MicroRNA-455-3p mediates GATA3 tumor suppression in mammary epithelial cells by inhibiting TGF-β signaling

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GATA3 is a basic and essential transcription factor that regulates many pathophysiological processes and is required for the development of mammary luminal epithelial cells. Loss-of-function GATA3 alterations in breast cancer are associated with poor prognosis. Here, we sought to understand the tumor-suppressive functions GATA3 normally performs. We discovered a role for GATA3 in suppressing epithelial-to-mesenchymal transition (EMT) in breast cancer by activating miR-455-3p expression. Enforced expression of miR-455-3p alone partially prevented EMT induced by transforming growth factor β (TGF-β) both in cells and tumor xenografts by directly inhibiting key components of TGF-β signaling. Pathway and biochemical analyses showed that one miRNA-455-3p target, the TGF-β-induced protein ZEB1, recruits the Mi-2/nucleosome remodeling and deacetylase (NuRD) complex to the promoter region of miR-455 to strictly repress the GATA3-induced transcription of this microRNA. Considering that ZEB1 enhances TGF-β signaling, we delineated a double-feedback interaction between ZEB1 and miR-455-3p, in addition to the repressive effect of miR-455-3p on TGF-β signaling. Our study revealed that a feedback loop between these two axes, specifically GATA3-induced miR-455-3p expression, could repress ZEB1 and its recruitment of NuRD (MTA1) to suppress miR-455, which ultimately regulates TGF-β signaling. In conclusion, we identified that miR-455-3p plays a pivotal role in inhibiting the EMT and TGF-β signaling pathway and maintaining cell differentiation. This forms the basis of that miR-455-3p might be a promising therapeutic intervention for breast cancer.

Among these transcription factors, GATA3 is the most highly expressed in the mammary epithelium and is expressed exclusively in the luminal epithelial cell population, where it not only specifies but also maintains luminal epithelial cell differentiation (3, 4). In addition, mutations in the zinc finger domains of GATA3 that diminish or abolish DNA-binding ability have been commonly identified in the human breast (5), and GATA3 was recently found to be one of three genes (with PIK3CA and TP53) mutated in >10% of all breast cancers (6). We have previously shown that GATA3 coordinates a program of transcriptional repression composed of G9A and the MTA3-Mi-2/ nucleosome remodeling and deacetylase (NuRD)2 complex, which represses a cohort of genes including ZEB2 and inhibits the invasive potential and metastasis of breast cancer (7). However, how GATA3 loss-of-function contributes to the expression of microRNAs (miRNAs), which are also important regulators of tumor progression and metastasis, is still poorly understood.

Transforming growth factor β (TGF-β) signaling is a multifunctional pathway that regulates the vast majority of physiological processes, including pluripotency, proliferation, migration, apoptosis, and differentiation in metazoan cells (8–10). TGF-β and downstream signaling play a dual role in carcinogenesis (11), and the effects of this pathway depend on the cellular context, which is particularly apparent in tumors. TGF-β from the inflammatory tumor microenvironment mediates cancer cell apoptosis and tumor suppression (12) or induces epithelial-to-mesenchymal transition (EMT), which promotes the separation, migration, invasion, and metastasis of epithelial cancer cells (13) or cancer stem cell heterogeneity and drug resistance (14). As a common and potent inducer of EMT, TGF-β induces this process through both Smad and non-Smad signaling pathways (15–17). In the Smad pathway, phosphory-

The GATA family of zinc finger transcription factors, comprising GATA1–GATA6, specifically binds the (T/A)GATA(A/G) consensus DNA sequence with high affinity (1, 2).

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‡¶The abbreviations used are: NuRD, nucleosome remodeling and deacetylase; miRNA, microRNA; TGF-β, transforming growth factor-β; EMT, epithelial-to-mesenchymal transition; qPCR, quantitative PCR; ER, estrogen receptor; qChIP, quantitative ChIP; SCID, severe combined immunodeficiency; GSEA, Gene Set Enrichment Analysis; MTA, metastasis-associated; EdU, 5-ethynyl-2’-deoxyuridine; GST, glutathione S-transferase; DAPI, 4’,6-diamidino-2-phenylindole.
GATA3 regulates miR-455-3p to inhibit TGF-β signaling

GATA3 is a transcription factor that has been functionally linked to estrogen receptor α (ERα) expression and activity in breast carcinoma; moreover, it is involved in a positive cross-regulatory loop with ERα, where each is required for the transcription of the other (31). Recently, Mair et al. (32) found that GATA3 interacts with the histone methyltransferases G9A and GLP independent of estrogen receptor signaling. Therefore, we investigated whether ERα plays a role in the regulation of miR-455-3p by GATA3. To this end, the putative promoter region (−2050 to +500 bp) of miR-455-3p was analyzed using the JASPAR database (http://jaspar.genereg.net)3 (79), and nine potential GATA3-binding sites were located; however, no ERα-binding sites were identified (relative profile score threshold = 90%; Fig. 2A). To gain a deeper insight into the mechanism through which GATA3 stimulates miR-455-3p transcription, we next conducted a quantitative ChIP (qChIP)-based promoter-walk using MCF-7 cells and mapped the enrichment of GATA3 to two regions of the miR-455-3p promoter, specifically at positions #4 and #6, as indicated. This is consistent with

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lated and activated Smad2 and Smad3 form a complex with Smad4 to promote the expression of the three families of EMT-inducing transcription factors, namely Snail/Slug, ZEB1/2, and Twist (15, 17, 18). These transcription factors, which have historically been implicated in lineage determination, repress the expression of epithelial markers while activating the expression of mesenchymal markers (18). Furthermore, TGF-β can also promote EMT by activating the PI3K/Akt/mTOR pathway and small GTPases (19, 20). In addition, it was noted that GATA3 inhibits the activity of TGF-β signaling and TGF-β-induced EMT (21). However, how GATA3 regulates TGF-β signaling is largely unexplored.

miRNAs, 19–22 nucleotides in length, are members of a large family of small noncoding RNAs that regulate a variety of cellular processes (22) and have concordantly been found to be dysregulated in diseases, including cancer (23, 24). As a class of post-transcriptional regulators, miRNAs modulate gene expression by binding complementary sequences in the 3′-UTR of mRNAs to either target them for degradation or inhibit their translation (22). Because the binding of miRNA to mRNAs is often achieved via imperfect complementarity, each miRNA can bind and regulate multiple protein-coding mRNAs and noncoding RNAs. Therefore, aberrant expression of a single miRNA can deleteriously affect the translation of multiple genes within a cell, leading to profound phenotypic effects (25). Moreover, miRNAs have established roles as oncogenes or tumor suppressors (23). Recent evidence indicates that some miRNAs, including miR-10b and miR-374a, act as oncogenes to promote breast cancer metastasis (26, 27), whereas miR-146 and miR-17/20 exhibit the opposite function (28, 29).

In this study, we addressed the issue of how GATA3 loss-of-function contributes to the expression of miRNAs in breast cancer. For this, we explored the molecular mechanism underlying the functional interplay among miR-455-3p, Smad2, ZEB1, and HDAC2 to investigate the role of miR-455-3p in EMT and breast cancer progression.

