miR-125b is Methylated and Functions as A Tumor Suppressor by Regulating the ETS1 proto-oncogene in Human Invasive Breast Cancer

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Running title: miR-125b suppresses breast cancer formation

Key Words: invasive breast cancer; miR-125b; methylation; cell cycle; ETS1;
Abstract

The microRNA miR-125b is dysregulated in various human cancers but its underlying mechanisms of action are poorly understood. Here we report that miR-125b is downregulated in invasive breast cancers where it predicts poor patient survival. Hypermethylation of the miR-125b promoter partially accounted for reduction of miR-125b expression in human breast cancer. Ectopic restoration of miR-125b expression in breast cancer cells suppressed proliferation, induced G1 cell cycle arrest \textit{in vitro} and inhibited tumorigenesis \textit{in vivo}. We identified the ETS1 gene as a novel direct target of miR-125b. siRNA-mediated ETS1 knockdown phenocopied the effect of miR-125b in breast cell lines and ETS1 overexpression in invasive breast cancer tissues also correlated with poor patient prognosis. Taken together, our findings point to an important role for miR-125b in the molecular etiology of invasive breast cancer, and they suggest miR-125b as a potential theranostic tool in this disease.
Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that modulate gene expression by binding to the 3'-untranslated region (3'UTR) of target mRNA, and promoting RNA degradation, inhibiting mRNA translation, and affecting transcription (1). Growing evidence indicates that miRNAs play important roles in biological processes including development (2), cell proliferation, apoptosis (3) and differentiation (4). miRNAs are aberrantly expressed or mutated in cancer, suggesting that they may function as either tumor suppressors or oncogenes (5).

miR-125b, a brain-enriched miRNA, is evenly distributed between neurons and astrocytes (6). Recent reports suggest that miR-125b might act as an oncogene or as a tumor suppressor, depending on the cellular context. Deregulation of miR-125b has been observed in invasive breast cancer (BC), ovarian carcinoma, hepatocellular carcinoma, and thyroid carcinoma (7-10), and it is also associated with clinical outcome in liver cancer patients (9). Expression of miR-125b is elevated in pancreatic cancer, oligodendroglial tumors, prostate cancer, myelodysplastic syndromes, and acute myeloid leukemia (11-14). miR-125b was reported to suppress cell proliferation by downregulation of ERBB2 and ERBB3 in BC, but to promote proliferation by downregulation of Bak1 in prostate cancer cells (15). miR-125b was also reported to negatively regulate p53, and suppress p53-dependent apoptosis in both zebrafish and humans (16).

In this study, we presented evidences that miR-125b was hypermethylated and functioned as a tumor suppressor by regulating the ETS1 proto-oncogene in invasive...
Materials and Methods

Tissue specimens and TMAs construction

All the BC tissues were obtained from Sun Yat-sen University Cancer Center (SYSUCC, Guangzhou, China), and classified according to the American Joint Committee on Cancer (AJCC) and tumor-lymph node-metastasis (TNM) classification system (17). For miR-125b quantitative analysis, formalin-fixed paraffin-embedded tissues (FFPETs) of 105 invasive BCs and 40 paired normal adjacent tissues (NATs, >2 cm from cancer tissue) were used. For DNA methylation and miR-125b quantitative analysis, as well as in situ hybridization (ISH) analysis, fresh samples from nine invasive BCs and paired NATs were used. For immunohistochemical (IHC) staining, FFPETs of 221 surgical patients with invasive BC were used for the TMA construction (18). The study was approved by the Research Ethics Committee of SYSUCC (Reference number: YP-2009174). Detailed patients recruitment and methodologic issues are described in Supplementary Data.

The clinicopathological characteristics and follow-up data of the patients were summarized Supplemental Table 1.

Locked nucleic acid (LNA)-based ISH

Detailed methods are described in Supplementary Data.

