supplementary Figure S1A, available at Carcinogenesis Online).

miR-720 suppresses cell invasion and migration in vitro

To determine the potential role of miR-720 in breast cancer metastasis, cell invasion and migration assays were performed. SKBR-3 and MDA-MB-231 cells were transfected with a miR-720 mimic, and the expression of mature miR-720 was confirmed by qRT–PCR (Figure 2A). Reexpression of miR-720 significantly inhibited cell viability in SKBR-3 cells but not in MDA-MB-231 cells (Figure 2B). Furthermore, miR-720 reexpression in both breast cancer cell lines remarkably suppressed migration ability as determined by transwell assays, showing that migrated cells are dramatically decreased in miR-720-expressed cells (Figure 2C). The inhibitory effect of miR-720 on cell migration was further confirmed by wound-healing assays (Figure 2D and Supplementary Figure S3A, available at Carcinogenesis Online). Consistently, miR-720 reexpression resulted in diminished invasive abilities in both cell lines (Figure 2E). These observations indicate that miR-720 inhibits metastasis in breast cancer by impeding cell invasion and migration.

miR-720 directly targets TWIST1

We next searched for the potential targets of miR-720 using two bioinformatic algorithms (TargetScan and micrometa.org). For both algorithms, TWIST1 was found as a potential effector of miR-720 based on the putative target sequence at 200–206 base pairs of the 3’UTR (Figure 3A). To determine whether TWIST1 is regulated by miR-720 via direct binding to its 3’UTR, luciferase reporter assays were performed in HEK-293T cells. Luciferase activity was reduced in cells cotransfected with luciferase reporter psi-TWIST1-3’UTR-WT and miR-720 mimic as compared with the negative control cells. This suppressive effect was abolished by mutation of the miR-720 target sequence (Figure 3B). On the other hand, reexpression of miR-720 led to a significant decrease in TWIST1 expression at both mRNA and protein levels (Figure 3C). In addition, inhibition of miR-720 resulted in the upregulation of TWIST1 protein (Supplementary Figure S2B, available at Carcinogenesis Online). In human primary breast cancer tissues, the expression of TWIST1 was markedly inversely correlated with the level of miR-720 (P = 0.038, r = –0.467; Figure 3D). Similar results were obtained by IHC (Figure 3E). In human breast cancer cell lines, a steady increase of TWIST1 correlated with a gradual decrease of miR-720 (Figure 3F). Consistent with the inhibition of metastasis, miR-720 overexpression resulted in the upregulation of epithelial markers (E-cadherin and β-catenin) and downregulation of TWIST1.
Fig. 2. Reexpression of miR-720 suppresses cell migration and invasion in vitro. (A) miR-720 was reexpressed in SKBR-3 and MDA-MB-231 cells after mimic transfection. miR-720 levels were determined by qRT–PCR 24 h after transfection. miR-720 expression (miR-720/U6) was calculated as the fold change relative to the negative control (NC). (B) Reexpression of miR-720 inhibits cell proliferation in SKBR-3 but not in MAD-MB-231 cells. Cell viabilities were determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay. (C) miR-720 expression decreased the number of migrated cells in transwell assays. Cells were seeded into transwell chambers. After 48 h, migrated cells that stained with 0.1% crystal violet were counted. Data represent the means ± SD from three independent experiments. (D) The inhibitory effect of miR-720 on cell migration was further confirmed by wound-healing assays. Data represent the means ± SD from three independent experiments. (E) miR-720 expression inhibits cell invasion in breast cancer cells. Data represent the means ± SD from three independent experiments (*P < 0.05).
miR-720 targets TWIST1