Results

GATA3 regulates miR-455-3p transcription

To identify miRNAs that are potentially regulated by GATA3, we utilized GATA3-positive, noninvasive (MCF-7) breast cancer cells to define the miRNA expression profile with respect to this marker (30). Morphologically, whereas control cells maintained organized cell-cell adhesion and polarity, GATA3-deficient MCF-7 cells exhibited the loss of cell-cell contacts and cells became dispersed; further, the cobblestone-like appearance was replaced by a more spindle-shaped, mesenchymal morphology, in addition to rearrangements of the actin cytoskeleton from a cortical to a stress fiber pattern (Fig. 1A, left), indicative of the characteristic morphological changes of EMT. Meanwhile, we found that MCF-7 cells with GATA3 depletion underwent EMT, as indicated by the loss of α-catenin and E-cadherin and the up-regulation of N-cadherin and fibronectin (Fig. 1A, right). However, morphological changes and EMT progression in MCF-7 cells caused by GATA3 deletion were rescued by WT GATA3 (Fig. 1A). These results demonstrated that GATA3 promotes the differentiation of breast cancer epithelial cells and inhibits EMT progression. To investigate the role of miRNA in this process, a miRNA microarray was used; miRNAs exhibiting a 2-fold or greater change in expression between GATA3-knockdown and control cells were chosen for further study ( GEO accession number: GSE129068). Analyses of these data identified a large number of miRNAs that were either up-regulated (n = 44) or down-regulated (n = 48) by GATA3 knockdown (Fig. 1B; see Data Set 1). Interestingly, 13 of the 48 down-regulated miRNAs were proven or suggested suppressors of EMT, based on previous studies (Table S1). These results implied that GATA3 can suppress EMT and maintain cell differentiation by activating miRNAs expression. To verify the results of microarray, we chose five up-regulated miRNAs (miR-503–5p, miR-486–5p, miR-450a-5p, miR-152, and miR-134) and five down-regulated miRNAs (miR-455-3p, miR-200b-5p, miR-25–5p, miR-7–1–3p, and let-7e-5p) and evaluated their expression by quantitative real-time PCR (qPCR) in MDA-MB-231 or MCF-7 cells, respectively. Consistent with the microarray, MCF-7 cells with GATA3 stably depleted (Fig. 1C) led to the induction of miR-503-5p, miR-486–5p, miR-450a-5p, miR-152, and miR-134 and a reduction of miR-455-3p, miR-200b-5p, miR-25–5p, miR-7–1–3p, and let-7e-5p (Fig. 1D). We then ectopically expressed GATA3 in MDA-MB-231 cells and found that a roughly 15-fold increase in GATA3 expression level (Fig. 1E) resulted in decreased expression levels of miR-503-5p, miR-486-5p, miR-450a-5p, miR-152, and miR-134 and a reduction of miR-455-3p, miR-200b-5p, miR-25–5p, miR-7–1–3p, and let-7e-5p (Fig. 1F). Of the 92 miRNAs screened, miR-455-3p stood out as the top down-regulated miRNA in GATA3-deficient MCF-7 cells (Fig. 1B) and was increased in MDA-MB-231 cells by GATA3 (Fig. 1F). We therefore focused on miR-455-3p as a potential target of GATA3.

GATA3 directly induces miR-455-3p expression independent of ERα signaling

GATA3 is a transcription factor that has been functionally linked to estrogen receptor α (ERα) expression and activity in breast carcinoma; moreover, it is involved in a positive cross-regulatory loop with ERα, where each is required for the transcription of the other (31). Recently, Mair et al. (32) found that GATA3 interacts with the histone methyltransferases G9A and GLP independent of estrogen receptor signaling. Therefore, we investigated whether ERα plays a role in the regulation of miR-455-3p by GATA3. To this end, the putative promoter region (−2050 to +500 bp) of miR-455-3p was analyzed using the JASPAR database (http://jaspar.genereg.net) (79), and nine potential GATA3-binding sites were located; however, no ERα-binding sites were identified (relative profile score threshold = 90%; Fig. 2A). To gain a deeper insight into the mechanism through which GATA3 stimulates miR-455-3p transcription, we next conducted a quantitative ChIP (qChIP)-based promoter-walk using MCF-7 cells and mapped the enrichment of GATA3 to two regions of the miR-455-3p promoter, specifically at positions #4 and #6, as indicated. This is consistent with
previous results that GATA3 promotes its expression by directly binding the promoter region of miR-455-3p. Meanwhile, there was no significant ERα enrichment in this putative region (Fig. 2B). Next, the putative promoter sequence, containing all predicted GATA3-binding sites, was cloned into the luciferase reporter vector pGL4, and GATA3-dependent activity was assessed in ER-positive (MCF-7, MCF 10A, and T-47D) and ER-negative cell lines (MDA-MB-231, MDA-MB-436, and MDA-MB-468). As shown in Fig. 2 (C and D), promoter activity was activated by GATA3 in a dose-dependent manner in both ER-positive and ER-negative cell lines. These results indicated that GATA3 induces the transcription of miR-455-3p in an ER-independent manner.

miR-455-3p inhibits the proliferation and metastatic potential of breast cancer cells

As reported previously, GATA3 can maintain the differentiation of luminal epithelial cells in the mammary gland and inhibit the metastasis and proliferation of breast cancer (4, 7, 33–35). Therefore, we postulated that GATA3 might affect the
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Prediction binding sites in the promoter region of MiR455 for GATA3 and ERα

| Name   | Score  | Relative score | Strand | Distance to TSS | Predicted sequence | Predicted DNA binding profile |
|--------|--------|----------------|--------|-----------------|--------------------|------------------------------|
| GATA3  | 8.38812| 0.9999999995  | +      | -799            | agatag             | GAT                            |
|        | 8.09247| 0.987997174   | -      | -872            | tgatag             | GATAA                          |
|        | 7.42236| 0.959484907   | +      | -644            | agatag             | GAT                            |
|        | 6.22367| 0.90919803    | +      | -1551           | agatag             | GATAA                          |
|        | 12.0141| 0.95803701    | -      | -354            | agatagaa           | GAT                            |
|        | 10.9871| 0.97384009    | +      | -1590           | agataaag           | GAT                            |
|        | 10.8641| 0.9710221     | +      | -926            | tgataagg           | GATAA                          |
|        | 10.8641| 0.9710221     | +      | 427             | tgataagg           | GATAA                          |
| ERα    | N/A    | N/A            | N/A    | N/A             | N/A                | N/A                            |

**MIR455 promoter region**

![Graph showing relative enrichment fold for IgG, GATA3, and ERα](image)