Quantitive real-time PCR (qRT-PCR) analysis for miR-125b

Total RNAs were isolated from FFPETs using Trizol reagent (Invitrogen, Carlsbad,
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CA) according to the manufacturer’s instructions. Quantitative analysis of miR-125b expression in 105 invasive BC tissues was assayed using a Hairpin-it miRNA real-time PCR Quantitation Kit (GenePharma, Shanghai, China). qRT-PCR analysis was performed on an ABI 7900HT instrument (Applied Biosystems, Foster, CA). Each sample was analyzed in triplicate, and U6 snRNA was used for normalization. The quantity of miR-125b in each invasive BC sample relative to the average expression in 40 NATs was calculated using the equation: relative quantity (RQ) = $2^{-\Delta \Delta CT}$ (19). The primers were in Supplemental Table 2.

**Bisulfite sequencing and methylation-specific PCR (MSP)**

The primers for bisulfite sequencing and methylation-specific PCR used in this study were designed by MethPrimer (20), and detailed methods are described in Supplementary Data.

**Cell culture and treatments**

Human BC cell lines (MCF-7, MDA-MB-231 and MDA-MB-453) were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China) and used within 2 months after resuscitation of frozen aliquots. MDA-MB-435 BC cell line was kindly provided by Dr. Xiao-Ming Xie (Department of Breast Oncology, Sun Yat-sen University, Guangzhou, China). Cell lines were authenticated based on viability, recovery, growth, morphology, and isoenzymology by the provider. Culture conditions are described in Supplementary Data.

For 5-aza-2’deoxycytidine (5-aza-CdR) treatment, human BC cell lines MCF-7, MDA-MB-435, MDA-MB-231 and MDA-MB-453 were seeded at $2 \times 10^5$ per well in
six-well plates and cultured with 5 μM 5-aza-CdR (Sigma-Aldrich, St Louis, MO) for 72 h respectively. The medium containing drug was replaced every 24 h. RNA was isolated and qRT-PCR was performed to evaluate the restoration of miR-125b after 5-aza-CdR treatment.

For cells transfection, pre-miR-125b precursor molecules and negative control (pre-control) were bought from Ambion Inc. (Ambion, Austin, TX). ETS1-siRNAs and control-siRNA were from GenePharma (GenePharma). The sequences of siRNAs were listed in Supplemental Table 2. Transfection of pre-miR-125b, pre-control, ETS1-siRNA, and control-siRNA were used with Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Transfected cells were incubated at 37°C for 24 h, followed by incubation with complete medium. Cells were harvested at indicated time points.

**Cell proliferation assay and colony-forming assay**

Detailed methods are described in Supplementary Data.

**Flow cytometry**

Detailed methods are described in Supplementary Data.

**In vivo tumorigenicity assays**

Detailed methods are described in Supplementary Data.

**cDNA microarray analysis**

Total RNA was isolated at 12 h or 24 h post-transfection from MCF-7 or MDA-MB-435 cells, using Trizol reagent (Invitrogen). The mRNA expression profile was performed using a human genome oligo array service V1.0 (Cat. No. 220010;
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CapitalBio, Beijing, China). Each sample was analyzed once, and data preprocessing, normalization, and filtering were conducted as previously described (21). All microarray data had been deposited to the Gene Expression Omnibus public database with accession number GSE22546.

**Validation of cDNA microarray**

qRT-PCR was conducted as previously described (22) and primers were in Supplemental Table 2. Relative expression was calculated using the equation $RQ = 2^{-\Delta\Delta CT}$ (19).

**Luciferase reporter assay**

Detailed methods are described in Supplementary Data.

**Immunoblot analysis**

Antibodies used for immunoblots were RPS6KA1 (1:1000 dilution, Cell Signaling Technology, Danvers, MA), C9orf86 (1:200 dilution, Abcam, Cambridge, MA), MANIB1 (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), ASB13 (1:500 dilution, Abcam), MKNK2 (1:500 dilution, Abcam), UBE2E3 (1:500 dilution, Abcam), ETS1 (1:1000 dilution, Abcam) and anti-GAPDH antibody (1:3000 dilution, Santa Cruz), as a loading control. All protein bands were detected using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

**Immunohistochemistry staining**

Dako Real Envision Kit (K5007, Dako, Carpinteria, CA) was used in IHC Staining analysis. Hormonal receptors were evaluated with the 1D5 antibody for estrogen receptor a (ER) and antibody PGR-1A6 for the progesterone receptor (PR; Dako).
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CerbB2 was detected with CB11 (Dako). ETS1 was detected with a primary antibody ETS1 (1:500 dilution, Abcam). Three observers independently determined consensus scoring of IHC staining using a semi-quantitative estimation, as described previously (23). Samples with scores lower than the median score were grouped as low protein expression (24).