Fig. 3. miR-720 directly targets TWIST1. (A) The putative miR-720-binding sequence in the 3′UTR of the TWIST1 mRNA is shown. Mutation was generated in the TWIST1 3′UTR sequence at the complementary site for the seed region of miR-720. Human TWIST1 3′UTR fragments containing either the wild-type or mutant miR-720-binding sequences were cloned downstream of the luciferase reporter gene. (B) Luciferase reporter assays show the miR-720-mediated suppression of the activity of the wild-type TWIST1 3′UTR luciferase, but not the mutant 3′UTR luciferase, in HEK-293 cells (**P < 0.01). (C) Reexpression of miR-720 in SKBR-3 and MDA-MB-231 cells attenuated the expression of TWIST1 at both mRNA and protein levels. The value under each lane indicates the relative expression level of TWIST1, which is represented by the intensity ratio between TWIST1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or 18s ribosomal RNA (rRNA) bands in each lane. (D) TWIST1 expression is inversely correlated with miR-720 expression in human primary breast cancer samples (n = 20). The expressions of TWIST1 and miR-720 were determined by real-time RT–PCR. The levels of TWIST1 and miR-720 were normalized to 18s rRNA and U6, respectively. (E) IHC staining shows TWIST1 expression in human primary breast cancer samples with low and high miR-720 expression. (F) Expression of TWIST1 in the human immortalized normal breast cell line (MCF-10A) and human breast cancer cell lines as assessed by western blotting. (G) Reexpression of miR-720 upregulates epithelial markers (E-cadherin and β-catenin) and downregulates mesenchymal markers (N-cadherin, fibronectin, vimentin and MMP-2).
of mesenchymal markers (N-cadherin, fibronectin, vimentin and MMP-2) (Figure 3G).

**miR-720 expression mimics metastatic inhibition caused by TWIST1 siRNA**

To evaluate whether miR-720 suppresses cell migration and invasion by downregulating TWIST1, cells were transfected with either TWIST1 siRNA or miR-720 mimic. Similar reductions in TWIST1 protein were induced by TWIST1 siRNA and miR-720 mimic in SKBR-3 and MDA-MB-231 cells (Figure 4A). Silencing of TWIST1 significantly suppressed cell migration and invasion. Strikingly, the reintroduction of miR-720 noticeably phenocopied the consequence of TWIST1 knockdown by siRNA (Figure 4B and C). These findings imply that miR-720 regulates tumor metastasis, at least in part, by suppressing TWIST1 expression.

**Reexpression of TWIST1 reverses the miR-720-induced inhibition of cell invasion and migration in vitro**

To determine whether in vitro phenotypes associated with miR-720 expression could be reversed via TWIST1 reexpression, plasmids expressing TWIST1 with [3′ UTR (+)] or without [3′ UTR (−)] its 3′ UTR were constructed. Reintroduction of TWIST1 3′ UTR (−) markedly reversed the inhibition of cell migration and invasion in the miR-720-expressing cells, whereas TWIST1 3′ UTR (+) expression had no effects on these traits (Figure 5A and B). Mechanistically, ectopic expression of TWIST1 3′ UTR (−) noticeably abrogated the miR-720-mediated induction of E-cadherin and the miR-720-mediated suppression of N-cadherin and MMP-2 (Figure 5C). Collectively, these data suggest that TWIST1 is a functionally relevant effector of miR-720-mediated ant metastatic activity.

**miR-720 expression inhibits metastasis in vivo**

To confirm that reexpression of miR-720 suppresses migration and invasion in vivo, nude mice were injected with MDA-MB-231 cells expressing miR-720 via the tail vein. After 6 weeks of injection, body weights of the mice did not differ (Figure 6A). However, the lungs from the miR-720 group were significantly lighter as compared with the control group (Figure 6B). On the other hand, only 3 of the 11 mice from the miR-720 group developed lung metastasis. In the control group, 9 of the 11 mice presented lung metastasis. Furthermore, the number of tumor foci found in the control group was much more than that in the miR-720 group (Figure 6C–E). IHC showed downregulation of TWIST1 in the tumors formed in the miR-720 groups (Figure 6F). These results indicate that reexpression of miR-720 suppresses breast cancer metastasis by downregulating TWIST1 in vivo.

**Discussion**

Tumor metastasis is one of the primary causes of death in breast cancer patients. Here, we demonstrate that downregulation of miR-720 is significantly associated with invasive features of breast cancer, and that it functions as an antimetastatic miRNA. These results elucidate a role for miR-720 deregulation in breast cancer metastasis and enrich...
miR-720 targets TWIST1

our understanding of the molecular mechanisms of breast cancer metastasis.