**ER positive breast cancer cells**

- **MCF-7**
  - Vector
  - GATA3

- **MCF 10A**
  - Vector
  - GATA3

- **T-47D**
  - Vector
  - GATA3

**ER negative breast cancer cells**

- **MDA-MB-231**
  - Vector
  - GATA3

- **MDA-MB-468**
  - Vector
  - GATA3

- **MDA-MB-436**
  - Vector
  - GATA3

![Bar charts showing relative miR-455-Luc expression](image)
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proliferation and metastasis of breast cancer by regulating miR-455-3p. To verify this hypothesis, we performed 5-ethyl-2'-deoxyuridine (EdU) assays to examine the role of miR-455-3p in the proliferation of breast cancer cells. The less-differentiated MDA-MB-231 cells had a much lower proportion of EdU-labeled cells after transfection with miR-455-3p mimics, whereas the number of positively labeled cells in the differentiated MCF-7 cell line obviously increased upon treatment with miR-455-3p inhibitors (Fig. 3A). Next, to further investigate the role of miR-455-3p in the invasion and metastasis of breast cancer, we detected the expression level of epithelial/mesenchymal markers in miR-455-3p mimic- or miR-455-3p inhibitor–transfected MDA-MB-231 or MCF-7 cells by qPCR and Western blotting. miR-455-3p gain-of-function in MDA-MB-231 cells resulted in the induction of epithelial markers (E-cadherin, α-catenin, and γ-catenin) and the reduction of mesenchymal markers (N-cadherin, fibronectin, and vimentin) at both the mRNA and protein level. Accordingly, miR-455-3p loss-of-function in MCF-7 cells was associated with an apparent reduction in epithelial markers and the significant induction of mesenchymal markers (Fig. 3, B and C). In addition, transwell invasion assays showed that forced expression of miR-455-3p significantly inhibited the invasive potential of MDA-MB-231 cells. Consistently, treatment with miR-455-3p inhibitors improved the invasive potential of MDA-MB-231 cells (Fig. 3D). However, the negative effect on invasion mediated by GATA3 was diminished by treatment with miR-455-3p inhibitors (Fig. 3E). Together, these results suggested that miR-455-3p can inhibit the proliferation, EMT, and invasion of breast cancer cells.

To investigate the role of miR-455-3p in tumor development and progression in vivo, MDA-MB-231 cells engineered to stably express firefly luciferase (MDA-MB-231-Luc-D3H2LN, Xenogen Corp.) were co-infected with lentiviruses harboring hsa-miR-455-3p. These cells were then orthotopically implanted into immunocompromised severe combined immunodeficiency (SCID) mice to assess tumorigenesis. Specifically, MDA-MB-231-Luc-D3H2LN cells were implanted into the abdominal mammary fat pad (n = 6) of 6-week-old female SCID mice. The growth of tumors was monitored weekly through bioluminescence imaging using an IVIS imaging system (Xenogen Corp.). Accordingly, orthotopic tumors were measured by quantitative bioluminescence imaging after 8 weeks. The results showed that, in the orthotopically implanted groups, forced expression of miR-455-3p resulted in a significant reduction in MDA-MB-231-Luc-D3H2LN tumor growth (Fig. 3F, top). The tumor specimens were surgically removed (Fig. 3F, bottom) and examined by in vitro bioluminescence imaging (Fig. 3G). Next, we analyzed published data downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). The results supported the idea that miR-455-3p expression was decreased in breast carcinoma relative to that in adjacent normal tissues (Fig. 3H). To further extend our observations to a clinical and pathologically relevant context, we analyzed the expression of miR-455-3p and its correlation with the clinical outcomes of breast cancer patients. Kaplan–Meier survival analysis of the relationship between survival time and the expression of miR-455-3p was performed with an online tool (http://kmplot.com/analysis/)3 (80). This showed that higher expression of miR-455-3p (p = 0.02) was associated with improved survival in breast cancer patients when the influence of systemic treatment, endocrine therapy, and chemotherapy were excluded (Fig. 3I). In conclusion, these experiments demonstrated that miR-455-3p inhibits the tumorigenesis and metastasis of breast cancer cells in vitro and in vivo.

miR-455-3p participates in the TGF-β signaling pathway

To explore the molecular mechanism through which miR-455-3p inhibits the proliferation and metastasis of breast cancer, an analysis of signaling pathways associated with miR-455-3p-target genes using the analysis site (http://snf-515788.vm.okeanos.grnet.gr/)3 (81) revealed that miR-455-3p is mainly implicated in multiple signaling pathways, such as p53, TGF-β, and cell cycle (Fig. 4A). Moreover, we investigated the genome-wide effect of miR-455-3p expression by high-throughput RNA deep sequencing (RNA-Seq; GEO accession number: GSE129146). In brief, total RNA was extracted from MCF-7 cells transfected with control or miR-455-3p mimics. RNA-Seq analysis identified 143 genes for which expression was down-regulated and 333 genes for which expression was up-regulated upon miR-455-3p treatment (-fold change > 1.5, probability > 0.8; Fig. 4B). Genes typically interact to play roles in certain biological functions. We thus performed pathway enrichment analysis on differentially expressed genes based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.kegg.jp/kegg/pathway.html)3 and revealed that miR-455-3p regulates target genes involved in TGF-β, PI3K-Akt, cell cycle, and other pathways closely related to tumor growth and metastasis (Fig. 4C). Further analysis using Gene Set Enrichment Analysis (GSEA) software also indicated that miR-455-3p significantly inhibits the cell cycle and TGF-β/Smad signaling pathway (Fig. 4, D and E). We next chose 15 representative genes implicated in the cell cycle and TGF-β/Smad signaling pathway, including CCNE1, CCNE2, CDK2N2D, CDK6, CHEK1, CHST11, FGF18, MCM5, MTMR4, MYC, SMAD2, TGFBR1, TGFBR3, TGFBR2, and TTK, and validated their responses to miR-455-3p in MCF-7 cells by qPCR (Fig. 4F). Taken together, these results suggested that miR-455-3p is

Figure 2. GATA3 promotes miR-455-3p transcription independent of ERα

Figure 2. GATA3 promotes miR-455-3p transcription independent of ERα. A, the online analysis tool JASPAR was used to predict the potential binding sites of GATA3 and ERα in the promoter region (~2000 to +500) of MIR455. B, primer pairs including #1 to #10 were synthesized to cover the promoter region of MIR455 as indicated. qChIP-based promoter-walk was performed using MCF-7 cells, and the enrichment of GATA3 was mapped to two regions of the MIR455 promoter. Error bars, S.D. of three independent experiments (*, p < 0.05; **, p < 0.01, two-tailed unpaired t test). C and D, ER-positive breast cancer cells (MCF-7, MCF 10A, and T-47D) (C) or ER-negative breast cancer cells (MDA-MB-231, MDA-MB-468, and MDA-MB-436) (D) were co-transfected with an miR-455-Luc reporter and FLAG-luciferase reporter with GATA3-binding sites deleted and FLAG-GATA3. The nonmutated (WT) reporter was used as the control. Firefly luciferase activities were normalized to Renilla luciferase activities and plotted relative to control levels. Error bars, S.D. of three independent experiments (*, p < 0.05; **, p < 0.01, two-tailed unpaired t test).
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A. EdU and DAPI staining of MDA-MB-231 and MCF-7 cells with control or miR-455-3p inhibitor.

B. Relative expression levels of E-cadherin, α-catenin, γ-catenin, N-cadherin, Fibronectin, and Vimentin in MDA-MB-231 and MCF-7 cells with control or miR-455-3p inhibitor.

C. Western blot analysis of E-cadherin, α-catenin, γ-catenin, N-cadherin, Fibronectin, and Vimentin in MDA-MB-231 and MCF-7 cells with control or miR-455-3p inhibitor.