Statistical analysis

Data were analyzed using SPSS16.0 software (SPSS Inc., Chicago, IL). Significant associations between miR-125b expression and clinicopathological parameters were assessed using a chi-square test. Survival curves were plotted by Kaplan-Meier analysis and compared by the log-rank test. Cox regression analysis was performed to assess the significance of variables for survival. Data were expressed as mean ± standard deviation (SD), and t-test was used to determine the significance of differences between two groups. All tests performed were two-sided. *P* <0.05 was considered statistically significant.

Results

miR-125b expression is downregulated in invasive BC, and correlated with poor prognosis and lymph node metastasis

A decrease in miR-125b staining intensity was observed in invasive BC tissues, compared to corresponding NATs by chromogenic *in situ* hybridization (CISH) (Fig. 1A). Parallel detection by fluorescent *in situ* hybridization (FISH) is shown in Supplemental Fig. S1. The expression of miR-125b was detected by qRT-PCR in the
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FFPETs of 105 invasive BCs and 40 NATs (Fig. 1B). Consistent with the results of CISH and FISH, miR-125b expression levels in BCs (-2.69 ± 0.49, mean ± SD) were significantly lower than that in NATs (1.10 ± 0.27, mean ± SD) (*P* < 0.001, Fig. 1B).

105 invasive BC tissues were used to analyze the correlation between miR-125b expression and clinicopathological characteristics of invasive BC (Table 1). Low miR-125b expression correlated with lymph node metastasis (*P* = 0.017), and no correlation was observed between miR-125b expression and age, pathologic grade, clinical stage, estrogen receptor (ER) status, progesterone receptor (PR) status, or CerbB2 status.

The 5-year overall survival rate of the 105 invasive BC patients was 68.6% (Fig. 1C). The 5-year survival rate of BC patients with low miR-125b expression was 53.8 % (n = 50), which was significantly lower than that in patients with high miR-125b expression (81.8%, n = 55; *P* = 0.002, Fig. 1C). When patients were stratified by lymph node status, low miR-125b expression exhibited a significant correlation with poor survival of patients without lymph node metastasis (*P* = 0.003, Fig. 1C). However among the patients with lymph node metastasis, miR-125b expression did not show a statistical correlation with survival (*P* = 0.232, Fig. 1C). In addition, in the BC patients with early clinical stage stratum, low CerbB2 stratum, PR positive stratum or treatment with surgery & adjuvant therapy stratum, miR-125b expression exhibited a significant correlation with survival (Supplemental Fig. S2).

Univariate Cox proportional hazard regression analysis revealed that low miR-125b expression was a significant predictive factor for poor prognosis (hazard ratio, HR =
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2.98; \( P = 0.004 \)). Multivariate Cox proportional hazard regression analysis revealed that low miR-125b expression (HR = 2.60, \( P = 0.012 \)) was also a significantly unfavorable independent prognostic factor in BC patients (Supplemental Table 3).

**miR-125b is methylated in BC**

Recent studies demonstrated that the methylation state of distal CpG-rich regions correlated with the expression pattern of miRNAs in cancer (25). Here, we defined a CpG-rich region as island length > 100 bp, GC percent > 50, Observe/Expect > 0.60. Two CpG-rich regions within 2000 bp upstream of the miR-125b-1 gene were found by using MethPrimer with the default criteria of the online tool (20). Recently, researchers revealed that the transcription factor NF-kappaB p65 binding site of the promoter element of miR-125b-1 resided in -1059 to -1050 region of the miR-125b-1 gene (26). Given that NF-kappaB p65 binding site of the promoter element of miR-125b-1 is close to CpG-rich region 1 (Fig. 2A), we carried out bisulfite sequencing to assess CpG methylation status within an -843 to -1052 region upstream of miR-125b-1 in two invasive BC tissues, paired NATs, as well as two BC cell lines MCF-7 and MDA-MB-435 cells (Fig. 2A). CpG-rich region 1 within upstream of miR-125b-1 was found to be heavily methylated in the two BC cell lines and invasive BC tissues, while NATs showed absence of methylation (Fig. 2A).