Differential expression of miR-720 has been reported in several human diseases. It is upregulated in myelodysplastic syndromes (26) and cutaneous malignant melanoma (27), but downregulated in rectal cancer (28) and esophageal squamous cell carcinoma (13). Interestingly, the potential value of miR-720 for diagnosis is emphasized in myeloma. miR-720 and miR-1308 in serum together provide a powerful diagnostic tool for distinguishing normal healthy controls from precancerous myeloma and myeloma patients (29). Our data showed that miR-720 is frequently downregulated, and its low expression is associated with aggressive clinicopathological features in breast cancer. Although there is no significant correlation between its expression and patient survival, high expression of miR-720 tends to improve the overall survival of breast cancer according to the statistical data of 105 patient cases.

Fig. 5. Expression of TWIST1 using a construct lacking its 3′UTR rescues the biological effect associated with reexpression of miR-720. (A and B) Ectopic expression of TWIST1 3′UTR (−) rescued the inhibition of cell migration and invasion associated with the reexpression of miR-720. Transwell assays were performed after the cotransfection of miR-720 mimics and the TWIST1 3′UTR (+) or 3′UTR (−) vectors (*P < 0.05, **P < 0.01). (C) TWIST1 3′UTR (−) reintroduction abrogated both miR-720-induced downregulation of N-cadherin and MMP-2, and miR-720-induced upregulation of E-cadherin.
As a critical epithelial–mesenchymal transition (EMT) and metastasis promoter, in the present study, TWIST1 was found to be directly regulated by miR-720 at the posttranscriptional level. It was recently reported that TWIST1 is directly regulated by some miRNAs. miR-580, CPEB1 and CPEB2 act as negative regulators of TWIST1 expression in a sequence-specific and additive/cooperative manner (30). miR-214 directly targets TWIST1 to suppress EMT and metastasis in intrahepatic cholangiocarcinoma (31). miR-124 was found to be involved in cellular differentiation through the suppression of TWIST1 and SLUG in glioma cells (32). Furthermore, during TWIST1-induced EMT, downregulation of E-cadherin and upregulation of N-cadherin and MMP-2 were observed (22,24,33). miR-214 can inhibit EMT and metastasis by targeting TWIST1 in intrahepatic cholangiocarcinoma; downregulation of miR-214 increases TWIST1 expression and decreases E-cadherin expression (31). Consistently, reexpression of miR-720 upregulated the expression of E-cadherin and downregulated the expressions of N-cadherin and MMP-2 in our study. In addition, the expression of TWIST1 using a construct lacking its 3′UTR reversed these alterations mediated by miR-720. We also provide evidence that miR-720, by targeting TWIST1, suppresses EMT and metastasis in breast cancer cells.

Fig. 6. Exogenous miR-720 expression suppresses breast cancer metastasis in vivo. Five-week-old female nude BALB/c mice were injected with $5 \times 10^5$ MDA-MB-231 cells expressing miR-720 via the tail vein. Six weeks later, the body weights (A) and the lung weights (B) of the mice were measured ($^{*}P < 0.05$). (C) Representative images of lung metastases are shown. (D) Representative hematoxylin and eosin staining pictures are shown. (E) The numbers of metastatic nodules in the lung were counted in 10 randomly selected high-power fields under a microscope. (F) IHC staining of TWIST1 in lung tissues from miR-720 and control groups is shown. ($^{**}P < 0.01$).
Our statistical analysis revealed that downregulation of miR-720 was significantly associated with positive expression of HER2 ("P = 0.030") and negative expression of VEGF ("P = 0.008"). The HER2 gene is a well-established therapeutic target for breast cancer. Overexpression of HER2 is found in 20–30% of breast cancer ("34") and correlates with an aggressive phenotype and poor prognosis in patients with breast cancer ("35"). TWIST1 has been reported to upregulate HER2 by enhancing Y-box binding protein ("YB-1") in breast cancer ("36"). This can, at least partly, explain the significant association that we observed between the downregulation of miR-720 and the positive expression of HER2 in breast cancer. Moreover, the impact of miR-720 on patient prognosis was further investigated based on HER2 expression. Results revealed that patients with low miR-720 expression in HER2-negative subgroup survived shorter than those with high miR-720 expression ("P = 0.025; Supplementary Figure S1C, available at Carcinogenesis Online"). This suggests patients with high miR-720 expression may receive more benefits from the loss of HER2 expression. The mechanisms of regulation among miR-720, TWIST1 and HER2 are worth investigation in future studies. In addition, our clinicopathological analysis showed that downregulation of miR-720 is significantly related to negative expression of VEGF. However, VEGF can be upregulated by TWIST1 in prostate cancer, bladder cancer and hepatocellular carcinoma ("33,37"). This result indicates that miR-720 may participate in the regulation of VEGF by mechanisms other than the TWIST1 pathway.