D. Fold of invasion of control and miR-455-3p inhibitor-treated MDA-MB-231 and MCF-7 cells.

E. Invasion assay of control and miR-455-3p inhibitor-treated MDA-MB-231 cells.

F. Luminescence images of luciferase activity in MDA-MB-231 cells with control or miR-455-3p inhibitor.

G. Radiance (p/sec/cm²/sr) and luminescence of mammmary tumors with plmiR-NC and plmiR-455-3p.

H. Relative miR-455-3p expression in normal and tumor samples from GSE68085.

I. Kaplan-Meier curve showing the expression of miR-455-3p in tumors.
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mainly involved in regulating the cell cycle and TGF-β/Smad signaling pathway. Detailed results of RNA-Seq experiments are provided in Data Set 2.

miR-455-3p directly targets Smad2, ZEB1, and HDAC2

To explore the molecular mechanism through which miR-455-3p regulates the cell cycle and TGF-β pathway, we predicted miR-455-3p targets using programs including DIANA, miRANDA, miRDB, miWalk, and TargetScan. It was found that miR-455-3p has 212 overlapping potential targets, including Smad2, ZEB1, HDAC2, and other genes closely related to tumorigenesis (Fig. 5A). As a key component of the TGF-β signaling pathway, phosphorylated Smad2, which is induced by TGF-β, combines with Smad3 and Smad4 to form trimeric Smad complexes that translocate to the nucleus to regulate gene expression (36). The ΔE1 family proteins, namely ΔE1F1 (ZEB1) and SIP1 (ZEB2), act as EMT transcription factors and are activated by TGF-β signaling (37). Meanwhile, the epigenetic modifier HDAC2 is up-regulated by TGF-β signaling during endothelial-to-hematopoietic transition (38), and TGF-β also activates HDAC2 in the kidneys (39). We used the public algorithm TargetScan (http://www.targetscan.org/vert_72/)(82) and identified the putative miR-455-3p-binding sites of these three targets, which were all conserved among Sus scrofa, Canis familiaris, Oryctolagus cuniculus, and Homo sapiens. To test whether Smad2, ZEB1, and HDAC2 are directly modulated by miR-455-3p, we performed reporter assays using HEK 293T cells, wherein the luciferase gene was driven by either WT or mutated 3’-UTR sequences. The results showed that luciferase activity was reduced with the WT 3’-UTRs, but not the mutant 3’-UTRs, of these targets when HEK 293T cells were transfected with miR-455-3p mimics. Consistently, treatment with miR-455-3p inhibitors led to the opposite effect on luciferase activity with WT and mutant 3’-UTRs (Fig. 5, B and C). However, neither mimics nor inhibitors of miR-455-3p had an effect on the luciferase activity of the other potential binding site of Smad2 3’-UTR at positions 7258–7264 (data not shown).

In MCF-7 and MDA-MB-231 cells transfected with miR-455-3p mimics, Western blot analysis showed that the protein levels of ZEB1, Smad2, and HDAC2 were significantly decreased; in contrast, in cells transfected with miR-455-3p inhibitors, the protein levels of all three were partially increased (Fig. 5D). Similarly, the depletion of GATA3 in MCF-7 cells resulted in the induction of ZEB1, Smad2, and HDAC2, whereas their expression was depressed in MDA-MB-231 cells overexpressing GATA3 (Fig. 5E). Importantly, these results suggest that miR-455-3p binds the 3’-UTR of Smad2, ZEB1, or HDAC2 and that these genes are the downstream targets of miR-455-3p.

miR-455-3p inhibits TGF-β–induced EMT

As stated, studies have shown that TGF-β is a common and potent inducer of EMT (15–17); thus, it was reasonable to speculate that miR-455-3p can inhibit EMT by disrupting the TGF-β signaling pathway. To this end, gain-of-function miR-455-3p experiments were performed, and morphological alterations and the expression of epithelial/mesenchymal markers were analyzed in MCF 10A nonmetastatic human mammary epithelial cells with TGF-β1 treatment. After treatment, MCF 10A cells acquired a spindle-shaped morphology, and the actin filaments that regulate cell migration were reorganized (Fig. 6A). Further, phenotypic changes in MCF 10A cells were accompanied by the up-regulation of mesenchymal markers (N-cadherin, ZEB1, and fibronectin) and the down-regulation of epithelial markers (E-cadherin and α-catenin) at both the mRNA and protein level (Fig. 6, B and C). The increased levels of Smad2 and Smad3 phosphorylation and up-regulation of ZEB1 and ZEB2 verified the activation of TGF-β signaling. Of the components of NURD, the metastasis-associated (MTA) family of proteins, including MTA1, MTA2, and MTA3, have been implicated in cancer progression and metastasis (40–42). MTA3 inhibits EMT and breast cancer metastasis, and its expression is increasingly lost during breast cancer progression (43), whereas MTA1 promotes breast tumor progression, and its expression gradually increases during the process (44). Hence, we investigated whether the MTA family is involved in TGF-β–induced EMT. However, we did not detect statistically changes in HDAC2, GATA3, MTA1, MTA2, and MTA3, which have been implicated in cancer progression and metastasis, in TGF-β–stimulated MCF 10A cells (Fig. 6C).

To verify the relationship between miR-455-3p and TGF-β signaling, we analyzed the effect of miR-455-3p on TGF-β/Smad-dependent transcription using the p3TP-Lux and ARE-Luc reporters in MCF 10A cells and found that miR-455-3p inhibited TGF-β–reporter activities (Fig. 6D). Consistent with the results of TGF-β inhibitor (SB-431542) treatment, we found that miR-455-3p could reverse, at least in part, TGF-β–induced EMT (Fig. 6E). Next, we examined cell morphological transitions in miR-455-3p mimic-transfected MCF 10A cells, with or without TGF-β1 treatment, by microscopy and found that upon exposure to TGF-β1, MCF 10A cells treated with miR-455-3p mimics had more epithelial morphological fea-

Figure 3. miR-455-3p inhibits the proliferation and metastasis of breast cancer cells. A, EdU cell proliferation assays using MDA-MB-231 cells transfected with control or miR-455-3p mimics and MCF-7 cells transfected with control inhibitor or miR-455-3p inhibitors. B and C, qPCR and Western blotting were performed to examine the expression levels of epithelial or mesenchymal markers in MDA-MB-231 cells overexpressing miR-455-3p or MCF-7 cells transfected with miR-455-3p inhibitors. D, transwell invasion assays of MDA-MB-231 cells transfected with miR-455-3p mimics or inhibitors. The invaded cells were stained and counted as shown. E, GATA3 activated the expression of miR-455-3p to inhibit the invasiveness of breast cancer cells. MDA-MB-231 cells were transiently transfected with expression vectors or miRNA inhibitors as indicated before performing transwell assays. In each experiment, at least six microscopic fields with a ×40 magnification were randomly selected for cell counting. Representative photographs are shown on the left, and statistical analysis is presented on the right. F and G, MDA-MB-231-Luc-D3H2LN cells were infected with lentiviruses harboring plmiR-NC or plmiR-455-3p and inoculated orthotopically into the abdominal mammary fat pad of 6-week-old female SCID mice (n = 6). Primary tumors were quantified from the region of interest (ROI) by performing bioluminescence imaging 6 weeks after initial implantation. Representative in vivo bioluminescent images are shown (F), and tumor specimens were examined by in vitro bioluminescent measurements (G). All scale bars, 1 cm. H, analysis of public dataset GSE68085 for the expression of miR-455-3p, based on a two-tailed unpaired t test. I, Kaplan–Meier survival analysis of the relationship between survival time and miR-455-3p signature in breast cancer using an online tool (http://kmplot.com/analysis/). Each bar (A, B, D, E, G, and H) represents the mean ± S.D. (error bars). *, p < 0.05; **, p < 0.01; ***, p < 0.001, two-tailed unpaired t test.
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A) Neurotrophin signaling pathway
B) miR-455-3p/Control
C) TGF-beta signaling pathway
D) KEGG_CELL_CYCLE
E) REACTOME_TGF_BETA_RECEPTOR_SIGNALING_ACTIVATES_SMADS
F) Relative mRNA levels