To investigate whether hypermethylation status of miR-125b-1 could affect miR-125b expression in BC, four BC cell lines MCF-7, MDA-MB-435, MDA-MB-231 and MDA-MB-453 were treated with a demethylating agent 5-aza-CdR, then methylation status and expression of the miR-125b were analyzed by
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MSP and qRT-PCR respectively. We found that miR-125b expression in the MCF-7, MDA-MB-435 and MDA-MB-231 cell lines, which showed hypermethylation in the promotor of miR-125b-1, were restored after treatment with the demethylating agent 5-aza-CdR for 72 h. However, MDA-MB-453 cell lines with unmethylation in the promotor of miR-125b-1, 5-aza-CdR treatment did not cause a obvious increase in miR-125b level (Figure 2B and 2C).

To determine the correlation between DNA methylation status and expression of the miR-125b in clinical samples, the methylation status of miR-125b-1 promoter and expression of miR-125b were analyzed in 9 invasive BC tissues and paired NATs by MSP and qRT-PCR respectively. The promoter of miR-125b-1 was hypermethylated in 55.6% (5/9) invasive BC tissues; however unmethylation in the promoter of miR-125b-1 was observed in all of 9 NATs. Furthermore, miR-125b levels in tissues with miR-125b promoter hypermethylation were significantly lower than those without miR-125b promoter hypermethylation ($P < 0.05$, Figure 2D). Taken together, these data suggested that hypermethylation of the CpG-rich region 1 within upstream of miR-125b-1 might be involved in miR-125b downregulation in invasive BC.

**miR-125b suppresses cell proliferation and clonogenicity, and induces G1 cell cycle arrest**

Overexpression of miR-125b in MDA-MB-435 and MCF-7 cells transfected with pre-miR-125b was confirmed by qRT-PCR and FISH (Supplemental Fig. S3). miR-125b overexpression in MDA-MB-435 and MCF-7 cells led to a decrease in growth and proliferation ($P < 0.05$, Fig. 3A). Evaluation of the effect of miR-125b on
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the clonogenicity of the two BC cell lines found that miR-125b-transfected cells displayed obvious colony inhibition compared to control transfectants (Fig. 3B). These results indicated that miR-125b may play an important role in tumor growth inhibition.

The cell cycle distribution of transfected cells was investigated using Flow cytometry. The proportion of G1 phase cells increased to 66.4 ± 2.9% in MDA-MB-435 cells transfected with pre-miR-125b, compared to 57.9 ± 4.8% in pre-control-transfected cells, at 48 h after transfection (Fig. 3C). Similarly, the proportion of G1 phase cells increased by 5.6% in MCF-7 cells transfected with pre-miR-125b compared to pre-control cells (Fig. 3C). No effect was observed on apoptosis or migration when MDA-MB-435 and MCF-7 cells were transfected with pre-miR-125b (Supplemental Fig. S4).

**miR-125b suppresses tumorigenicity in vivo**

To address the potential effects of miR-125b in vivo BC cell growth, equal numbers (5 x 10^6) of MDA-MB-435 cells transfected with pre-miR-125b or the pre-control were injected into female nude mice. As shown in Fig. 3D, MDA-MB-435 cells transfected with pre-miR-125b showed significantly reduced tumor growth compared to those transfected with pre-control. On day 18, the mean tumor volume in the pre-miR-125b transfection group (253.9 ± 183.7 mm^3) was smaller than that in the pre-control transfection group (486.0 ± 146.4 mm^3, P < 0.05). Moreover, the mean tumor weight of the pre-miR-125b group (0.085 ± 0.079 g) was lower than that of the pre-control group (0.165 ± 0.083 g, P = 0.045). These in vivo studies indicated that
miR-125b could inhibit the tumorigenicity of MDA-MB-435 cells in nude mice.