In most previous studies, miR-720 has been studied as an miRNA. It participates in epithelial development by targeting p63 in adult mouse keratinocytes ("12") and inhibits cell proliferation by targeting p63 in esophageal squamous cell carcinoma cells ("13"). However, it was recently reported that miR-720 is probably a fragment from transfer RNA ("tRNA"), rather than an miRNA ("38"). Over the last several years, a significant number of tRNA-derived RNA fragments ("tRFs") have been identified in several independent next-generation sequencing studies ("39–41"). These tRFs are derived from precise processing at the 5‘ or 3’ end of mature or precursor tRNAs. The tRFs from the 3’ end of mature tRNAs ("3′CCA tRFs") contain a tRNA-like ‘CCA’ motif at their 3’ ends ("42"). Accumulating evidence suggests that tRFs participate in several biological functions such as the cellular stress response ("43"), cell proliferation ("39") and the regulation of gene expression ("41,44"). However, investigations on the function of tRFs in cancer are limited. Yong et al. ("17") identified 17 abundantly cloned tRFs in prostate cancer cell lines. One of these tRFs, tRF-1001, was found to correlate with the cellular proliferation rate, and knockdown of tRF-1001 by siRNA resulted in impaired cell proliferation ("39"). The mature sequence of miR-720 has a ‘CCA’ motif at its 3’ end as the 3′CCA tRFs matches the 3’ end sequence of tRNA™. Moreover, the predicted score of miR-720 by the miPred algorithm is 64.4%, which is below the threshold of 73.3% ("38"). These lead an argument on whether miR-720 is a miRNA or a tRF. Thus, further studies are required to clarify the origin of miR-720.

In the present study, stem-loop RT primers were designed and used to quantify miR-720 expression. Although it is a well-developed way to amplify and quantify mature miRNAs, the qRT–PCR strategy for quantifying miR-720 may crosswise react with its precursor or tRNA fragments. Tang et al. ("45") showed that the level of pre-miR-720 was much higher than that of the mature miR-720, and that pre-miR-720 was able to react with its miRNA probes. In another study, the mature sequence of miR-720 matches the 3’ end sequence of tRNA™ ("38"). It is therefore possible that the stem-loop RT primer used to detect mature miR-720 in our study may misprimer to its precursor or tRNA fragments, which may consequently result in cross detections. Thus, it should be noticed that the signal detected in our assay could be obtained from the mixture of mature miR-720, pre-miR-720 and tRNA fragments. However, our data may, to some extent, reflect the clinical significance of miR-720.

In summary, our study provides experimental evidence that miR-720 suppresses cell migration and invasion in breast cancer by directly targeting TWIST1. miR-720 is identified as an important antimetastatic miRNA that is frequently downregulated in breast cancer. The present study implies the intriguing possibility of using this miR-720 as an innovative and promising therapeutic target for breast cancer.

**Supplementary material**

Supplementary Tables S1 and S2 and Figures S1–S3 can be found at http://carcin.oxfordjournals.org/

**Funding**

National Natural Science Foundation of China (81372572, 81172345, 81201717); the Project of State Key Laboratory of Oncology in South China.

**Acknowledgement**

We thank Prof. Xin-Yuan Guan for kindly providing the human breast cancer cells MDA-MB-231 and the immortalized mammary cell line MCF-10A.

**Conflict of Interest Statement:** None declared.

**References**


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Received March 28, 2013; revised August 28, 2013; accepted September 23, 2013.