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**GATA3 regulates miR-455-3p to inhibit TGF-β signaling**

It has been reported that the EMT-activator ZEB1 can repress expression of the miR-200 family, which also directly targets ZEB1 (46–48). Accordingly, we examined whether ZEB1, which is also a target of miR-455-3p, could regulate the expression of miR-455-3p. Scanning the **MIR455** promoter region using the JASPAR database identified ten putative binding sequences (relative profile score threshold = 90; (Fig. 7A)). This implied that ZEB1 might also directly suppress the transcription of **MIR455**. We previously reported that ZEB2 physically associates with the NuRD (MTA1/2) complex (7), but it was unclear whether ZEB1 could also interact with this complex. Immunoprecipitation assays with an anti-ZEB1 antibody followed by immunoblotting using antibodies against Mi-2, MTA1, MTA2, MTA3, HDAC1, HDAC2, RbAp46/48, or MBD2/3 indicated that all of these proteins were efficiently co-immunoprecipitated with ZEB1 in MDA-MB-231, MCF-7, and T-47D cell lines (Fig. 7B). In addition, GST pulldown with GST-fused MTA1, MTA2, MTA3, HDAC1, HDAC2, RbAp46, RbAp48, or MBDB3 and in vitro-transcribed/translated ZEB1 revealed that ZEB1 interacts with MTA1 and MTA2, but not with MTA3 and other tested components of the NuRD complex (Fig. 7C). These experiments indicated that ZEB1 can also associate with the NuRD complex. Interestingly, HDAC2 is also a direct target of miR-455-3p, and thus, ZEB1 might interact with the NuRD complex to repress the expression of **MIR455**.

We next confirmed the role of ZEB1/NuRD in the transcription of **MIR455**. For this, quantitative ChIP analysis was performed using MDA-MB-231 cells and specific antibodies against ZEB1, MTA1, or HDAC2 to test ten promoter regions of **MIR455**. Results showed that ZEB1, MTA1, and HDAC2 were strongly enriched at the #4 and #7 regions of **MIR455** (Fig. 7D). To further support the proposition that ZEB1 nucleates NuRD to form one protein complex at target promoters, sequential ChIP or ChIP/re-ChIP experiments were performed on the #4 and #7 regions of **MIR455**. For these experiments, soluble chromatin was first immunoprecipitated with antibodies against ZEB1, MTA1, or HDAC2. The immunoprecipitates were subsequently re-immunoprecipitated with appropriate antibodies. The results showed that the #4 and #7 promoter regions of **MIR455** that were immunoprecipitated with antibodies against ZEB1 could be re-immunoprecipitated with antibodies against MTA1 or HDAC2 (Fig. 7E). Similar results were obtained when the initial ChIP was performed with antibodies against MTA1 or HDAC2 (Fig. 7F).

Next, we investigated whether ZEB1, MTA1, and HDAC2 directly suppress transcriptional activity of the **MIR455** miRNA gene. Luciferase assays showed that the promoter activity of **MIR455** was suppressed by ZEB1, MTA1, or HDAC2 overexpression in MCF-7 cells (Fig. 7F, top). In contrast, knockdown of either HDAC2, MTA1, or ZEB1 in MDA-MB-231 cells resulted in enhanced promoter activity when compared with that in controls (Fig. 7F, top). Moreover, gain-of-function and loss-of-function ZEB1, MTA1, or HDAC2 experiments were performed, and the expression of miR-455-3p was examined in MCF-7 or MDA-MB-231 cells. We found that the overexpression of either ZEB1, MTA1, or HDAC2 could down-regulate miR-455-3p expression in MCF-7 cells (Fig. 7F, bottom). Consistently, the individual depletion of ZEB1, MTA1, or HDAC2 resulted in the induction of miR-455-3p expression (Fig. 7F, bottom). These results support the contention that a reciprocal feedback regulatory loop exists between GATA3 and the ZEB1-nucleated repression program to regulate the expression of miR-455-3p, which ultimately inhibits TGF-β signaling to control EMT and the metastasis of breast cancer cells (Fig. 7G).

**Discussion**

It is widely recognized that the loss of or mutation in GATA3 is tightly linked to the progression of breast cancer (32, 33) and that this cooperates with ERα (31). Because miRNAs are widely involved in the regulation of biological processes (22, 49), the transcription factor GATA3 modulates its own biological func-

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**Figure 4. miR-455-3p is associated with the TGF-β signaling pathway.** A, pathway analysis of the potential target genes of miR-455-3p arranged into functional groups. B, volcano plot of RNA-Seq data comparing miR-455-3p versus control-treated MCF-7 cells and showing 143 and 333 genes significantly up- and down-regulated, respectively, with a fold-change higher than 1.5 and probability > 0.8. C, scatter plot of the top 10 enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways comprising the up-regulated or down-regulated genes regulated by miR-455-3p. The RichFactor is the ratio of the number of differentially expressed genes annotated in a pathway term to the number of all genes annotated in that pathway term. A greater RichFactor indicates greater intensity. The Q-value is the corrected p value ranging from 0 to 1, and a lower Q-value indicates greater intensity. D and E, GSEA analysis of RNA-Seq data. F, qPCR analysis of indicated mRNAs in cells treated with control or miR-455-3p mimics. Error bars, S.D. of three independent experiments (*, p < 0.05; **, p < 0.01, two-tailed unpaired t test).
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| Gene      | Aggregate PCT | Context Score | miR SVR | miR DB score | miT TG score |
|-----------|---------------|---------------|---------|--------------|--------------|
| SMAD2     | 0.33          | -0.28         | -0.8472 | 60           | 0.962255851  |
| HDAC2     | 0.31          | -0.39         | -0.4591 | 59           | 0.988831554  |
| ZEB1      | 0.3           | -0.16         | -0.8486 | 70           | 0.51011366   |
| SUV39H1   | 0.29          | -0.22         | -0.3628 | 66           | 0.973264647  |
| RICTOR    | 0.28          | -0.04         | -0.2815 | 73           | 0.644478681  |
| PRKD3     | <0.1          | -0.43         | -1.1377 | 90           | 0.97280799   |
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tions by regulating the expression of miRNAs. As a transcription factor, GATA3 plays a dual role in regulating microRNA expression. On one hand, GATA3 increases the expression of miR-29b and miR-503, which promote cell differentiation and suppress tumor metastasis (21). On the other hand, GATA3 can also down-regulate levels of miR-183 to induce adipogenesis in 3T3-L1 cells (50). In this study, we first revealed that the depletion of GATA3 leads to multiple changes in miRNA expression, such as up-regulation of miR-134, miR-152, and miR-486-5p and down-regulation of miR-455-3p, miR-200b-5p, and miR-7-1-3p; moreover, miR-455-3p was found to be the most dysregulated miRNA in these cells. Furthermore, GATA3 was found to significantly increase the transcription of MiR455 in either ER-positive or ER-negative cells, which indicates that GATA3 promotes the expression of miR-455-3p, independent of the function of ERs. These results indicated that GATA3 has an additional mechanism that does not rely on ERs to regulate gene expression.