**miR-125b target gene screening and prediction**

Differential genes influenced by miR-125b in BC were evaluated by cDNA microarray analysis using CapitalBio chips. MDA-MB-435 and MCF-7 cells were transfected with pre-miR-125b or pre-control, and then microarray analyses were performed. We found 53 genes (22 upregulated and 31 downregulated) that were differentially expressed in both cell lines (Fig. 4A). Among these 53 candidate genes, six genes, which were predicted by at least one of TargetScan 5.1, miRBase Targets V.5, miRNAMap 2.0, PicTar, and miRanda 3.0 programs, were selected for further validation by qRT-PCR. Consistent with the microarray results, UBE2E3 showed increased mRNA levels, while MAN1B1, C9orf86, MKNK2, ASB13 showed decreased mRNA levels in both cell lines with miR-125b overexpression. However, in the MCF-7 cell line, the microarray and qRT-PCR results for PRS6KA1 were incompatible (Fig. 4B). We further examined the effect of miR-125b on the endogenous protein expression of UBE2E3, MAN1B1, C9orf86, MKNK2, ASB13, and PRS6KA1 by Western blot. Transfection of miR-125b induced an obvious decrease in MAN1B1, C9orf86, and MKNK2 proteins in both MDA-MB-435 and MCF-7 cells. UBE2E3 protein increased in MCF-7 transfected with miR-125b, but not in MDA-MB-435 cells. RPS6KA1 protein decreased in MDA-MB-435 transfected with miR-125b, but not in MCF-7 (Fig. 4C). In addition, the ETS1 protein decreased dramatically in both cell lines transfected with miR-125b, while its mRNA showed no obvious difference by microarray.
miR-125b directly inhibits ETS1 expression through the 3’UTR

To determine whether ETS1 responded to miR-125b through direct 3’UTR interaction, we cloned the 3’UTR of ETS1 into a reporter plasmid downstream of the luciferase gene to generate pMIR-report-ETS1 3’UTR and pMIR-report-vector. These plasmids, or miR-125b precursor or precursor control and pCMV-Renilla (internal control) were transiently transfected into HEK 293T cells. At 48 h after transfection, a dual-luciferase reporter assay system was used to detect luciferase expression. Overexpression of miR-125b using a miRNA precursor resulted in a significant decrease in luciferase expression in pMIR-report-ETS1 3’UTR-transfected cells, but not in pMIR-report-vector-transfected cells. In addition, transfection of pMIR-report-ETS1 3’UTR resulted in a decrease in luciferase expression (precursor control) compared to cells transfected with the pMIR-report-vector under the same conditions, suggesting a negative effect of the endogenous miR-125b on the ETS1 3’UTR (Fig. 5A). Taken together, these results indicated that ETS1 was a direct target of miR125b.

Knockdown of ETS1 suppresses cell growth, proliferation and induces G1 cell cycle arrest in vitro

To address the function of ETS1 in BC, MCF7 and MDA-MB-435 cells were transfected with ETS1-specific siRNAs. Immunoblot analysis indicated that when cells were transfected with an ETS1-specific siRNA (100 nM), at 48 h post-transfection the ETS1 protein was decreased significantly both in MCF7 and MDA-MB-435 cells (Fig. 5B). Knockdown of ETS1 led to inhibition of cell growth
and proliferation both in MCF-7 and MDA-MB-435 cells ($P < 0.05$, Fig. 5C). A colony-forming assay was performed to evaluate the effect of ETS1 on the clonogenicity ability of BC cells. ETS1-specific siRNA-transfected MCF-7 and MDA-MB-435 cells displayed fewer and smaller colonies compared to control siRNA-transfectants (Fig. 5C). Flow cytometry analysis revealed that the proportion of G1 phase cells increased to $75.7 \pm 0.5\%$ in MCF-7 cells transfected with ETS1-specific siRNA, compared to $60.5 \pm 1.5\%$ in control siRNA-transfected cells. The proportion of G1 phase cells increased to $66.4 \pm 1.4\%$ in MDA-MB-435 cells transfected with ETS1-specific siRNA, compared to $52.3 \pm 3.2\%$ in control siRNA-transfected cells (Fig. 5D).