miR-455-3p has been reported to suppress the expression of ROCK2, HOXBS, hTERT, and RUNX2, which are involved in multiple biological processes including renal fibrosis, chondrogenic development and differentiation, Alzheimer’s disease, preeclampsia, and tumorigenesis (51–57). EMT has been described as the transformation of epithelial cells to mesenchymal cells and is associated with embryonic development, tissue repair, and carcinoma progression (58, 59). During cancer pathogenesis, EMT is induced by pleiotropic signals and then triggers the dissociation of cells from primary carcinomas, which subsequently migrate and disseminate to distant sites (60). Here, we showed that miR-455-3p acts as an inhibitor of EMT in breast cancer cells and that miR-455-3p loss-of-function promotes the acquisition of cell invasive properties and the ability to metastasize. Simultaneous inhibition of miR-455-3p function in GATA3-positive cells was found to attenuate the inhibitory effect of GATA3 on cell metastasis and dedifferentiation. These results indicate that miR-455-3p represents an important node that inhibits breast cancer metastasis.

The TGF-β signaling pathway activates the expression of multiple transcription factors, such as ZEB1, ZEB2, and SNAIL1, and induces the initiation of EMT (15, 18, 61). miRNAs usually play a role in transcriptional regulation by inhibiting the expression of target genes (22). As mentioned under “Results,” miR-455-3p broadly inhibited TGF-β-induced EMT by repressing the expression of Smad2, ZEB1, and HDAC2. Smad2 as well as Smad3 are phosphorylated and activated by TGF-β receptors and are referred to as R-Smads; these signaling molecules initiate TGF-β signal transduction and translocate to the nucleus to activate or inhibit target gene transcription (36, 62, 63). Further, ZEB1 is a potent promoter of EMT. Acting as a transcriptional repressor of E-cadherin and the microRNA-200 family and an activator of mesenchymal markers (47, 48, 64), ZEB1 positively affects cellular signaling pathways including TGF-β and the Wnt pathway to promote EMT and metastasis. Moreover, the chromatin remodeler HDAC2, which is a subunit of the NuRD complex, acts as a corepressor to repress the transcription of E-cadherin and miR-200 and regulate cancer metastasis (65–67). In collaboration with HDAC1, HDAC2 can also modulate TGF-β signaling and promote hematopoiesis in the hemogenic endothelium (38). Thus, the suppression of Smad2, ZEB1, and HDAC2 by the GATA3/miR-455-3p axis is of particular significance. Taken together, it is obvious that miR-455-3p inhibits the TGF-β signaling pathway, maintains cell differentiation, and inhibits EMT. Previous reports indicate that GATA3 can also inhibit the TGF-β signaling pathway (21); together with our results indicating that the signaling protein Smad2 and the effector protein ZEB1 are also targets of miR-455-3p, this suggests that GATA3 can inhibit this signaling pathway at the transcriptional and post-translational levels. This complex regulatory network would ensure that GATA3 can efficiently regulate the TGF-β signaling pathway.

The NuRD complex, which consists of Mi-2, MTA1, MTA2, MTA3, HDAC1, HDAC2, RbAp46, and MBD3, possesses histone deacetylation activity and primarily functions in transcriptional repression programs to regulate breast cancer metastasis (68). Our results showed that TGF-β could induce the expression of N-cadherin, fibronectin, ZEB1, and ZEB2, in addition to the phosphorylation of Smad2 and Smad3, while down-regulating levels of E-cadherin and α-catenin in MCF 10A cells. Interestingly, we did not observe statistically significant changes in the expression of MTA family proteins, including MTA1, MTA2, and MTA3, after TGF-β1 treatment. However, these TGF-β1-mediated alterations could be reversed by miR-455-3p treatment. Specifically, miR-455-3p dramatically restored the loss of E-cadherin and α-catenin and reversed the up-regulation of N-cadherin, fibronectin, and ZEB1, which respond to TGF-β1 stimulation. It is worth noting that we only observed a decrease in Smad2 expression and phosphorylation, without changes in Smad3. These results demonstrate that miR-455-3p could not only repress the direct TGF-β signaling mediator Smad2, but also inhibit the downstream target gene of TGF-β signaling pathway ZEB1 and the epigenetic regulator HDAC2, thus disturbing the TGF-β signaling pathway at multiple levels. Therefore, miR-455-3p could be regarded as an inhibitor of the TGF-β/Smad signaling pathway and a potential new therapeutic target for breast cancer.

Figure 5. Smad2, ZEB1, and HDAC2 are downstream targets of miR-455-3p. A, the intersection of the Venn diagram (left) displays potential targets of miR-455-3p predicted by mirDB, DIANA, mirNANDA, TargetScan, and miWalk algorithms with high-confidence scores. Of these, 212 common targets that are involved in the TGF-β signaling pathway or cell cycle are shown in the table (right). B, sequence alignment of Smad2, ZEB1, and HDAC2 3′-UTRs among human (H. Sapiens), pig (S. scrofa), dog (C. familiaris), and rabbit (O. cuniculus). Mutations generated within the 3′-UTRs of Smad2, ZEB1, or HDAC2 are shown in red. C, WT and mutant (mut) 3′-UTR of Smad2 (bases 1–330 of the 3′-UTR), ZEB1 (bases 1074 to 3230 of the 3′-UTR), and HDAC2 (bases 1–500 of the 3′-UTR) were cloned into pmiR-Reporter vectors, and luciferase reporter assays were used to identify binding between miR-455-3p sites and indicated 3′-UTRs. After treatment with negative control miR, miR-455-3p mimics (miR-455-3p), or miR-455-3p inhibitors, HEK 293T cells were transfected with WT or the indicated mutant 3′-UTR luciferase reporters and a plasmid encoding Renilla luciferase. Normalized luciferase activity in the control group was set as the relative luciferase activity. Error bars, S.D. of three independent experiments (*, p < 0.05, two-tailed unpaired t test). D, miR-455-3p represses the expression of Smad2, ZEB1, and HDAC2 at the protein level. MDA-MB-231 and MCF-7 cells were transfected with control miR, miR-455-3p mimics, or miR-455-3p inhibitors for 48 h and subjected to Western blot analysis. E, Western blot analysis of Smad2, ZEB1, and HDAC2 protein levels in GATA3-depleted MCF-7 and GATA3-overexpressing MDA-MB-231 cells.
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As previously described, GATA3 physically associates with the G9A/NuRD (MTA3) complex to repress ZEB2, which in turn suppresses the expression of G9A and MTA3 through the recruitment of G9A/NuRD (MTA1) (7). Here, we reported that GATA3, through activation of miR-455-3p, inhibits expression of the EMT activators ZEB1 and HDAC2 and maintains differentiation of the mammary epithelium. Upon GATA3 loss-of-function, due to down-regulation or mutation, which occurs frequently in breast cancer (3, 69, 70), the expression of ZEB1 and HDAC2 are derepressed; consequently, elevated ZEB1 and HDAC2 can promote EMT and metastasis. Surprisingly, we found that ZEB1 represses the expression of miR-455-3p through recruitment of the MTA1-NuRD complex. Apparently, a reciprocal feedback regulatory loop exists in which GATA3 loss-of-function in breast cancer leads to the elevated expression of ZEB1 and HDAC2, which in turn further down-regulates miR-455-3p to maintain high levels of these markers; this promotes EMT and eventually leads to breast cancer metastasis. Meanwhile, these results showed that GATA3 and the ZEB1/NuRD (MTA1) complex occupy similar promoter regions of MIR455, which suggests possible competition between GATA3 and ZEB1 for the transcriptional regulation of miR-455-3p based on their abundance and binding to the promoter. In this loop, miR-455-3p acts as an integral component and represents a rate-limiting factor. Generally, GATA3 separately suppresses the expression of ZEB1 and ZEB2 at the transcriptional or post-translational level, thereby inhibiting breast cancer progression.

In summary, our study demonstrated that a reciprocal feedback loop between the GATA3/miR-455-3p and TGF-β/ZEB1/NuRD (MTA1) axes regulates mammary epithelial cell fate and dynamically regulates epithelial cell plasticity; further, dysfunction of this loop affects the fate of mammary epithelial cells and promotes breast cancer metastasis. Our research further revealed the molecular mechanism through which GATA3 loss-of-function affects the progression of breast cancer and enhances our understanding of the complexity of EMT hierarchical regulatory networks. Several studies have indicated that GATA3 is mutated or deleted in many types of diseases including tumors; however, it was not found to act as a classic tumor suppressor (5, 71–73). miRNAs are considered potential anticancer drugs (74, 75), and thus the activation or expression of miR-455-3p in tumors exhibiting a loss of GATA3 function represents a new potential therapeutic approach.

Experimental procedures

Antibodies and reagents

The antibodies used in this study are listed in Table S2. Dynabeads Protein G was obtained from Invitrogen by Thermo Fisher Scientific (Waltham, MA), and the protease inhibitor mixture was from Roche Applied Science. GSH Sepharose™ 4B beads were purchased from GE Healthcare (Uppsala, Sweden). Recombinant Human TGF-β1 protein was obtained from R&D Systems (Minneapolis, MN). The synthesized mimics/inhibitors of miR-455-3p and short hairpin RNAs were from GenePharma Co., Ltd. (Shanghai, China). The sequences are listed in Table S3.

Cell culture and transfection

MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (Manassas, VA). T-47D, MCF 10A, MDA-MB-436, and MDA-MB-468 cell lines were obtained from the Chinese Academy of Medical Sciences. T-47D and MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco, Invitrogen), whereas MDA-MB-231, MDA-MB-436, and MDA-MB-468 cells were maintained in Leibovitz’s L-15 medium (Gibco, Invitrogen) without CO₂. MCF 10A cells were cultured with the mammary epithelium growth factor medium kit supplemented with growth factors (cc-3150, Lonza, Switzerland). In addition to mammary epithelium growth factor medium, all of the other medium were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (Gibco, BRL); the complete medium for MDA-MB-436 cells also contained 10 µg/ml insulin and 16 µg/ml GSH. Transfections were carried out using Lipofectamine 2000 or Lipofectamine® RNAiMAX Reagent (Invitrogen) according to the manufacturer’s instructions.

siRNA transfection

Specific MTA1 siRNA and negative control siRNA were obtained from Dharmacon (Lafayette, CO), and siRNAs against HDAC2, ZEB1, SMAD2, and SMAD3 were purchased from Sigma-Aldrich. The transfection of siRNA was performed with Lipofectamine® RNAiMAX Reagent (Invitrogen) according to the manufacturer’s instructions. The sequences are listed in Table S3.

RT-qPCR

Total cellular RNA was isolated with TRIzol reagent following the manufacturer’s instructions (Invitrogen). Any potential DNA contamination was avoided with RNase-free DNase treat-
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**A**

| Name   | Score  | Relative score | Strand | Distance to TSS | Predicted sequence | Predicted DNA binding profile |
|--------|--------|----------------|--------|-----------------|---------------------|------------------------------|
| ZEB1   | 13.786 | 0.9769469565   | +      | -1116           | Gcacacg            |
|        | 13.2187| 0.9698306566   | +      | -1608           | Tlcacacg           |
|        | 11.541 | 0.950031047    | +      | -1113           | Cacacgctgga        |
|        | 10.9219| 0.938610669    | +      | -1605           | Ctcacacgtgaa       |
|        | 10.243 | 0.926088198    | +      | -1103           | Atcacaacgcg        |
|        | 10.2056| 0.925396743    | +      | -1209           | Atcacaacgcagggcc   |
|        | 6.68302| 0.858869653    | -      | -445            | Ccacactg          |
|        | 5.86756| 0.841650732    | -      | -264            | ggcacactgcattctg   |
|        | 5.84138| 0.841207842    | +      | -446            | cacacgctgagggg     |
|        | 5.1849 | 0.832787612    | +      | -443            | gtcagctggcgaag     |

**B**

- MDA-MB-231
- MCF-7
- T-47D

**C**

- Mi-2
- MTA1
- MTA2
- MTA3
- HDAC1
- HDAC2
- RbAp46
- RbAp48
- MBD2/3

**D**

**E**

- #4
- ZEB1
- MTA1
- HDAC2

**F**

- MCF-7
- MDA-MB-231

**G**

- MCF-7
- MDA-MB-231

**Figure Legends**

- **A**: Prediction binding sites in the promoter region of MIR455 for ZEB1
- **B** and **C**: Western blot analysis showing protein expression levels
- **D**: Enrichment fold of MIR455 promoter region
- **E**: ChIP-Seq results
- **F and G**: Luciferase reporter assay and qPCR for miR-455-3p expression
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Plasmid construction and luciferase assays

The WT promoter or mutant promoters with GATA3-binding sequences in MIR455 deleted (nucleotides −2050 to +500) were amplified by PCR and cloned into pGL4 (Promega). Then the pGL4-miR-455-3p-Luc reporter was co-transfected with a vector encoding Renilla luciferase (pRL-TK) and either a vector control or FLAG-GATA3 into ER-positive or ER-negative cells. Human WT and mutant Smad2, ZEB1, and HDAC2 3′-UTRs were amplified by PCR and cloned into the Spl−HindIII sites of the pMIR-REPORT luciferase vector (Ambion, Grand Island, NY). The TGF-β reporter p3TP-Lux (J. Massagué, Memorial Sloan-Kettering Cancer Center, New York; plasmid 11767) was obtained from Addgene. The p3TP-Lux reporter was co-transfected with pRL-TK into MCF 10A cells. Three repeat sequences of the activin response element were artificially synthesized and inserted into the pGL4 vector (ARE-Luc). For the ARE-luc experiments, the cells were co-transfected with FLAG-FoxH1 (76). Then cells were serum-starved overnight and stimulated with 5 ng/ml TGF-β1 (R&D Systems) for 24 h. HEK 293T cells treated with controls, miR-455-3p mimics, or miR-455-3p inhibitors were transfected with WT or mutant Smad2, ZEB1, or HDAC2 3′-UTR luciferase reporters together with a vector encoding Renilla luciferase. All luciferase assays were performed as described previously (77). In brief, 48 h after transfection, lysates were collected, and firefly and Renilla luciferase activities were detected using the Dual-Luciferase Reporter System and a GloMax luminometer according to the manufacturer’s protocol (Promega). Firefly luciferase activity was normalized to that of Renilla luciferase. Each experiment was repeated in triplicate.