**ETS1 is overexpressed in invasive BC and correlated with poor prognosis of BC patients**

To investigate ETS1 protein expression in invasive BC, IHC was performed to detect ETS1 expression in invasive BC tissues. ETS1 was found predominantly in the cytoplasm of BC tumor cells (Fig. 6A). Patients were divided into high ETS1 expression ($n = 118$, score $> 6$) and low ETS1 ($n = 103$, score $\leq 6$) expression. No significant correlation was observed between ETS1 and clinicopathological parameters (Supplemental Table 4). The 5-year overall survival rate of the 221 BC patients was $80.5\%$. The 5-year overall survival rate of patients with high ETS1 expression ($71.0\%$, $n = 118$) was significantly lower than those with low ETS1 expression ($91.1\%$, $n = 103$, $P < 0.001$, Fig. 6B). When patients were stratified by clinical stage, Kaplan–Meier survival estimates revealed that early stage (I-II) patients...
with high ETS1 expression (78.6%, n = 94) had inferior survival to those with low ETS1 expression (93.0%, n = 87; \( P < 0.001 \), Fig.6B). Similarly, late stage (III-IV) patients with high ETS1 expression (40.9%, n = 24) had inferior survival to those with low ETS1 expression (80.8%, n = 16; \( P < 0.001 \), Fig.6B). In patients subjected to only surgery stratum and adjuvant therapy stratum, ETS1 levels exhibited a significant correlation with survival (Supplemental Fig. S5).

Univariate Cox proportional hazard regression analysis revealed that high ETS1 expression was a significant predictive factor for poor prognosis (HR = 3.69, \( P = 0.001 \)). Multivariate Cox proportional hazard regression analysis revealed that high ETS1 expression (HR = 3.92, \( P < 0.001 \)) was also a significantly unfavorable independent prognostic factor (Supplemental Table 5).

**Discussion**

Consistent with previous report of miR-125b in BC (7), we showed that miR-125b was frequently downregulated in invasive BC tissues, and downregulation of miR-125b was associated with poor prognosis of the patients. These results suggested that miR-125b downregulation may play an important role in the development and progression of invasive BC. However, the underlying mechanism responsible for miR-125b dysregulation in invasive BC is still unknown. Recent studies showed that miRNAs can be dysregulated by promoter methylation in human malignacies (27, 28). In this investigation, we computationally mapped a CpG-rich region in the promoter region of the miR-125b-1. Hypermethylation of miR-125b-1 promoter was found in
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BC, and it was correlated with low levels of miR-125b expression. Among 4 cases of invasive BC tissues without methylation in CpG-rich region 1 within upstream of miR-125b-1, the expression of miR-125b in 3 cases was still downregulated, compared to paired normal adjacent tissues repectively. Taken together, these data revealed that DNA methylation of CpG-rich region 1 partially accounted for the reduction of miR-125b expression in invasive BC, and meanwhile other unknown factors might be also involved in the downregulation of miR-125b in BC.

The role of miR-125b in malignancies is controversial. miR-125b acts as a tumor suppressor in BC and hepatocellular carcinoma (7, 9), but acts as an oncogene to promote proliferation in prostate cancer (13). Consistent with previous findings in BC (13), our functional studies indicated that overexpression of an miR-125b precursor in MCF-7 and MDA-MB-435 BC cell lines significantly suppressed proliferation and colony formation ability of the cells in vitro, and inhibited tumor growth in vivo of mice xenografts.