EdU assays

After respective treatments, MCF-7 and MDA-MB-231 cells were seeded in 6-well plates and subjected to EdU assays to detect proliferation based on fluorescence detection (Thermo Fisher Scientific). Before fixation, the cells were incubated in conditioned medium from the kit, and EdU assays were performed according to the manufacturer’s instructions.

Cell invasion assays

Transwell invasion assays were performed using transwell chambers (BD Biosciences) coated with Matrigel. After appropriate treatment, cells were resuspended in serum-free medium, and then 2.5 × 10⁴ cells were seeded into the upper chamber in a volume of 500 µl. The chamber was then transferred to a well containing 500 µl of medium with 10% FBS at 37 °C for 18–20 h. Cells on the upper side of the chamber were removed by wiping the top of the membrane with cotton swabs. Cells on the lower side were then stained and counted. Three high-powered fields were counted for each membrane.

Immunoprecipitation

For immunoprecipitation assays, cellular extracts were harvested as described previously (78) and incubated with a specific anti-ZEB1 antibody or normal mouse/rabbit IgG antibody at 4 °C overnight with constant rotation. Samples were then incubated with Dynabeads Protein G for 2 h at 4 °C and, after washing with cell lysis buffer, were subjected to SDS-PAGE. Immunodetection was performed using the enhanced chemiluminescence (ECL) system (Amersham Biosciences) according to the manufacturer’s instructions.

GST pulldown assays

The coding sequences of GST-fused proteins were amplified and cloned into the pGEX 4T-3 vector (Amersham Biosciences). GST-fused proteins were expressed in Escherichia coli (BL-21 strain) at the appropriate temperature, followed by ultrasonic cell disruption in cold PBS in the presence of a protease inhibitor mixture, and purified using GSH Sepharose 4B beads according to the manufacturer’s instructions (GE Healthcare). The beads were co-incubated with the in vitro-transcribed/translated proteins, which were transcribed and translated using rabbit reticulocyte lysate (Promega), and then subjected to further washing with binding buffer (75 mM NaCl, 50 mM HEPES, pH 7.9) five times to prevent nonspecific interactions. The beads were resuspended in 30 µl of 2× SDS-PAGE loading buffer and then analyzed by Western blotting.

ChIP and re-ChIP

ChIP and re-ChIP experiments were performed with MCF-7 cells as previously described (68). In brief, the cells were cross-linked with 1% formaldehyde, sonicated, precleared, and incubated with 5–10 µg of antibody per reaction, which was fol-

Figure 7. Reciprocal Regulation of the ZEB1/NuRD complex and miR-455-3p. A, the online analysis tool JASPAR was used to predict the potential binding sites of ZEB1 in the promoter region (−2050 to +500) of MIR455. B, association between ZEB1 and NuRD complex members including Mi-2, MTA1, MTA2, MTA3, HDAC1, HDAC2, RbAp46/48, and MBD2/3 in MDA-MB-231, MCF-7, and T-47D cells. Whole cell lysates were immunoprecipitated (IP) with an antibody against ZEB1, and immunocomplexes were immunoblotted (IB) using an antibody against subunits of the NuRD complex as indicated. C, GST pulldown assays with GST-fused ZEB1 and in vitro transcribed/translated components of the NuRD complex, as indicated. D, qChIP-based promoter-walk in MDA-MB-231 cells to map ZEB1, MTA1, and HDAC2 enrichment to two regions of the MIR455 promoter. Error bars, S.D. of three independent experiments (*, p < 0.05; **, p < 0.01, two-tailed unpaired t test). E, ZEB1, MTA1, and HDAC2 complexes were found to exist in the same protein complex at the #4 (left) and #7 (right) regions of the MIR455 promoter. ChIP and re-ChIP experiments were performed in MDA-MB-231 cells with the indicated antibodies. F, luciferase activities were measured in MCF-7 and MDA-MB-231 cells to examine the effect of ZEB1, MTA1, and HDAC2 on the transcriptional activity of the MIR455 promoter. Firefly luciferase activities were normalized to Renilla luciferase activities and plotted relative to control levels (top). The expression of the miR-455-3p was measured by qPCR in MCF-7 cells overexpressing ZEB1, MTA1, or HDAC2 and MDA-MB-231 cells with ZEB1, MTA1, or HDAC2 depletion (bottom). Error bars, S.D. of three independent experiments (*, p < 0.05; **, p < 0.01, two-tailed unpaired t test). G, graphic model as discussed under “Results.” A feedback loop between GATA3 and the ZEB1-nucleated repression program is involved in regulating the expression of miR-455-3p to control EMT and the metastasis of breast cancer cells.
lowed by the addition of Dynabeads Protein G. The beads were then washed in buffers of high and low salt concentrations, and DNA was eluted and purified with the QiAquick PCR purification kit. For re-ChIP assays, immune complexes were eluted from the beads with 20 mM DTT at 37 °C for 30 min. The eluents were then diluted 30-fold with ChIP dilution buffer, which was followed by re-immunoprecipitation with the second antibodies. The final elution step was performed with 1% SDS solution in Tris-EDTA buffer, pH 8.0. Enrichment of the DNA template was analyzed by qPCR using primers specific for each target gene promoter. The primer pairs used are listed in Table S5.

**Mouse xenograft models**

MDA-MB-231 cells stably expressing firefly luciferase (Xenogen Corp.) were infected with lentiviruses encoding control plmiR-Control or plmiR-455-3p (GenePharma). These cells were inoculated into the left abdominal mammary fat pad (5 × 104 cells) of 6-week-old female SCID mice. For bioluminescence imaging, mice were injected abdominally with 200 mg/kg d-luciferin in PBS. At 15 min after injection, mice were anesthetized and bioluminescence was determined using a charge-coupled device camera (IVIS; Xenogen Corp.). Bioluminescence images were obtained with a 15-cm field of view, binning (resolution) factor of 8, 1/f stop, open filter, and an imaging time of 30 s to 2 min. Bioluminescence from relative optical intensity was measured manually. Photon flux was normalized to the background, which was based on relative optical intensity from a mouse not administered luciferin. The volume of tumors was measured using a Vernier caliper and calculated according to the formula, \( \frac{1}{6} \times \text{length} \times \text{width}^2 \). Animal handling and procedures were approved by Tianjin Medical University Institutional Animal Care.

**Statistical analysis**

Results are reported as mean ± S.D. unless otherwise noted. Comparisons were performed using two-tailed paired \( t \) tests based on a bidirectional hypothesis for continuous variables. Tumor data sets were downloaded from [http://www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo), and GSE numbers are shown in the figures. Data for Kaplan–Meier survival analysis were from [http://kmplot.com](http://kmplot.com)/analysis.3

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