Dysregulation of the cell cycle is involved in cell proliferation and tumorigenesis. Growing numbers of miRNAs have been implicated in the regulation of the cell cycle, for instance, the miR-15a/16-1 cluster affects the G0/G1-S phase transition in B cells (29). miR-125b was reported to induce cell cycle arrest at G1/S transition by suppressing CDK6 and CDC25A expression in CD133-positive U251 cells (30). In this study, overexpression of an miR-125b precursor increased G1 cell cycle arrest in BC cell lines, consistent with previous studies (30).

Identification of miR-125b target genes is critical for understanding its role in
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tumorigenesis, and is important for defining novel therapeutic targets. To date, ERBB2/ERBB3, Bak1, CYP24, NR2A, TNF-α Bmf, Smo, and p53 have been identified as targets of miR-125b (13, 15, 16, 31-35). We report that ETS1 is a target gene of miR-125b. ETS1 is a member of the ETS family of transcription factors, located on the long arm of chromosome 11 (11q23-q24), which is involved in regulating a wide variety of biological processes, including cellular growth, migration, and differentiation (36-38). ETS1 is required for angiogenesis under both physiological and pathophysiological conditions, such as chronic inflammatory reactions and tumor-associated angiogenesis. High ETS1 expression is associated with invasion and metastasis, and predicts poorer prognosis of cancer patients (39, 40). This study revealed that ETS1 was overexpressed in invasive BC, and overexpression of ETS1 predicted poor prognosis. Moreover, our data provides the first evidence that miR-125b decreases protein levels of ETS1 by targeting its 3’UTR. Functional studies by knockdown of ETS1 phenocopied overexpression of miR-125b in BC cells, resulting in G1 cell cycle arrest, cell growth suppression, proliferation inhibition in vitro, and suppression of the tumorigenicity of mice xenografts, suggesting that miR-125b acted as tumor suppressor by regulating the ETS1 target gene in the development and progression of BC. miR-125b overexpression or siRNA-mediated downregulation of the target gene ETS1 is a potential BC therapy.

In conclusion, we report that miR-125b expression was downregulated in invasive BC tissues and hypermethylation of miR-125b promoter partially accounted for the reduction of miR-125b expression. miR-125b is a tumor suppressor in the
miR-125b is methylated and regulate Ets-1 proto-oncogene development and progression of invasive BC, as it suppressed tumorigenesis, and induced G1 cell cycle arrest through its target gene ETS1. Low expression of miR-125b and overexpression of its target gene ETS1 predicted poor survival of BC patients. These data suggest that miR-125b plays an important role in BC carcinogenesis, and may have prognostic or therapeutic implications for the clinical management of BC patients.

Acknowledgments

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### Table 1. Relationship between miR-125b expression level and clinicopathologic parameters of invasive breast cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of cases</th>
<th>miR-125b (%)</th>
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<td>≥ 48</td>
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<td>24 (52.2)</td>
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miR-125b is methylated and regulate Ets-1 proto-oncogene

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ER, estrogen receptor; PR, progesterone receptor.
miR-125b is methylated and regulate Ets-1 proto-oncogene

Figure Legends

Figure 1. miR-125b was overexpressed in invasive BC and correlated with poor prognosis. A, Detection of miR-125b by CISH at 400× magnification. Positive in situ hybridization signals are in blue. miR-125b expression levels in invasive BC tissues is much lower than in corresponding NATs. B, Real-time PCR shows that expression of miR-125b is reduced in invasive BC tissues compared to NATs (P < 0.001). C, Kaplan–Meier survival curves and log-rank test for BC patients with high or low miR-125b expression and negative or positive lymph node status.

Figure 2. miR-125b was methylated in invasive BC tissues and BC cell lines. A, Methylation status of 8 CpG sites within the miR-125b-1 gene promoter was analyzed by bisulfite sequencing. Five clones were picked out and sequenced for two breast cancer tumors (BC1 and BC2), two normal adjacent tissues (NAT1 and NAT2) and two breast cancer cell lines (MDA-MB-435 and MCF7). Open and filled circles represented unmethylated and methylated CpG sites, respectively. Circles were partially filled according to the percentage of methylation of the CpG site. The frames showed the CpG pairs covered by MSP primers. B, MSP and qRT-PCR analysis were used to determine the methylation status and expression of miR-125b in BC cell lines MCF7, MDA-MB-435, MDA-MB-231 and MDA-MB-453. C, Relative expression of miR-125b in breast cancer cell lines MCF7, MDA-MB-435, MDA-MB-231 and MDA-MB-453, which were treated with 5 uM 5-aza-CdR. D, MSP and qRT-PCR analysis were used to determine the methylation status and expression of miR-125b in nine breast cancer tumors (BC1-9) and nine normal adjacent tissues (NAT1-9), then
miR-125b is methylated and regulate Ets-1 proto-oncogene
correlation of methylation status and expression of miR-125b was analyzed. (M: methylated alleles; U: unmethylated alleles).

**Figure 3.** miR-125b suppresses cell growth and proliferation, induces G1 cell cycle arrest *in vitro* and suppresses tumor growth *in vivo*. A, MTT assay showed that MDA-MB-435 and MCF-7 cells transfected with pre-miR-125b grew slower than cells transfected with pre-control. Data are expressed as absorbance. Results are means ± SD. *P* < 0.05. B, Colony formation assay with fewer colonies in MDA-MB-435 (*P* = 0.002) and MCF-7 (*P* = 0.046) cells transfected with pre-miR-125b than cells transfected with pre-control. C, Cell cycle distribution monitored by flow cytometry. D, Effect of miR-125b on tumor formation in nude mice. The volume of MDA-MB-435/pre-miR-125b-tumor (n = 9) was significantly less than those of MDA-MB-435/pre-control-tumor (n = 10). The weight of tumors in MDA-MB-435/pre-miR-125b-injected mice were significantly decreased compared to pre-control (*P* = 0.045).

**Figure 4.** mRNA profiling of miR-125b overexpression in BC cell lines. A, Unsupervised hierarchical cluster analysis of differentially expressed genes screened by cDNA microarray. Rows, mRNAs; columns, biological samples; red, expression value higher than average expression across all samples; green, expression value lower than average. B, Real-time PCR validation of six genes from an independent transfection experiment. C, UBE2E3, MAN1B1, C9orf86, MKNK2, ASB13, PRS6KA1 and ETS1 protein detected by immunoblot from BC cell lines transfected with pre-miR-125b and pre-control.
Figure 5. ETS1 is a direct target of miR-125b and knockdown of ETS1 suppresses cell growth, proliferation, and induces G1 cell cycle arrest. 

A, pMIR-Report vectors containing the 3’UTR of wild-type ETS mRNA (ETS1) or no 3’UTR (Vector), and miR-125b precursor or precursor control were cotransfected into HEK293T cells. The overexpression of miR-125b by the miR-125b precursor resulted in a significant decrease in luciferase signals from ETS1, but not Vector-transfected cells. 

B, MCF-7 and MDA-MB-435 cell lines were transfected with ETS1-siRNA (100 nM) or control siRNA (100 nM). Cells lysates were generated at 48 h post-transfection, followed by immunoblot to determine ETS1 expression, with GAPDH was used as the loading control. 

C, The functional role of ETS1 on BC cell growth was analyzed by MTT and colony formation at very low cell density. 

D, Cell cycle distribution monitored by flow cytometry.

Figure 6. Overexpression of ETS1 in BC and its correlation with BC patient survival. 

A, ETS1 overexpression was observed predominantly in the cytoplasm of carcinoma cells (immunohistochemical staining) at 40× and 200× magnification. 

B, Kaplan–Meier survival curve and log-rank test for BC patients with high or low ETS1 expression.
Figure 1

Overall survival rate of 105 cases

Overall survival rate of different miR-125b expression levels (n = 105)

Absence of lymph node metastasis (n = 43)

Presence of lymph node metastasis (n = 62)

P = 0.003

P = 0.002
Figure 6
miR-125b is Methylated and Functions as A Tumor Suppressor by Regulating the ETS1 proto-oncogene in Human Invasive Breast Cancer

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